Molecular Basis of T Cell-mediated Recognition of Pancreatic Cancer Cells

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ABSTRACT

Pancreatic cancer continues to be a major unsolved health problem in the world. The prognosis of pancreatic cancer is extremely poor with a median survival of 3–4 months and the 5-year survival being 1–4%. This poor prognosis is primarily because of a lack of effective therapies, and thus development of new treatment modalities is needed. One of these treatments could involve specific immunotherapy, for which elucidation of the molecular basis of T cell-mediated recognition of cancer cells is required. We report here six different genes and 19 immunogenic epitopes from pancreatic adenocarcinoma cells and T-cell receptor β usage of HLA-A2-restricted CTL clones reacting to some of these epitopes. Sixteen of 19 epitopes were found to possess the ability to induce HLA-A2-restricted restricted CTL activity in the peripheral blood lymphocytes of patients with pancreatic and also colon adenocarcinomas. These results should provide a scientific basis for the development of specific immunotherapy for pancreatic and colon cancer patients.

INTRODUCTION

Pancreatic cancer is one of the major causes of cancer death in the world, with ~27,000 deaths annually in the United States and 50,000 deaths in Europe (1). Only 1–4% of pancreatic cancer patients survive the disease; the incidence rates are virtually identical to the mortality rates. Therefore, the development of new treatment modalities is needed; one of which could involve specific immunotherapy. Molecules involved in specific immunity, including tumor rejection antigen genes, HLA class I-binding immunogenic epitopes capable of inducing CTLs, and TCRs have been identified in melanomas and other cancers in the past decade (2–10). These results have opened the door to peptide-based specific immunotherapy for advanced or metastatic cancer patients (11–13). However, little information is available regarding pancreatic cancer cells (14, 15). Here, we report on the molecular basis of T cell-mediated recognition of pancreatic cancer cells. These results should provide a scientific basis for the development of specific immunotherapy for pancreatic cancer patients.

MATERIALS AND METHODS

Generation of HLA-A2-restricted CTLs. The HLA-A2-restricted and tumor-specific CTL (OK-CTL) line was established from tumor-infiltrating lymphocytes of a patient with colon cancer (HLA-A0207/3101, HLA-A23/51, HLA-Cw1) by incubation with interleukin 2 (100 units/ml) for >50 days as reported previously (10). Their CTL activity against various cancer and normal cells was tested by a 6-h 

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The abbreviations used are: TCR, T-cell receptor; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; SCC, squamous cell carcinoma; EBV-B, EBV-transformed B cell; PHA, phytohemagglutinin; CDR, complementarity-determining region; MFI, mean fluorescence intensity.

I of PBMCs was serotyped by the conventional method, whereas HLA-A2 subtypes were determined by the sequence-specific oligonucleotide probe method and also by direct DNA sequencing. The surface phenotypes of CTLs were investigated by a direct immunofluorescence assay with FITC conjugated anti-CD3, anti-CD4, or anti-CD8 mAb (Nichirei, Tokyo, Japan) or anti-TCRb mAb (WT31; Becton Dickinson, Mountain View, CA). To inhibit CTL activity, 20 μg/ml of anti-HLA-class I (W6/32, IgG2a), anti-HLA-A2 (B7.2, IgG2b), anti-CD8 (Nu-Ts/c, IgG2a), anti-HLA-class II (H-DR-1, IgG2a), and anti-CD4 (Nu-Thi, IgG1) mAbs were used. Anti-CD13 (MCS-2, IgG2a) and anti-CD14 (JML-H14, IgG1) served as isotype-matched control mAbs.

Identification of the cDNA Clones. A previously reported expression gene cloning method (8 –10) was used to identify genes coding for tumor antigens recognized by the OK-CTLp. In brief, cDNA of Panc-1 was inserted into the expression vector pCMV-SPORT-2 (Life Technologies, Inc., Rockville, MD). HLA-A0207, HLA-A2301, or HLA-A2601 was amplified by reverse transcription-PCR and cloned into the pCR3 (Invitrogen, San Diego, CA). COS-7 cells (5 × 105) were cotransfected with the Panc-1 cDNA library and HLA-A0207 plasmid DNA by Lipofectamine (Life Technology) and cultured for 2 days. The cells were analyzed by an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster, CA). The two clones of WHSC were obtained from a Panc-1 cDNA library by the standard colony hybridization method using the 32P-labeled cDNA clone no. 3 as a probe.

CTL Activity and TCR β Usage of OK-CTL Clones. The OK-CTL clones were obtained from the parental OK-CTLp line by methods reported previously (10). Cloned CTLs were tested for CTL activity to tumor cells, peptides were loaded on T2 cells and also to the COS-7 cells cotransfected with each of the six genes and HLA-A0207 plasmid DNA. Total RNA was isolated from CTL clones (5 × 105 cells), and cDNA was prepared. Single-stranded cDNA was amplified using one of the 22 different Vβ primers (Vβ1–20) and 3 Cβ primer (16). PCR was performed for 35 cycles (at 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min). The PCR product was inserted into pCR2 (Invitrogen, San Diego, CA), followed by transformation into competent Escherichia coli cells, selection of colonies, and plasmid preparation for sequencing.

Peptides, CTL Assay, and CTL Induction. Peptides capable of binding to the HLA-A2 molecules were searched at the literature level with regard to peptides for HLA-A2 binding motifs (17), and 128 different peptides (≥70% purity) were synthesized for screening. For further studies, 26 peptides with >95% purity were synthesized. The binding activity of peptides to HLA-A0201 molecules on T2 cells was tested by methods reported previously (18). Briefly, T2 cells were incubated with a peptide (10 μM) for 3 h at 26°C, followed by incubation for 3 h at 37°C in the cells. The cells were incubated with the anti-HLA-A2 mAb (BB7.2) and then stained with the phycoerythrin-conjugated F(ab’2) rabbit antimonosial immunoglobulin (Dako, Copenhagen, Denmark), followed by analysis of the expression pattern with FACScan (Becton Dickinson). A MAGE3-derived HLA-A2 binding peptide (FLGPRALV) or SART1-derived HLA-A26 binding peptide (RALTGVGGSK) was used as a positive or negative control, respectively. Peptide-binding affinity to HLA-A0201 molecules was expressed as the relative MFI of the HLA-A2 molecules. The MFI of the positive or negative control was 898 or 490, respectively. To test for recognition of the peptides by the CTLs, the CTLs were incubated for 18 h with T2 cells pulsed with the peptides (10 μM) for 2 h, followed by harvesting of supernatant to measure IFN-γ by ELISA. The method used to induce CTLs by a peptide has also been described elsewhere (8–10). Briefly, PBMCs from cancer patients were incubated with a peptide (10 μM). After 7 and 14 days of culture, the cells were restimulated with irradiated (30 Gy) autologous PBMCs as antigen-presenting cells that had been pulsed with the
same peptide at the same dose for 2 h. Effector cells were harvested at day 21 of culture and tested for both the surface phenotypes and the activity to produce IFN-γ in response to various target cells by ELISA. These PBMCs were further tested in the presence of autologous antigen-presenting cells, interleukin 2, and a corresponding peptide, and the cells were retested for their surface phenotypes and CTL activity at around days 21–28 of reculture.

RESULTS

HLA-A2-restricted CTL Subline Used for Study. The HLA-A2-restricted CTL line (OK-CTL) was established from tumor-infiltrating lymphocytes of a patient HLA-A0207/3101 with colon adenocarcinoma. One of the sublines (OK-CTLp) with an 80% CD3+CD4+CD8+ phenotype (the remaining cells were CD3+CD4+CD8+) showed HLA-A2-restricted and tumor-specific CTL activity at day 58 of culture, as measured by both 51Cr release (Fig. 1A) and IFN-γ production assays (Fig. 1B). The OK-CTLp showed significant levels of cytotoxicity to HLA-A0201+ Panc-1 pancreatic adenocarcinoma cells and SW620 colon adenocarcinoma cells, HLA-A0206+ KE3 esophageal SCC cells, and HLA-A0207+ CA9–22 oral SCC cells but failed to lyse either HLA-A2+ tumor cells or autologous EBV-B cell line and PHA-activated T cells (Fig. 1A). The OK-CTLp also lysed all of the HLA-A2+ tumor cells tested (HLA-A0201+ R27 breast adenocarcinoma, HAK-2 hepatocellular carcinoma, SK-MEL-5 melanoma, SF126 astrocytoma, HLA-A0206+ PC9 lung adenocarcinoma, HLA-A0207+ 1–87 lung adenocarcinoma, and OMC-4 cervical SCC cells; data not shown). The CTL activity was inhibited by anti-HLA-class I, anti-CD8, or anti-HLA-A2 mAb but not by any other mAbs tested (Fig. 1B). This OK-CTLp, which showed the highest response to Panc-1 (Fig. 1B), was used as an indicator in the following experiments.

Identification of Genes. A total of 1 × 10¹⁰ cDNA clones from the cDNA library of Panc-1 and HLA-A0207 cDNA was cotransfected to COS-7 cells, followed by a test of their ability to stimulate IFN-γ production by the OK-CTLp. After repeated experiments, six cDNA clones, no. 1 to no. 6, were identified (Fig. 2A). This CTL line, however, did not react with COS-7 cells transfected with HLA-A0207 cDNA and cDNA clone no. 7 taken as a negative control or with COS-7 cells transfected with one of the cDNA clones no. 1 to no. 6 and HLA-A2402 or HLA-A2601 cDNA (Fig. 2A). The sequence of cDNA clone no. 1 registered in the GenBank (accession no. AB044550) was almost identical to that of the ubiquitin-conjugated enzyme variant Kua (UBE2V) gene (accession no. AF155120), and its deduced amino acids are slightly longer (3 amino acids at positions 109–111) than those of the UBE2V gene, the biological function of which has not yet been reported. The sequence of cDNA clone no. 2 (accession no. AB044547) was almost identical to that of the heterogeneous nuclear ribonucleoprotein L gene (HNRPL gene; accession no. NM001533), but its deduced amino acids are longer at the NH₂-terminal at positions 1 to 31 than those of the HNRPL gene, which is one of the heterogeneous nuclear ribonucleoprotein complexes providing a substrate for the processing events that pre-mRNAs undergo before becoming functional and translatable mRNAs in the cytoplasm (19, 20). The sequence of cDNA clone no. 3 was 25 bp shorter at the 5’-end region than that of the Wolf-Hirschhorn syndrome candidate 2 protein (WHSC2) gene (accession no. AK001304). Subsequently, a full-length gene (accession no. AB044549) was obtained by the colony hybridization method using 32P-labeled cDNA no. 3 as the probe. The deduced amino acid sequence of the obtained gene is 21 amino acids longer at positions 30–50 than that of the WHSC2 gene. The WHSC2 gene seems to play a role in the phenotype of WHS, a multiple malformation syndrome characterized by mental and developmental defects resulting from a partial deletion of the short arm of chromosome 4 (21). The nucleotide sequence of cDNA no. 4 (accession no. AB044548) was identical to that of eIF-4E-binding protein 1 gene (EIF4EBP1, accession no. NM004095). This protein is known as a translation initiation factor that initiates insulin-dependent phosphorylation of 4E-BP1, making it a substrate for the processing events that pre-mRNAs undergo before becoming functional and translatable mRNAs in the cytoplasm.

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reading frames were all recognized by the OK-CTLp in a dose-dependent fashion (Fig. 2B). mRNA of these genes except for pp-MAPkkk showed a similar expression pattern by Northern blot analysis. These genes were ubiquitously expressed in both cancer and normal cells, and their expression levels in cancer cells, including Panc-1, SW620, and CA9–22 tumors, were significantly higher than those in the normal cells, including PHA-blastoid T cells and EBV-B cells (data not shown). However, mRNA expression of ppMAPkkk was scarcely detectable under the conditions used, which could have been attributable to rare expression, because only three clones were detected in the approximately $1 \times 10^6$ cDNA library by colony hybridization using $^{32}$P-labeled cDNA clone no. 5.

**Determination of Epitopes and TCR Usage.** Three-hundred T-cell clones were established from the parental OK-CTLp line. Eighty CTL clones among them showed HLA-A2-restricted and tumor-specific CTL activity (data not shown). All of these 80 CTL clones expressing CD3 $^+$CD4 $^-$CD8 $^+$ and TCR $\alpha \beta ^+$ phenotypes (data not shown) were tested for their reactivity to the six gene products. Among them, 2, 3, 1, 3, 2, and 4 CTL clones were reactive to UBE2V, HNRPL, WHSC2, EIF4EBP1, ppMAPkkk, and 2-5 OAS3 gene products, respectively. Representative data are shown in Table 1. Each of 27, 17, 21, 5, 19, or 39 different synthesized peptides with HLA-A2 molecule-binding motifs derived from the six gene products, respectively, was loaded onto the T2 cells, followed by testing for their ability to induce IFN-\(\gamma\) release by the OK-CTLp and its CTL clones. Five peptides of UBE2V at positions 43–51, 64–73, 85–93, 201–209, and 208–216 were recognized by the OK-CTLp, whereas the two peptides at positions 43–51 and 64–73, but not any of the other 25
peptides, were strongly and dimly recognized by the CTL clone 2-2-H3, respectively (Fig. 3A, upper left column). Four peptides of HNRPL at positions 140–148, 404–412, 443–451, and 501–510 were recognized by the OK-CTLp, whereas one peptide at positions 140–148, but not any of the other 16 peptides, was recognized by the CTL clone 1-2-D12 (Fig. 3A, middle left column). Similarly, the peptides recognized by the OK-CTLp were as follows: four peptides of WHSC2 at positions 103–111, 141–149, 157–165, and 267–275; two peptides of EIF4EBP1 51–59 and 52–60; three peptides of ppMAPkkk 290–298, 294–302, and 432–440; and one 2-5 OAS3 peptide 666–674. Furthermore, CTL clones 4-2-A11, 4-2-B3, 0.5-1-H2, and 2-1-H4 recognized a WHSC2 peptide 103–111, an EIF4EBP1 51–59, and ppMAPkkk 294–302; and one 2-5 OAS3 peptide 666–674 peptides, were strongly and dimly recognized by the CTL clone 2-2-H3, respectively (Fig. 3A, upper left column). Four peptides of HNRPL at positions 140–148, 404–412, 443–451, and 501–510 were recognized by the OK-CTLp, whereas one peptide at positions 140–148, but not any of the other 16 peptides, was recognized by the CTL clone 1-2-D12 (Fig. 3A, middle left column). Similarly, the peptides recognized by the OK-CTLp were as follows: four peptides of WHSC2 at positions 103–111, 141–149, 157–165, and 267–275; two peptides of EIF4EBP1 51–59 and 52–60; three peptides of ppMAPkkk 290–298, 294–302, and 432–440; and one 2-5 OAS3 peptide 666–674. Furthermore, CTL clones 4-2-A11, 4-2-B3, 0.5-1-H2, and 1-2-D1 recognized a WHSC2 peptide 103–111, an EIF4EBP1 51–59, a ppMAPkkk 432–440, and a 2-5 OAS3 666–674, respectively (Fig. 3A). A dose-dependent reaction was observed between the loaded amounts of peptides and the amounts of IFN-γ produced by these peptide-specific CTL clones (Fig. 3B). TCR Vβ usage of these peptide-specific CTL clones was determined by amplification of the TCR Vβ chain by the RT-PCR method. Two each of the CTL clones reacting to a UBE2V, an HNRPL, and a 2-5 OAS3 peptide used the different CDR3, respectively (Table 2). Similarly, each CTL clone reacting to a WHSC2, an EIF4EBP1, or a ppMAPkkk peptide used the different CDR3, respectively (Table 2). These amplified products were further provided for direct sequencing of the TCR β chain to address whether these CTL clones reacting to different tumor epitopes possess similar or different CDR3, an element responsible for binding to antigenic epitopes on the groove of HLA class I molecules (24). Two each of CTL clones recognizing a UBE2V, an HNRPL, or a 2-5 OAS3 peptide used the different CDR3, respectively (Table 2). Similarly, each CTL clone reacting to a WHSC2, an EIF4EBP1, or a ppMAPkkk peptide used the different CDR3, respectively.

**Immunogenic Epitopes Capable of Inducing CTLs.** Nineteen peptides recognized by the OK-CTLp were tested for their ability to induce HLA-A2-restricted and tumor-specific CTLs from the autologous PBMCs (OK) and the two HLA-A2001+ patients (one with pancreatic cancer and one with colon cancer). These PBMCs produced significant levels of IFN-γ in response to HLA-A2+ SW620, CA9–22, and Panc-1 but not to the HLA-A2− tumor cells when stimulated three times in vitro with the following 13 peptides: UBE2V 43–51, 85–93, and 208–216; HNRPL 140–148, 443–451, and 501–510; WHSC2 103–111, 141–149, and 267–275; EIF4EBP1 51–59 and 52–60; and ppMAPkkk 294–302 and 432–440. These CTL activities were inhibited by anti-HLA-class I, anti-CD8, or anti-HLA-A2 mAb but not by any of the other mAbs tested. Similar results were obtained for all three patients, and representative results from the autologous PBMCs are shown in Table 3. An HNRPL 404–412 or a WHSC2 157–165 peptide induced the CTLs reactive to only SW620 or Panc-1 tumor cells, respectively. A ppMAPkkk 290–298 peptide induced the CTL-reactive CA9–22 and Panc-1 but not SW620 (Table 3). In contrast, no CTL activity was induced by the UBE2V 64–73 and 201–209, and 2-5 OAS3 666–674 peptides. The levels of binding affinity for these 19 peptides, although different from each other, did not correlate well with the ability to induce CTLs (Table 3). The peptide-induced CTL activity was confirmed by a 6-h 51Cr-release assay, and representative results are shown in Fig. 4A. The PBMCs stimulated by each of UBE2V 43–51, 85–93, 208–216, HNRPL 140–148 and 501–510, and WHSC2 103–111 and 141–149 peptides showed significant levels of cytocytotoxicity against HLA-A2+ Panc-1 and SW620 but failed to lyse HLA-A2+ RERF-LC-MS, the autologous EBV-B cells, and the PHA-blastoid T cells (Fig. 4B). A total of 2041
of immunological ignorance, with these molecules in turn possibly being recognized by the T cells.

Among the 16 peptides with the ability to induce CTLs, 13 induced the HLA-A2-restricted CTLs reacting to all of the three tumor cell lines tested, with the remaining 3 inducing the CTLs reactive only to certain tumors. This difference might have in part been attributable to the number of epitopes on the HLA-A2 molecules of certain tumor cells. Alternatively, it could have been attributable to a lack of...
tumor-cell molecules involved in the processing and export of these particular epitopes (31). In contrast to the 16 peptides, the three peptides failed to induce CTLs. This difference might have in part been attributable to the different levels of CTL precursors reacting to peptides in circulation. Relatively large numbers of CTL precursors recognizing each of the 16 peptides with the ability to induce CTLs might be present in the circulation of cancer patients, whereas the number of precursors reacting to the latter three peptides might have been too small to be detected by the used assay for CTL induction. Alternatively, the difference might have been attributable to an immunological tolerance to certain peptides. The binding affinity of each peptide to HLA-A2 molecules might not significantly influence the ability to induce CTLs, because these binding affinities, although different from each other, did not well correlate with the ability to induce CTLs.

SW620 cells were more susceptible to lysis by the peptide-induced CTLs and also by the OK-CTLp as compared with the susceptibility of Panc-1. This might have in part been attributable to the higher expression levels of HLA-A2 molecules on SW620 tumor cells (data not shown). In addition, HLA-A2 molecules have been found to be undetectable on the cell surface of some (10–20%) Panc-1 cells (data not shown). HLA-A24+ EBV-B cells were susceptible to lysis by the CTLs induced by the peptides from EIF4EBP1, ppMAPkkk, and 2-5 OAS3 but not any of the other peptides tested. We reported previously that the HLA-A24-restricted CTLs induced by a cyclophilin B peptide, but not those by a SART3 peptide, were cytotoxic to autologous EBV-B cells (9, 10). The mechanisms of the different susceptibilities of EBV-B cells to these peptide-induced CTLs are presently unknown. In contrast to EBV-B cells, PHA-activated T cells were resistant to lysis by the peptide-induced CTLs in all of the cases tested. These results are also consistent with our previous observations on the peptide-induced, HLA-A24-restricted CTLs (9, 10). Several molecules in activated T cells, including a family of serpins, might be involved in their resistance to CTL-mediated lysis (32, 33).

We have reported in this study the six genes and 16 immunogenic epitopes capable of inducing HLA-A2-restricted and tumor-specific CTLs in PBMCs from pancreatic and/or colon cancer patients. Both Panc-1 and SW620 tumors were found to be susceptible to lysis by both the OK-CTLp and the peptide-induced CTLs. These results suggest that pancreatic and colon cancers share the same tumor epitopes recognized by the host CTLs. The incidence and number of cancer deaths from colon cancer are five to six times and two to three times higher than those of pancreatic cancers (34, 35), respectively. Although resection for cure is possible in 70–75% of all colon cancer patients, 50% still die from their disease regardless of the many different treatments, and thus, the development of new treatment...
Table 3  Induction of HLA-A2-restricted CTL activity by the peptides in PBMCs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MFI</th>
<th>CD4(%)</th>
<th>CD8(%)</th>
<th>QG56 (HLA-A26/26)</th>
<th>RERF-LC-MS (HLA-A11/11)</th>
<th>COLO320 (HLA-A24/24)</th>
<th>SW620 (HLA-A0201/24)</th>
<th>CA9-22 (HLA-A0207/24)</th>
<th>Panc-1 (HLA-A0201/11)</th>
<th>Panc-1 + anti-classI</th>
<th>Panc-1 + anti-classII</th>
<th>Panc-1 + anti-CD4</th>
<th>Panc-1 + anti-CD8</th>
<th>Panc-1 + anti-HLA-A2</th>
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<td>571</td>
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<td>81</td>
<td>492</td>
<td>59</td>
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<td>UB2E2 64-73</td>
<td>607</td>
<td>12.0</td>
<td>83.9</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>53</td>
<td>0</td>
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<td>UB2E2 85-93</td>
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<td>44</td>
<td>188</td>
<td>58</td>
<td>289</td>
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<tr>
<td>UB2E2 201-209</td>
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<td>81.2</td>
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<td>60</td>
<td>0</td>
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<td>UB2E2 208-216</td>
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<td>38</td>
<td>500</td>
<td>96</td>
<td>638</td>
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*Percentage of CD3+CD4+CD8+ or CD3+CD4+CD8-T cells of the peptide-stimulated PBMCs at the time of assay.

The PBMCs of a patient with colon adenocarcinoma, from which the OK-CTL clones were established, were stimulated in vitro with a peptide (10 μM) three times every 7 days, followed by a test for their ability to produce IFN-γ at day 21 of culture in response to various target cells at an E:T ratio of 5.

For inhibition assay, the OK-CTL-mediated IFN-γ production by recognition of Panc-1 tumor cells at an E:T ratio of 5 was tested in the presence of 20 μg/ml of mAbs shown in the Table.
Fig. 4. Induction of CTLs by peptides. A, PBMCs from the cancer patients were stimulated without peptides or with one of the 11 different peptides (10 μM) shown in the figure, and then there was a test of their cytotoxicity against HLA-A2<sup>+</sup> Panc-1 and SW620, HLA-A2<sup>+</sup> RERF-LC-MS, the autologous EBV-B cells, and PHA-blastoid T cells by a 6-h <sup>51</sup>Cr-release assay at three E:T ratios. Values are the means of triplicate assays. B, some of the peptide-induced CTLs used in A were tested by a 6-h <sup>51</sup>Cr-release assay for their cytotoxicity to T2 cells or the autologous PHA-blastoid T cells that were pulsed with various doses of a corresponding peptide used for stimulation.
modalities is needed. The HLA-A2 allele is found in 23% of African Blacks, 53% of Chinese, 40% of Japanese, (36) and 50% of Caucasians (37). The information presented in this study should provide a better understanding of the molecular basis of T-cell-mediated recognition of pancreatic cancer cells and also of colon cancer cells. Furthermore, the molecules identified in this study could be an appropriate target for use in specific immunotherapy for a large numbers of cancer patients.

REFERENCES


