An HLA-A2 Polyepitope Vaccine for Melanoma Immunotherapy

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An HLA-A2 Polyepitope Vaccine for Melanoma Immunotherapy

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Epitope-based vaccination strategies designed to induce tumor-specific CD8+ CTL are being widely considered for cancer immunotherapy. Here we describe a recombinant poxvirus vaccine that codes for ten HLA-A2-restricted epitopes derived from five melanoma Ags conjoined in an artificial polyepitope or polytope construct. Target cells infected with the melanoma polytope vaccinia were recognized by three different epitope-specific CTL lines derived from HLA-A2 melanoma patients, and CTL responses to seven of the epitopes were generated in at least one of six HLA-A2-transgenic mice immunized with the construct. CTL lines derived from vaccinated transgenic mice were also able to kill melanoma cells in vitro. Multiple epitopes within the polytope construct were therefore shown to be individually immunogenic, illustrating the feasibility of the polytope approach for melanoma immunotherapy. Tumor escape from CTL surveillance, through downregulation of individual tumor Ags and MHC alleles, might be overcome by polytope vaccines, which simultaneously target multiple cancer Ags. *The Journal of Immunology, 1999, 163: 4058–4063.

A common feature of malignant melanoma is the expression of multiple Ags, which are recognized by ab CD8+ CTL. Recent human therapeutic vaccine trials, which utilize the epitopes recognized by such CTL, have illustrated the potential for CTL epitope-based immunotherapeutic vaccine strategies (1, 2). Such strategies do not require surgical removal and culture of autologous tumor cells from the patient, and the use of autologous dendritic cells might also be avoided if effective, safe vaccine vectors can be developed (3). CTL epitope-based approaches offer a number of potential advantages over whole Ag-based cancer vaccines: 1) they can focus immunity toward optimal (4) and/or cryptic protective epitopes (5); 2) sequences that have oncogenic activity (6) or contain targets for autoimmune CD4+ cells (7) are omitted; and 3) sequences that are the target of preexisting CD4+ T cells or B cell responses are avoided. Such preexisting responses have the potential to deviate (8, 9) or inhibit (10, 11) effective CTL induction by a therapeutic vaccine.

Single epitope-based approaches have the disadvantage that an HLA-restricted CTL response can be raised to only one Ag. CTL responses specific for multiple Ags and restricted by multiple HLA alleles would clearly be desirable for cancer immunotherapy, given the variable expression of tumor Ags (12, 13) and MHC alleles (14) by melanomas and their metastases. Targeting multiple Ags and MHC alleles might be achieved by using multiple recombinant Ags or mixtures of synthetic peptide epitopes. The former loses the advantages of epitope-based approaches and would require complex recombinant vaccine Ag mixtures or constructs. The latter is complicated by adjuvant considerations and by problems associated with peptide solubility, chemical modifications of certain amino acids, and interpeptide interactions (15). Here we describe the construction and testing of a melanoma polyepitope or polytope poxvirus vaccine that contains ten conjoined minimal HLA-A2-restricted CTL epitopes, derived from five melanoma Ags, in a single recombinant construct. Despite the large number of epitopes restricted by the same allele, multiple epitopes within the vaccine construct were either recognized by epitope-specific CTL from melanoma patients and/or generated epitope-specific CTL in HLA-A2-transgenic mice. The polytope approach thus allows multiple Ags to be simultaneously targeted and should increase a patient’s spectrum of antitumor CTL responses.

Materials and Methods
Construction of the melanoma polytope recombinant vaccinia

A synthetic oligonucleotide fragment (see Fig. 1) was constructed from two 70-mer and four 67-mer synthetic oligonucleotides using Splicing by Overlap Extension and PCR (16). The nucleic acid sequence of the fragment coded for (from the 5' end) a cap, a BamHI restriction site, a Kozak sequence, a methionine start codon, 10 contiguous minimal melanoma CTL epitopes (see Table I), a stop codon, and a Kozak sequence, a methionine start codon, 10 contiguous minimal melanoma CTL epitopes (see Table I), a stop codon, and a SaI site and a cap at the 3' end. The amino acid sequences of the CTL epitopes were converted to DNA sequence using universal codon usage but were designed to avoid inclusion of unwanted restriction sites. Dimers were made of synthetic oligonucleotides 1 and 2 (reaction A), 3 and 4 (reaction B), and 5 and 6 (reaction C) (0.4 μg each) in 40-μl reactions containing standard 1 × Pfu PCR buffer, 0.5 mM dNTPs, and 1 U of cloned Pfu DNA polymerase (hot start at 94°C), using the thermal program 94°C for 10 s, 52°C for 20 s, and 72°C for 20 s for five cycles. At the end of 5 cycles, the PCR program was paused at 72°C, and 20-μl aliquots of the dimer reactions were subjected to a further 5 cycles (94°C for 10 s, 58°C for 20 s, and 72°C for 20 s). At cycle 10, the program was paused again; 20 μl of reaction C was added to 20 μl from the A + B mix; and a further 5 cycles was completed (94°C for 10 s, 52°C...
for 20 s, and 72°C for 20 s). Two 20-mer oligonucleotides (matching the first and last 20 bp of the sequence shown in Fig. 1) were used to PCR amplify the gel purified full-length product using the reaction mixed above at an annealing temperature of 52°C for 25 cycles. The full-length gel-purified PCR fragment was cloned into the EcoRV site of pBluescript II KS+.

A correct DNA insert was cloned behind the vaccinia P7.5 promoter in the plasmid shuttle vector pBCB06 using BamHI/SalI restriction enzymes. Construction of a TK recombinant virus was then conducted using marker rescue recombination as described previously (16, 17), generating the recombinant melanoma polytope (rVV.mel.pt) coding for 10 HLA-A2 melanoma epitopes (see Table I).

**Human CTL lines**

HLA-A2-positive patients, P5 and P11, had confirmed cutaneous malignant melanoma and were enrolled in a therapeutic vaccination trial at the Ludwig Institute Oncology Unit (18). CTL lines specific for AAGIGILTV and YLEPGPVTA were established from PBMC by sensitizing half the PBMC with peptide (Chiron Technologies, Clayton, Australia; or made in-house at Queensland Institute of Medical Research (QIMR)) (10 μg/ml, 2 h, 37°C followed by two washes) and adding back to the remaining cells in a 24-well plate. The cells were cultured in RPMI 1640 media supplemented with 10% FCS (QIMR), 2 mM glutamine (ICN Biomed. Aust. Pty., Seven Hills, Australia), 100 μg/ml streptomycin, and 100 μIU/ml penicillin (CSL, Melbourne, Australia), and 1 ml of medium containing 5 U/ml recombinant human IL-2 (kindly provided by Cetus, Emeryville, California) was added on day 3. On day 7, IL-2 and peptide were added to a final concentration of 25 U/ml and 1 μg/ml, respectively. Partial medium changes with 25 U/ml IL-2, but no peptide, were given as necessary. On day 14, the cultures were used as effectors in standard chromium release assays.

The LLDGTATLRL-specific line was generated by restimulation of PBMC (derived from leukapheresis) from patient A02, with the autologous irradiated (8000 rad) A02-Mb melanoma cells (two times, 7 days apart), followed by two restimations (7 days apart) with peptide-sensitized (10 μg/ml, 37°C, 1 h), washed, irradiated (8000 rad) HLA-A2 lymphoblastoid cell lines (LCLs) (responder to stimulator ratio throughout, 20:1). IL-2 (250U/ml) was added on day 7, and the effectors were used on day 35.

**Human target cells for murine and human CTL**

An EBV (B95.8)-transformed LCL from a homozygous HLA-A2 healthy individual (HLA-A2* LCL) was 1 infected with rVV.mel.pt or a control vaccinia recombinant vaccinia expressing an unrelated polytope construct (rVV.Cont.) (16) (multiplicity of infection 10:1) overnight, before 3H thymidine labeling, or 2) sensitized with peptide (10 μg/ml) at the same time as 3H thymidine labeling. The following cell lines were also used in standard 6-h 51 Cr release assays against 1) human cell lines (see above) and 2) LCLs sensitized with each peptide, re-

**Vaccination and CTL assays using HHD transgenic mice.**

HHD mice have a transgene comprising the α1 and α2 domains of HLA-A2 linked to the α3 transmembrane and cytoplasmic domains of H-2D^d. This transgene was introduced into murine β2-microglobulin and H-2D^d double knockout mice; thus, the only MHC expressed by the HHD mouse was the modified HLA-A2 molecule (19).

HHD mice were vaccinated i.p. with 1 × 10^7 PFU recombinant vaccinia virus coding for the melanoma polytope (rVV.mel.pt) or a control polytope vaccinia virus coding for a series of EBV epitopes (17). Naïve control mice animals were not vaccinated. After 3 wk, splenocytes were harvested, and 5 × 10^6 cells were restimulated in 24-well plates with 1 × 10^6 LPS blasts

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### Table I. HLA A2-restricted melanoma epitopes included in the melanoma polytope construct

<table>
<thead>
<tr>
<th>Melanoma Ag</th>
<th>Peptide Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mart-1 (27-35)</td>
<td>AAGIGILTV</td>
<td>46</td>
</tr>
<tr>
<td>MAGE-3 (271-279)</td>
<td>FLWGPRALV</td>
<td>47</td>
</tr>
<tr>
<td>Tyrosinase (1-9)</td>
<td>MLLAVLCL</td>
<td>48</td>
</tr>
<tr>
<td>gp100 (457-466)</td>
<td>LLDGATATLRL</td>
<td>49</td>
</tr>
<tr>
<td>gp100 (154-162)</td>
<td>KTWGGWQV</td>
<td>25</td>
</tr>
<tr>
<td>Tyrosinase (368-376)</td>
<td>YMVWV/2/5TMGSQV</td>
<td>48</td>
</tr>
<tr>
<td>gp100 (209-217)</td>
<td>ITDQVPFSV</td>
<td>50</td>
</tr>
<tr>
<td>gp100 (280-288)</td>
<td>YLEPPGTV</td>
<td>50</td>
</tr>
<tr>
<td>Mart-1 (34-40)</td>
<td>ILTVILGVL</td>
<td>51</td>
</tr>
<tr>
<td>N-acetylglucosaminyl-transferase</td>
<td>VLPDFVIRC</td>
<td>52</td>
</tr>
</tbody>
</table>

*V gene intron*  

**Results**

A recombinant melanoma polytope vaccinia virus (rVV.mel.pt) was constructed that coded for ten conjoined HLA-A2 melanoma epitopes (Table I). The artificial recombinant insert (Fig. 1) was generated by using synthetic oligonucleotides and PCR. The DNA and protein sequence of the melanoma polytope construct is shown in Fig. 1.

**Melanoma-specific CTL lines recognized the melanoma polytope construct**

Three epitope-specific CTL lines from three melanoma patients (P5, P11, and A02) were generated and were shown to be specific for AAGIGILTV, YLEPGPVTA, and LLDGTATLRL by their ability to lyse HLA-A2* LCLs sensitized with each peptide, respectively (Fig. 2, HLA-A2* LCL+/– peptide). The melanoma specificity of the CTL lines from donors P5 and P11 was illustrated by their ability to recognize HLA-A2* melanoma lines (LAR1 and HTB64), but not HLA-A2-negative melanoma lines (ME235 and A12-M) or, in the case of P11, a fibroblast line (HTB-102) derived from the same individual as the HTB-64 melanoma line (Fig. 2, melanoma lines). (Although donor A02 had CTL reactivity against LLDGTATLRL, the melanoma line A02-Mb derived from the bowel metastasis of this patient did not appear to present gp100; data not shown).

Each of the epitope-specific CTL lines was capable of recognizing LCLs infected with rVV.mel.pt (Fig. 2; HLA-A2* LCL+/– rVV.mel.pt), but not a control rVV (Fig. 2; rVV.Cont.), illustrating that each of these three epitopes was individually processed from the melanoma polytope construct and presented to melanoma-specific CTL.
Mice vaccinated with the melanoma polytope generated CTL specific for multiple epitopes

To determine whether the polytope construct was capable of raising CTL responses in vivo, HHD-transgenic mice were vaccinated with the rVV.mel.pt. CTL responses were generated to AAAGIGILTV, LLDGTATLRL, KTWGQYWQV, YMDGTMSQV, ITDQVPFSV, YLEPGPVTA, and VLPDVFIRCV (Fig. 3). Not all the mice tested generated response to all the epitopes; five of the 6 (5/6) mice vaccinated with the rVV.mel.pt generated responses to AAAGIGILTV, 2/6 mice tested generated responses specific for LLDGTATLRL, 1/7 for KTWGQYWQV, 3/6 for YMDGTMSQV, 2/6 for ITDQVPFSV, 2/6 for YLEPGPVTA, and 6/7 for VLPDVFIRCV. Fig. 3 shows the average lysis of CTL effectors generated from responder mice, which were defined as mice with effector populations giving peptide-specific lysis of more than 10%. None of the mice tested generated CTL specific for FLWGPRALV, MLLAVLYCL, and ILTVILGLV following rVV.mel.pt immunization (Fig. 3). The total number of mice tested for these epitopes was 13, and Fig. 3 illustrates the mean lysis values for all these effector populations (n = 13 for each). Immunization of HHD mice with FLWGPRALV, MLLAVLYCL, and ILTVILGLV peptide-based vaccines also failed to induce CTL responses
FIGURE 4. Bulk effectors from rVV.mel.pt-immunized mice were restimulated in vitro and used against (first column) EL4S3 RobHHD cells sensitized with peptide (●) or the same cells without peptide (□); (second column) HLA-A2+ LCLs sensitized with the indicated peptide (●) or the same LCL without peptide (□); and (third column) melanoma cell lines expressing HLA-A2 (A02-Mb and A09-M; ●) and control lines, which are HLA-A2 negative (HTB73 and A12-M; □) or are HLA-A2+ but do not present the target Ag (HTB64; △).

Discussion

This paper illustrates the feasibility of delivering multiple HLA-A2-restricted melanoma CTL epitopes using the polytope vaccination strategy. The melanoma polytope vaccine induced epitope-specific CTL of multiple specificities in HLA-A2-transgenic mice and was recognized by CTL lines from HLA-A2 melanoma patients, arguing that multiple epitopes from the melanoma polytope vaccine can be simultaneously processed and presented. Polytope vaccine-induced CTL were also able specifically to kill human melanoma cells, suggesting that polytope vaccination can induce CTL of sufficient affinity to kill physiologically relevant target cells. This is likely to be a critical feature for cancer vaccines, given the down-regulation of HLA by melanoma cells (14) and the potential for induction of low affinity CTL by peptide vaccination (22, 23).

The HHD mouse system represents a useful model for preclinical and quality control testing of vaccines designed to induce HLA-A2-restricted CTL responses in humans. However, as reported previously, HLA-A2-transgenic mice appear unable to respond to some known HLA-A2-restricted epitopes (24). In this study, HHD mice failed to respond to FLWGPRALV, MLLAVLYCL, and ILTVILGVL following rVV.mel.pt immunization. In addition, variable induction of CTL specific for some epitopes was also observed in individual transgenic mice (Ref. 24, Fig. 3 legend). These deficiencies may reflect 1) a limited and variable TCR repertoire in HLA-A2-transgenic mice (discussed below) and/or 2) the poor immunogenicity of individual epitopes. MLAVLYCL and ILTVILGVL bind poorly to HLA-A2.1, and polytope vaccination may provide insufficient amounts of these epitopes to promote efficient priming. The HLA-A2.1 binding and immunogenicity of ITDQVPFSV, KTWGQYWQV, and YLEPGPVTA peptides have been improved by changing the anchor residues to IMDQVPFSV, KLWGQYWQV, and YLEPGPVTV (2, 25, 26). A polytope vaccine’s ability to prime responses to poorly immunogenic epitopes might be improved if such epitopes were replaced with anchor-modified epitopes, which have higher HLA-A2-binding affinities.

As noted previously (24), a contributing factor to 1) the inability of HLA-A2-transgenic mice to respond to some HLA-A2 epitopes, and 2) the variable responses seen with other epitopes may be a limited and variable TCR repertoire educated on the HLA-A2 transgene in these animals. Murine TAP proteins appear to be more selective than their human equivalents (27), and other murine proteins involved in processing and presentation may also be inefficient at delivering some peptides for HLA binding (28, 29). Additionally, variable induction of CTL specific for some epitopes was also observed in individual transgenic mice (Ref. 24; Fig. 3 legend). These deficiencies may reflect 1) a limited and variable TCR repertoire in HLA-A2-transgenic mice, which is likely to limit the diversity of the HLA-A2-restricted CTL repertoire in the periphery of these animals (30). The intermouse variation in responses to some peptides may reflect a heterogeneous TCR repertoire, which could arise from minor histocompatibility differences between individual HHD mice (19). Negative selection or deletion of CTL (as opposed to TCR+) may be a restricted and variable TCR repertoire. A reduced quantity and/or diversity of self epitopes loaded onto the A2K2 or HHD transgene in the thymus will limit positive selection of HLA-A2-restricted CTL, which is likely to limit the diversity of the HLA-A2-restricted TCR repertoire in the periphery of these animals (30). The intermouse variation in responses to some epitopes may reflect a heterogeneous TCR repertoire, which could arise from minor histocompatibility differences between individual HHD mice (19).
to lack of positive selection) by murine equivalents of the melanoma epitopes is unlikely to be responsible for the inability of HHD mice to respond to certain epitopes. The sequence of the murine equivalent of FLWGRALV is FLWGPRALVA and of MLLAVLYCL is MFLAVLTYCL; thus, both murine homologues have changes in the anchor residues (underlined), which should prevent efficient binding to HLA-A2 (24). The ITDQVPFSV epitope, to which a response was generated, is equivalent in the mouse and the human gp100 melanocyte protein. However, autoimmunity against melanocytes could not be readily detected. If the patients’ tumor-specific CD4 T cell responses are deleted (like rVV.mel.pt) would induce CD4 responses specific for viral protein are processed and presented to CD8+ cytotoxic T cells: implications for vaccine design. Proc. Natl. Acad. Sci. USA 92:5845.


References


