707-AP Peptide Recognized by Human Antibody Induces Human Leukocyte Antigen A2-restricted Cytotoxic T Lymphocyte Killing of Melanoma

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ABSTRACT

We recently identified a tumor-associated antigen that was recognized by human monoclonal antibody L94. The antibody-reactive 707-AP sequence RVAALARAP, cloned from a melanoma cDNA library, was also found to be recognized by peripheral blood lymphocytes (PBLs) from melanoma patients. In this study, 707-AP was used to stimulate melanoma patients’ PBLs for the establishment of peptidesspecific CTL cell lines. CTL cell lines derived from 258 melanoma patients of different human leukocyte antigen (HLA)-A and HLA-B allele expressions were assessed by a 51Cr cytotoxicity assay against the peptide-pulsed autologous B lymphoblastoid cells and T2 HLA-A2 antigen-presenting cells and autologous and allogeneic melanoma cell lines. The analysis of 707-AP CTL activity demonstrated that only HLA-A2 patients’ PBLs could be stimulated with 707-AP. 707-AP CTLs were able to specifically lyse HLA-A2 autologous and allogeneic melanoma cell lines. This verified the endogenous processing and presentation of 707-AP by melanoma cells. 707-AP CTL cytotoxicity against peptide-pulsed autologous HLA-A2 B lymphoblastoid cells and T2 HLA-A2 cells was also demonstrated. The killing activity of HLA-A2 707-AP CTL cell lines (CD8+ CD3+) was inhibited by anti-HLA class and anti-HLA-A2 monoclonal antibodies. The amino acid substitution or deletion analysis of the 707-AP sequence in CTL stimulation and recognition confirmed that position 2, amino acid V and position 9, amino acid A were essential. Both positions are known as supermotif anchors for HLA-A2 peptide sequences. Our studies demonstrated that 707-AP is a potent stimulator of CTLs that can induce peptide-specific HLA-A2 melanoma cell killing. The recognition of 707-AP by both antibody and CTLs suggests its potential significance as a peptide immunotherapeutic.

INTRODUCTION

Human cutaneous melanoma has been proven to be antigenic through analysis of patients’ sera or PBLs. This has led to the identification of many immunogenic TAAs (1-9) using primarily human CTLs (10-17) or human and mouse antibodies (7-9). However, the characterization of individual TAAs and the classification of specific antigenic peptide sequences are still limited. This may be due to the tedious and extensive screening needed for identification.

The identification of antigenic TAAs has become popular because of their potential in cancer vaccines and as target antigens to monitor active-specific immunotherapy. Melanoma vaccines have shown promising results for the betterment of patient survival and control of tumor progression (18-20). This improvement has been correlated to antibody and T-cell responses to specific TAAs in melanoma vaccine clinical trials (18, 20). These findings indicate that the identification of TAA antigenic peptide sequences for both antibody and T-cell recognition may be of therapeutic benefit.

We recently reported the identification of a TAA and its peptide sequence reactive to HumAb L94 (3, 4). L94 was used to isolate TAA cDNA clone 707 from two separate melanoma cell line cDNA libraries (3, 4). The epitope analysis revealed that L94 HumAb recognizes the COOH-terminal alanine-proline (AP) of 707 peptide (707-AP) sequence RVAALARDAP. This peptide sequence was derived from cDNA cloned protein 707 expressed by a melanoma cDNA library. In preliminary studies, 707-AP was also shown to be recognized by T cells of PBLs from melanoma patients (3). In this study, the short-term stimulation of PBLs with 707-AP showed no specific HLA allele restriction in CTL killing. The recognition of a TAA peptide sequence by both antibody and CTL is uncommon. Although it has been suggested that both antibody and T cells can recognize linear peptide sequences, the current dogma separates peptides into either T-cell or antibody-specific recognition. The reason for this is that T-cell lines established to peptide sequences are well documented, but the development of HumAb to study peptide specificity is still in its infancy.

We have previously demonstrated the distribution of 707-AP peptide/protein expression by in situ hybridization with a specific oligonucleotide probe and by immune adherence

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The abbreviations used are: PBL, peripheral blood lymphocyte; BLC, B lymphoblastoid cell line; mAb, monoclonal antibody; HumAb, human mAb; TAA, tumor-associated antigen; HLA, human leukocyte antigen; IL, interleukin; AP, alanine-proline.
assay using L94 HumAb (4). The antigen is expressed at a high frequency in human melanoma cell lines (3, 4). In assessment of other human cancer cell lines, it was found in lung, colon, gastric, and breast carcinomas by immune adherence assay and by in situ hybridization. However, the frequency of expression among nonmelanoma tumor cell lines is much lower than in melanoma. The antigen was not expressed by normal human monocytes, T cells, and B cells (4).

The majority of the studies that define antigenic peptide sequences of TAAs identify specific HLA class I allele motif sequences within TAAs and stimulate T cells to determine whether they are antigenic in vitro. The limitation of this approach is that peptide motifs for all HLAs have not yet been defined, and individual HLA-A and -B alleles can have multiple anchor motif sequences (21–23). Specific anchoring motif sequences correspond to the binding of the B and F pocket of the HLA-A and -B molecule. The binding motifs are located near the NH₂-terminal and COOH-terminal of the peptide sequence, respectively (23). Although motif sequences may guide the identification of potential antigenic peptide sequences, this algorithmic approach is far from efficient; other factors such as hydrophobicity, conformation, electric charge, binding affinity, and so forth play a role in the HLA binding antigenicity of the peptide. The use of synthetic peptides from antigens with binding motifs to HLA molecules can be misleading and may not be relevant to the presence of natural ligands (22). In our studies, we identified a natural immunogenic peptide sequence using HumAb. Using the base sequence of the HumAb-recognizing peptide, we assessed the natural 9–10-mer peptides of TAA sequences for their ability to activate CTLs. The identification of 707-AP and 810 (gp43) peptide (1, 2) of specific cloned TAA is an example of our strategy.

The peptides that can be recognized by both T cell and antibody are very significant in immunotherapy. In this study, we characterize the generation of CTL cell lines toward 707-AP, demonstrating their HLA-A2-restricted killing and specific recognition of 707-AP-pulsed antigen-presenting cells and HLA-A2+ autologous and HLA-A2+ allogeneic melanoma cells.

MATERIALS AND METHODS

Synthetic Peptides. COOH-terminal decapeptides and nonapeptides were synthesized; peptide 707-AP sequence was RVAALARDAP. Various 707-AP peptide analogues with amino acid deletions or substitutions were synthesized (VAAALARDAP, RNAALARDAP, RVNALARDAP, RVAALANDAP, RVAALARNAP, RVAALDRDNP, and RVAALARDAN). The nonamer peptide, HLA-A2 peptide sequence influenza A virus matrix₅₈₋₆₆ peptide GILGFVFTL (24), was used as a control. All peptides were synthesized on solid phase using N-(9-fluorenyl)methoxycarbonyl (Fmoc) for transient NH₂-terminal protection and then purified (Research Genetics, using N-(9-fluorenyl)methoxycarbonyl (Fmoc) for transient

Hepatitis A virus matrix58_66 peptide GILGFVFTL (24), was

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Melanoma Patients. CTL analysis was carried out on American Joint Committee on Cancer stage II–IV melanoma patients (n = 258) seen at John Wayne Cancer Clinic. Heparinized blood (25–30 ml) was drawn from melanoma patients as described previously (2). PBLs were separated by Ficoll-Hypanque (Pharmacia, Piscataway, NJ) gradient centrifugation. PBLs were washed several times and resuspended in culture medium. An aliquot of cells was cryopreserved in liquid nitrogen. HLA typing was performed on patients’ PBLs using a complement-mediated microcytotoxicity assay in the laboratory of Dr. Paul Terasaki (University of California at Los Angeles, Los Angeles, CA), as described previously (25). The number of patients expressing specific HLA-A and HLA-B alleles was as follows (parentheses contain the number of patients assessed): A1 (78), A2 (113), A3 (65), A11 (35), A23 (9), A24 (32), A25 (13), A26 (18), A28 (16), A29 (19), A30 (8), A31 (15), A33 (8), A34 (2), A66 (1), A68 (10), A69 (1), B7 (56), B8 (57), B13 (9), B14 (23), B18 (25), B27 (18), B35 (53), B37 (3), B38 (10), B39 (16), B41 (7), B44 (65), B45 (2), B47 (1), B48 (1), B49 (5), B50 (4), B51 (22), B52 (5), B53 (2), B55 (9), B56 (2), B57 (19), B58 (5), B60 (24), B61 (11), B62 (36), B63 (3), B70 (2), B73 (2), and B75 (1).

Cell Lines and Primary Cell Cultures. Melanoma cell lines were established from metastatic lesions as described previously (26) and passaged in RPMI 1640 supplemented with 10% FCS heat-inactivated medium (Gemini Bioproducts, Califasbasas, CA) and antibiotics (Life Technologies, Inc., Grand Island, NY). Primary fibroblast cell lines were established from the biopsy tissue of several patients and cultured in the same manner as melanoma cell lines. Lymph nodes were obtained from melanoma patients undergoing elective surgery at Saint John’s Hospital (Santa Monica, CA). Lymph nodes were obtained from the operating room and made into a single-cell suspension (27), washed, and resuspended in culture medium. Epidermal cells were prepared from skin obtained from patients undergoing elective lymphadenectomy (28). The epidermis was cultured in RPMI 1640 containing 0.3% trypsin for 2 h. The epidermal layer was then separated from the dermis, minced in the presence of trypsin and collagenase for several hours, washed in medium, and then cultured in RPMI 1640 containing 10% FCS heat-inactivated medium. Cells were cultured as adherent cells for one to two passages before use. EBV-transformed BLC lines were established from all 258 melanoma patients as described previously (29). Briefly, PBLs were cultured overnight at 37°C in EBV-containing supernatant, which was collected from the culture media (RPMI 1640 + 10% FCS heat-inactivated medium) of the EBV-producing marmoset cell line B958. Transformed cells were then routinely passaged to establish cell lines. The BLCs and HLA-A2-transfected T2 mutant lymphoblastoid cell line (24) were used as targets for peptide pulsing studies in the cytotoxicity assay. Other target cell lines for the cytotoxicity studies were the natural killer/lymphokine-activated killer cell-susceptible cell line K562 and a negative control target, leukemia cell line 8392 (26).

Generation of CTL Cell Lines. Isolated PBLs from melanoma patients were seeded at 3 × 10⁶ cells/well in 24-well culture plates in AIM-V media (Life Technologies, Inc.), containing 10% human AB serum (Gemini Bioproducts) heat-inactivated medium and antibiotics (referred to as CTL medium). Cell cultures were pulsed with 10 μg/ml synthetic peptide. After 2 days of culture, half of the CTL media was changed and replaced with fresh medium, supplemented with 15 IU/ml IL-2 (R&D Systems, Inc., Minneapolis, MN) and 25 IU/ml IL-7 (R&D Systems, Inc.). Cultured cells were transferred to new
24-well plates at medium changes and peptide stimulation. At day 6, 3 × 10^6 autologous PBLs, which had been cryopreserved, were used as feeder cells. Briefly, cells were thawed, washed, and incubated for 6 h with CTL medium containing 500 IU/ml granulocyte macrophage colony-stimulating factor (R&D Systems, Inc.) and 10 µg/ml peptide. These cells were then irradiated at 5000 rads and allowed to adhere in a culture flask, and nonadherent cells were removed. Subsequently, the peptide-stimulated T cells were added to the adherent cells with CTL medium. Two days later, half of the medium was changed with fresh medium containing IL-2, IL-7, and responder cells. This procedure was repeated throughout CTL culturing. Activated peptide-specific CTL cell lines could be maintained for more than 18 weeks by restimulation with peptide and cytokines every 6 days. The cultures without peptide stimulation died rapidly in culture.

**Cytotoxic Assay.** Target cells were labeled with 100 µCi of Na^51^CrO_4_ (Amersham, Arlington Heights, IL) for 1 h, as described previously (2). After two washings with culture medium containing 10% FCS heat-inactivated medium, target cells were seeded at 5 × 10^3 cells/well in 96-well round-bottomed microtiter plates, and effector cells were added at the desired concentrations. The plates were incubated at 37°C for 4 h, and supernatant was removed and counted in a Beckman gamma counter (Fullerton, CA). In CTL peptide recognition assays, \(^{51}\text{Cr}\)-labeled T2 HLA-A2 or B lymphoblastoid target cells were pulsed with 10 µg/ml peptide for 2 h at 37°C before effector cells were added. The percentage of specific lysis was calculated as follows: % of specific lysis = 100 × [(experimental release - spontaneous release)/(maximum release - spontaneous release)] (2). Spontaneous release was determined by incubating target cells with medium only, and maximum release was determined with 3% Triton X-100. All values represent the mean of triplicate samples. Several E:T ratios were studied. For presentation purposes, the optimal and most relevant E:T ratio of 40:1 was shown. In peptide-pulsed target cell experiments, spontaneous release and maximum release without effector cells were assessed for both T2 HLA-A2 and BLCs with and without peptide. No difference was observed.

**mAb Antibody Blocking.** Target cells labeled with Na^51^CrO_4_ were incubated for 30 min at 37°C with 10 µg/ml murine IgG2 anti-HLA class I mAb, anti-HLA-DR mAb, anti-HLA-DQ mAb (AMAC, Inc., Westbrook, ME), or anti-HLA-A2 mAb (clone BB7.2; American Type Culture Collection) before the addition of effector cells (2). A standard cytotoxicity assay was then performed. The optimal concentration of antibody for blocking was determined previously.

**Cold-Target Inhibition Assays.** Effector cells were incubated with unlabeled cold targets at specific cold:hot target ratios in 96-well microplates for 1 h at 37°C (25). \(^{51}\text{Cr}\)-labeled hot targets were then added to the wells and incubated for 4 h at 37°C. Effector:hot target ratio was constant at 40:1.

**Flow Cytometric Analysis.** PBLs or CTL cell lines were washed twice with phenotyping buffer containing RPMI 1640 supplemented with 5% FCS and 0.2% sodium azide and then incubated with FITC-conjugated murine IgG1 mAb to human CD3, CD4, CD8, or CD16. Negative controls consisted of matched murine IgG (Becton Dickinson, Mountain View, CA). Cells were incubated for 30 min on ice with mAb and washed twice, and fluorescent intensity was analyzed by FACScan (Becton Dickinson, San Jose, CA), as described previously (30).

**Antibody Analysis by ELISA.** Melanoma patients' sera and L94 HumAb were assessed by ELISA. The highly purified L94 HumAb (IgM) was derived from an established cloned EBV BLC (4) and was assessed at several log dilutions. ELISA was performed by coating Reacti-Bind 96-well microplates (Pierce, Rockford, IL) with 1 µg of 707-AP peptide/well in PBS and incubating plates overnight at room temperature. Microplate wells were blocked with SuperBlock blocking buffer (Pierce) for 5 h at room temperature. The plates were incubated with sera or HumAb for 2 h, washed, and incubated with goat anti-human IgM (μ-chain specific) conjugated with horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN). Color development and absorbance readings were as described previously (31).

**RESULTS**

**Human Antibody Recognition of 707-AP Peptide.** 707-AP peptide was originally isolated and identified by L94 HumAb reactivity (3, 4). L94 HumAb log dilution and reactivity to 707-AP peptide in ELISA was demonstrated. L94 HumAb reactivity to 707-AP peptide was shown to be significantly reactive at 0.1 µg/ml. Binding activity was demonstrated to be enhanced with higher concentrations of the antibody. The antigenic epitope for L94 HumAb has been previously determined to be the COOH-terminal AP of the 707-AP sequence (4). Melanoma patients' sera were assessed for activity against 707-AP peptide by ELISA. At a 1:50 dilution of sera, significant activity to 707-AP peptide could be demonstrated. Antibody titer levels to 707-AP peptide varied among melanoma patients with disease levels. COOH-terminal AP was demonstrated to be the epitope recognized by antibody-positive sera. These studies indicated that the COOH-terminal AP is antigenic in melanoma patients and that melanoma can induce 707-AP-specific antibody responses.

**HLA Restriction of CTLs Induced by 707-AP Peptide.** PBLs from 258 melanoma patients were stimulated with 707-AP decapeptide. After 4 weeks of stimulation, CTL assays were performed using autologous BLCs of corresponding patients as target cells. CTL activity was assessed at an E:T ratio of 40:1 for screening cytolytic activity. HLA phenotype was correlated to individual patients after CTL analysis was performed. In 65 of 258 patients, the percentage of specific cytolysis to 707-AP peptide-pulsed BLCs was greater than 10% above that of controls (BLCs not pulsed with peptide). All 65 positive patients had a phenotype of HLA-A2 (data not shown). These findings strongly suggested that CTL recognition of 707-AP peptide was in a HLA-A2-restricted manner.

Additional studies were performed to confirm HLA restriction of 707-AP CTL cell lines. CTL cell lines were established by repetitive stimulation with 707-AP peptide for more than 8 weeks. The peptide-pulsed and nonpulsed autologous BLCs and T2 HLA-A2 cell lines were used as target cells. To verify the specificity of 707-AP peptide recognition by CTLs, we assessed both T2 HLA-A2 and autologous BLCs. In Table 1, representative examples of HLA-A2+ CTL cell line recognition of peptide- and non-peptide-pulsed T2 HLA-A2 and BLCs show 707-AP peptide-specific recognition. PBL cultures did not stimu-
Tumor Peptide 707-AP Induces CTL Killing of Melanoma

Table 1

<table>
<thead>
<tr>
<th>Patients (HLA-A)</th>
<th>BLC 707-AP (+)</th>
<th>BLC 707-AP (-)</th>
<th>T2 HLA-A2 707-AP (+)</th>
<th>T2 HLA-A2 707-AP (-)</th>
</tr>
</thead>
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<tr>
<td>1 (A1,2)</td>
<td>22.9</td>
<td>4.3</td>
<td>20.4</td>
<td>3.8</td>
</tr>
<tr>
<td>2 (A2)</td>
<td>21.8</td>
<td>2.2</td>
<td>19.7</td>
<td>1.5</td>
</tr>
<tr>
<td>3 (A2,3)</td>
<td>18.5</td>
<td>2.9</td>
<td>18.0</td>
<td>3.1</td>
</tr>
<tr>
<td>4 (A2,23)</td>
<td>23.4</td>
<td>3.0</td>
<td>18.1</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Representative CTL (>8 weeks of stimulation with 707-AP peptide) cytotoxicity was assessed at an E:T ratio of 40:1. Lymphoblastoid cell targets were 707-AP-pulsed (+) or were not pulsed (-).

HLA-A2-restricted 707-AP CTL killing was assessed using autologous and allogeneic melanoma cell lines. Allogeneic melanomas consisted of HLA-A2+ and HLA-A2− phenotypes. Only patients whose PBLs and autologous melanoma cell lines available were tested. Previously, we demonstrated that 707-AP peptide is expressed in >90% of human melanoma cell targets (3). In the 32 patients whose CTLs were studied, 13 were HLA-A2+ and 19 were HLA-A2−. In these experiments, HLA-A2+ patients’ 707-AP CTLs killed HLA-A2+ autologous and HLA-A2+ allogeneic melanoma cell lines significantly in the range of 25.9–50.4%. HLA-A2+ patients’ 707-AP-stimulated CTLs showed no significant killing of allogeneic HLA-A2− melanoma cell lines (1.0–5.7%). The HLA-A2+ CTL killing against HLA-A2+ melanoma cell lines varied with each individual melanoma cell line. The insignificant killing activity of 7562 and 8392 control target cell lines ruled out nonspecific lymphokine-activated killer cell/natural killer effector activity. HLA-A2− patient PBLs stimulated with 707-AP died off rapidly. Representative HLA-A2+ patients’ CTL killing activity against HLA-A2+ and HLA-A2− melanoma cell lines is shown in Table 2.

The 707-AP-specific CTL cytotoxicity demonstrated against two different target cell lines, T2 HLA-A2 and a melanoma cell line, was abrogated by anti-HLA class I mAb and anti-HLA-A2 mAb (Fig. 1). No significant inhibition was observed with anti-HLA-DR or anti-HLA-DQ mAb. These results confirmed the 707-AP peptide CTL cytotoxicity was HLA-A2 restricted.

Antigen Specificity for 707-AP Peptide-specific CTLs. CTL cell lines specific to 707-AP peptide were passaged for more than 4 months. In general, these CTL cell lines maintained specificity to 707-AP peptide and cytotoxic activity after 18 weeks of stimulation and passaging. Studies assessing CTL activity were performed biweekly during passaging. Cytolytic activity was assessed against autologous melanoma and 707-AP peptide-pulsed and nonpulsed autologous BLCs and T2 HLA-A2 cells. A representative 707-AP-specific CTL cell line is shown in Fig. 2. The kinetics of cytotoxic activity was similar over time: 4–6-week cytotoxicity increased steadily and then leveled off. As a positive control for analysis of several HLA-A2+ patients, HLA-A2 influenza-matrix nonamer peptide (GILGFVFTL) was used to stimulate patients’ PBLs. The influenza-matrix peptide has been demonstrated to induce a strong peptide-specific HLA-A2+ CTL response (24). The cytotoxic activity of influenza peptide-stimulated CTLs was higher than that of 707-AP-stimulated CTLs against respective peptide-pulsed autologous BLCs. This demonstrated that the difference in BLC killing activity was not due to defective peptide presentation and cytolysis. Respective studies with T2 HLA-A2 cells showed similar effects (data not shown).

The phenotyping of multiple 707-AP-specific CTL cell lines at week 18 by flow cytometry analysis revealed that >90% of cells had a CD3+ CD8+ phenotype and that a negligible level of CD4+ or CD16+ cells was present (data not shown).

Analysis of Amino Acid Substitution and/or Deletion of 707-AP. To demonstrate the relevance of individual amino acids in 707-AP for CTL recognition, several related peptide analogue sequences were synthesized by amino acid substitution or by deletion of 707-AP RVAALARDAP. HLA-A2+ melanoma patients’ (good responders to 707-AP) PBLs were stimulated for 4 weeks with 707-AP or 707-AP analogues and tested for cytotoxicity against autologous BLCs pulsed with respective peptides and autologous melanoma (Fig. 3). Deletion of the NH2-terminal end amino acid R of 707-AP did not significantly change the level of cytotoxic activity. Analogue 707-AP peptides with substitution of V at position 2 or A at position 9 with N amino acid abolished CTL activity completely against melanoma and peptide-pulsed to autologous BLCs. Peptide substitution of A at position 3, R at position 7, and P at position 10 with N amino acid did not induce CTL activity against melanoma cells. However, CTL stimulation with these 707-AP analogues induced cytotoxic activity against respective peptide-pulsed BLCs. When PBLs of HLA-A2+ melanoma patients were stimulated (4 weeks) by 707-AP with any amino acid substitution and assessed for specific cytotoxicity against 707-AP-pulsed BLCs, no significant cytotoxicity was observed (data not shown). In general, most of these PBL cultures died off. Stimulation with the nonamer VAALARDAP of PBL induced significant CTL activity against 707-AP peptide-pulsed BLCs.

When long-term established 707-AP CTL cell lines were stimulated with the above 707-AP analogues for several passages, CTL growth ceased, whereas the positive control cell lines that were continuously stimulated with 707-AP proliferated. These studies further validated the specificity of the 707-AP CTL cell lines and the dependency of specific peptide stimulation.

CTL Activity against Normal Cells. We investigated the cytotoxic activity of 707-AP CTL cell lines against normal cells targets. There was no significant CTL activity against HLA-A2+ autologous PBLs, fibroblasts, or epidermal cells (Fig. 4).

707-AP CTL killing against autologous melanoma cells in the presence of normal cells was investigated. Cold target inhibition studies were set up with normal autologous cells to assess the blocking of CTL killing of autologous melanoma (Fig. 5). Fibroblasts have been shown to be good targets in cytotoxicity assays. No inhibition by cold target autologous fibroblasts, PBLs, and lymph node cells was seen in CTL activity toward
Represents CTL (>8 weeks of stimulation with 707-AP) activity was assessed at an E:T ratio of 40:1. Melanoma line MA and patient 1 were autologous. PBLs of non-HLA-A2+ melanoma patients stimulated with 707-AP died off early in culture.

<table>
<thead>
<tr>
<th>Patients (HLA-A)</th>
<th>MA (A1,2)</th>
<th>MB (A2,24)</th>
<th>MC (A2,28)</th>
<th>MD (A1,11)</th>
<th>ME (A24,31)</th>
<th>MF (A28,32)</th>
<th>K562</th>
<th>MELANOMA TARGET CELL LINES</th>
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<tr>
<td>1 (A1,2)</td>
<td>41.3</td>
<td>27.2</td>
<td>45.9</td>
<td>2.7</td>
<td>5.4</td>
<td>4</td>
<td>5.1</td>
<td>1.6</td>
</tr>
<tr>
<td>2 (A2)</td>
<td>37.3</td>
<td>23.3</td>
<td>50.4</td>
<td>4.8</td>
<td>4.2</td>
<td>3.8</td>
<td>6.6</td>
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</tr>
<tr>
<td>3 (A2,3)</td>
<td>30.9</td>
<td>26.5</td>
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<tr>
<td>4 (A2,25)</td>
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<td>3.9</td>
<td>5.3</td>
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![Graph](image_url) Fig. 1 HLA restriction defined by mAb inhibition of 707-AP CTL cytotoxicity. Cytotoxicity assay (percentage of specific cytotoxicity) was performed at an E:T ratio of 40:1. Targets were autologous BLCs pulsed with 707-AP (upper group) and autologous melanoma cells (lower group). Columns, mean; bars SE.

The overall objective of this study was to determine whether 707-AP peptide could induce CTL cell lines and to analyze the specificity of those cell lines. It was demonstrated that 707-AP peptide stimulation induces 707-AP CTL-specific responses in HLA-A2+ patients only. The specificity of CTL cell lines was demonstrated to be HLA-A2 restricted. Most importantly, 707-AP induces specific CD8+ CD3+ CTLs that can kill HLA-A2+ autologous and HLA-A2+ allogeneic melanomas. We also showed that 707-AP is a common TAA peptide that is expressed by melanomas from different patients and recognized by CTLs. The recognition of melanoma cells by 707-AP CTLs indicated that 707-AP was derived from a protein that was endogenously processed and naturally presented by HLA class I molecules. These studies provided strong evidence of 707-AP CTL recognition on melanoma. The extensive screening of the PBLs of non-HLA-A2 melanoma patients substantiated that there was no other major HLA class I that could significantly present and induce 707-AP-specific CTL cell lines. The specificity of 707-AP recognition was demonstrated using two different types of antigen-presenting cells, BLCs and T2 HLA-A2. Cytotoxic activity of the established CTL cell line
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Fig. 3 CTL specificity of amino acid substitutions or deletions of 707-AP RVAALARDAP (individual amino acid changes are underlined). PBLs from HLA-A2+ melanoma patients were stimulated with 707-AP or 707-AP analogues for 4 weeks, and CTLs generated were tested against respective 707-AP or 707-AP analogue-pulsed autologous BLCs (□) or autologous melanoma (■). Cytotoxicity was performed at an E:T ratio of 40:1. The results shown are the mean percentage of specific cytotoxicity (±SE) values of six independent experiments from six different melanoma patients.

Fig. 4 Cytotoxicity of 707-AP CTLs against normal cells. 707-AP CTL cell lines were stimulated for 18 weeks and tested against autologous or allogeneic HLA-A2 normal cells. K562 was used as a control target. The percentage of specific cytotoxicity represents the mean values with SE of three independent cytotoxicity assays at an E:T ratio of 40:1. Auto, autologous to the CTL cell line. Allo, allogeneic to the CTL cell line. The HLA-A phenotype of the target is shown in parentheses.

could be maintained by stimulation with 707-AP for 4–6 months. Previously, we had suggested that 707-AP could activate CTL-like activity in short-term (5-day) culture from melanoma patients’ PBLs. It is now clear that after repetitive stimulation, 707-AP induces a specific HLA-A2+-restricted T-cell killing.

In general, 707-AP-specific CTLs showed greater cytolytic activity to autologous melanomas as compared with respective autologous peptide-pulsed T2 HLA-A2 target cells. The higher cytolytic activity toward melanoma cell lines than T2 cells could be due to a number of factors: (a) the level of natural peptide expression may be higher; (b) the composition of the natural peptide expressed by HLA-A2 molecules on melanoma cells may be different; at this time, we do not know the natural peptide composition of 707-AP expressed by HLA-A2 molecules on melanoma cells; (c) higher level of HLA-A2 and accessory cell surface molecules for CTL recognition; and (d) enhanced susceptibility to CTL lysis in general. All or a combination of these factors may play a role in the differences in lysis.

Specificity studies performed by manipulating the individual amino acid sequences in 707-AP showed that amino acids in position 2 and position 9 were very critical for inducing HLA-A2-restricted CTL killing of autologous melanoma and autologous BLC peptide-pulsed target cells. These results are supported by other studies that demonstrated that the peptide supermotif for HLA-A2 restriction can have V and A anchor motifs at the same positions (23). However, anchor determinant positions are not absolute and can vary. It is also well known that peptides with specific HLA-A2 anchor motifs are not always antigenic or expressed by melanomas. HLA-A2 is considered a peptide-binding supertype molecule (22, 23) in which the supermotif for HLA-A2 restriction can have V and A anchor motifs at the same positions (23). However, 707-AP sequence is not a peptide-binding supertype molecule.

Melanoma is a cancer that predominantly affects Caucasians. HLA-A2 is one of the most frequently expressed HLA class I alleles in Caucasians (32). The development of peptide immunotherapy toward HLA-A2 melanoma patients, who make up approximately 46% of this population, would be extremely useful (33). As more peptides are characterized with specific HLA class I restriction, a better-defined list of anchor motifs for individual HLA class I alleles will become known. However, there are peptides reported that do not have known anchor motifs but are still antigenic. For example, Muc-1 A55 peptide, PDTPAPGSTAPPA or PDTPAPGSTAP, yielded by digestion of protein, showed a HLA A2-restricted recognition by CTL regardless of its lack of a typical HLA-A2 anchor motif.
residues, other amino acid residues of 707-AP were also shown.molecules that are related to each other (33). Besides the anchor residues, other amino acid residues of 707-AP were also shown to be necessary for generating CTLs capable of killing the respective peptide-pulsed BLCs. Conversely, the 707-AP analogue did not activate PBL cultures very well. 707-AP CTLs could not recognize BLC target cells pulsed with 707-AP analogue having any one amino acid substituted. This suggests that the melanoma cells may have a more restricted requirement of peptide presentation or that the level of natural peptide expression by melanoma cells is modified through endogenous processing. It is possible that the amino acid-substituted peptide acts as an agonist of T-cell responses and inhibits recognition of melanoma cells presenting 707-AP.

707-AP could induce antigen-specific CTLs against HLA-A2+-expressing autologous melanoma and HLA-A2+-allogeneic melanoma. Many tumor antigen-derived peptides have been reported, but few can generate CTLs that effectively kill autologous and allogeneic tumor cells in a HLA class I-restricted manner. One of the major problems encountered in T-cell assessment of antigen recognition is the low level of antigen expression by target tumor cells. Although peptide stimulation can induce specific CTLs in vitro, they may not kill tumor cells effectively. Studies often rely on peptide stimulation of CTLs and measurement of activity against peptide-pulsed antigen-presenting cells. However, this merely demonstrates peptide specificity, not efficacy against tumor. To date, peptide immunogenicity has not been linked to peptide binding to HLA molecules or to CTL specificity toward a peptide in vitro. To consider peptide sequences useful for effective immunotherapy, they must be able to consistently activate a large patient population and kill different patients' tumors (36–38). Lack of consistent killing by CTLs of melanomas from a wide patient population will eventually translate into poor therapeutic efficacy. In this study, we have shown sufficient preclinical data on 707-AP-induced CTLs from a large patient population. Future studies will involve the designing of 707-AP peptide immunotherapies.

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