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*J Immunol* 1999; 163:4058-4063; 
http://www.jimmunol.org/content/163/7/4058

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An HLA-A2 Polyepitope Vaccine for Melanoma Immunotherapy

Luis Mateo,* Joy Gardner,* Qiyuan Chen,† Christopher Schmidt,* Michelle Down,* Suzanne L. Elliott,* Stephanie J. Pye,* Hüseyin Firat,‡ Francois A. Lemonnier,‡ Jonathon Cebon,‡ and Andreas Suhrbier2*

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 down regulation 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 αβ 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 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 7-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 Kozac 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 of 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 A and B were mixed (reaction C was left in the PCR machine) and 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), which were sensitized with peptide (10 μg/ml) at the same time as 105 Mβ-mercaptoethanol (Sigma, St. Louis, MO), 100 μg/ml streptomycin, and 100 U/ml penicillin (CSL, Melbourne, Australia), and 1 ml of medium containing 5 U/ml recombinant human IL-2 (kindly provided by Cetus, Emeryville, California). This cell line 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 restimulations (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 0Cr labeling, or 2) sensitized with peptide (10 μg/ml) at the same time as 1Cr labeling. The following cell lines were also used in standard 6 h 1Cr release assays: EL4S3-ITDQVPFSV, ATCC HTB-73 (HLA-A2-negative melanoma); ATCC HTB-64 (HLA-A2-positive melanoma) and HTB-102, a skin fibroblast line ([HHL].mel.2) from the same patient; A02-Mb and A09-M, melanoma lines from HLA-A2-negative patients enrolled in a therapeutic clinical trial of GM-CSF; and YLEPGPVTA, a D (generated by deamidation) in this position (53). The polytope codes for D in this position.

### Vaccination and CTL assays using HHD transgenic mice

Transgenic HHD mice have a transgene comprising the α1 and α2 domains of HLA-A2 linked to the α3, transmembrane, and cytoplasmic domains of H-2Dd with the β1 domain linked to human β2-microglobulin. This transgene was introduced into murine β2-microglobulin and H-2Dd double knockout mice; thus, the only MHC expressed by the HHD mouse was the HLA-A2 linked to the human β2-microglobulin and H-2Dd double knockout background; LCL lymphoblastoid cell line: EL4S3 RobHHD, murine β2-microglobulin-deficient EL4 cells transfected with the HHD transgene.

### 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+ LCLs sensitized with each peptide were used to stimulate the CTL 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 same individual 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+ LCLs 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.

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

<table>
<thead>
<tr>
<th>Melanoma Ag</th>
<th>Peptide Sequence</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Mart-1 (27-35)</td>
<td>AAGIGILTV</td>
<td>46</td>
</tr>
<tr>
<td>MAGE-3 (271-279)</td>
<td>FLWGPRLAV</td>
<td>47</td>
</tr>
<tr>
<td>Tyrosinase (1-9)</td>
<td>MLLAVLYCL</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>KTWGQYQV</td>
<td>25</td>
</tr>
<tr>
<td>Tyrosinase (368-376)</td>
<td>VN(V/D)GTMGSQV</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>YLEPGPVTA</td>
<td>50</td>
</tr>
<tr>
<td>Mart-1 (34-40)</td>
<td>ILTVLIGVL</td>
<td>51</td>
</tr>
<tr>
<td>N-acetylgalactosaminyl-transferase</td>
<td>VLPDVIFRCV</td>
<td>52</td>
</tr>
</tbody>
</table>

V gene intron

Position 370 is an N in the native protein but the epitope recognized by CTL has a D (generated by deamidation) in this position (53). The polytope codes for D in this position.
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 AA GIGILTV, 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 AAGIGILTV, 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 FLWGPRLAV, MLLAVLYCL, and ILTVILGVL 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 FLWGPRLAV, MLLAVLYCL, and ILTVILGVL peptide-based vaccines also failed to induce CTL responses.
FIGURE 4. Bulk effectors from rVV.med.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; △).

These data illustrated that the melanoma polytope vaccine had induced CTL capable of recognizing melanoma Ags processed and presented by human melanoma cells.

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 FLWGPRLAV, MLVVLYCL, and ILTVILGWVL following rVV.med.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. MLVVLYCL and ILTVILGWVL 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 YLEPGVPVTa peptides have been improved by changing the anchor residues to IMDQVPFSV, KLWGQYWQV, and YLEPGVPVTa (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).

Although these factors may result in the inefficient processing and presentation of some vaccine Ags, the inability of all HLA-A2 peptide epitope immunogens to induce efficiently CTL responses in all HLA-A2-transgenic mice (24) suggests that the main problem may be a restricted and variable TCR repertoire. A reduced quantity and/or diversity of self epitopes loaded onto the A2/Kd 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). Negative selection or deletion of CTL (as opposed
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 FLWGPRAV is FLWGPRAHA and of MLLAVLYCL is MFLAVLYCL; 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 vicinity of the SJL-mouse’s mutated tyrosinase gene, so all HHD mice are albinos (19).

A potentially important question for future polytope cancer vaccines is the source of CD4 T cell help. Should the vaccine induce CD4 helper responses specific for tumor Ags (33) or might vaccine induced CD4 help best be obtained from unrelated Ags (1, 34)? CD4 help is often required for optimal CTL induction but is also likely to be required for the maintenance of ongoing antitumor CTL responses (35). A virus-vectored melanoma polytope vaccine (like rVV.mel.pt) would induce CD4 responses specific for viral Ags and would not induce, or rely on, melanoma-specific CD4 responses. This may actually be advantageous in a clinical setting if the patients’ tumor-specific CD4 T cell responses are deleted (36), anergized (37), or Th2 deviated (8, 9) by the tumor. In contrast, vaccine-induced melanoma-specific CD4 responses may synergize with vaccine-induced CTL, resulting in improved antitumor responses (33). Apoptotic tumor cells killed by vaccine-induced CTL are also likely to induce tumor-specific CD4 responses (38), which may also influence vaccine-induced antitumor CTL.

As more melanoma Ags and target epitopes are identified, a panel of polytope vaccines might be envisaged, with each vaccine containing multiple epitopes restricted by one HLA allele. An appropriate HLA-matched mixture might be then delivered to cover all the HLA alleles expressed by any individual patient. Downregulation of some or all HLA alleles by the melanoma cells should increase their susceptibility to NK/LAK lysis (39). A variety of delivery modalities might be used for human melanoma polytope vaccines; these include attenuated poxvirus vectors (40), adenovirus (41), naked DNA (42), or transfected dendritic cells (43). CTL induction might also be enhanced by codelivery of cytokines (44, 45) and/or prime boost strategies (40).

Acknowledgments

We thank Drs. Thomson (Australian National University, Canberra) and B. Coupar (CSIRO, AAHL, Geelong, Australia) for their help in the construction of the melanoma polytope vaccinia.

References


