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Improved Immunogenicity of an Immunodominant Epitope of the Her-2/neu Protooncogene by Alterations of MHC Contact Residues

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The HER-2/neu (HER-2) oncogene is expressed in normal epithelial surfaces at low levels and overexpressed in several types of tumors. The low immunogenicity against this self tumor Ag can be improved by developing epitopes with amino acid replacements in their sequences. In this study, three HER-2/neu.369 (HER-2.369) analogue peptides, produced by modifying both anchor positions by introducing L, V, or T at position 2 and V at the C terminus, were analyzed for their capacity to induce CTLs in vitro from human PBMC and in vivo in HLA-A2.1/Kb transgenic mice. One of the analogues (HER-2.369 V2V9) sensitized target cells for HER-2-specific recognition by human CTLs and induced specific CTLs in vitro at 100-fold lower concentrations than the HER-2.369 wild-type epitope. These CTLs were also able to recognize the wild-type epitope and HER-2-expressing tumors in an MHC-restricted manner. Furthermore, a 100-fold lower amount of the HER-2.369 V2V9 analogue compared with the wild-type epitope was required to induce CTLs in HLA-A2.1/Kb transgenic mice. However, the V2V9 analogue demonstrated only marginally better binding to the MHC class I A2 allele compared with wild type. To establish thermodynamic parameters, we developed radiolabeled F3*Y analogues from both the HER-2.369 epitope and the V2V9 analogue. Our results indicate that the high biological activity of the HER-2.369 V2V9 epitope is associated with a slower dissociation kinetic profile, resulting in an epitope with greater HLA-A2 stability. The Journal of Immunology, 2004, 172: 3501–3508.

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3Abbreviations used in this paper: TAA, tumor-associated Ag; PSA, prostate-specific Ag; SM, standard medium.

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the immunodominant HER-2.369–377 epitope (17). We found that one of the analogues (HER-2.369 V2V9) can induce wild-type specific CTLs in vitro in HLA-A2-positive human donors and in vivo in HLA A2.1/Kb transgenic mice (HLA-A2.1/Kb tg) at mark-
edly lower concentrations than the HER-2.369 wild-type type. This analogue bound HLA-A2 only marginally better than the wild-type peptide, but resulted in HLA complexes associated with higher stability. We speculate that the higher biological activity of the HER-2.369 V2V9 epitope could result from a higher HLA-A2 stability and/or subtle conformation alterations of the peptide/HLA complex.

Materials and Methods

Cell lines

The TAP-defective HLA-A2.1 T2 cell line derived from the human T cell leukemia/b cell LCL Hybrid 174 was a gift from Dr. P. Cresswell (Yale University School of Medicine, New Haven, CT). The 221A2.1 cell line was produced by transfecting the HLA-A2.1 gene into the HLA-A,-B,-C-null mutant human B lymphoblastoid cell line 221. The colon adenocarcinoma cell lines SW403 and HT-29 were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 medium supplemented with antibiotics, sodium pyruvate, nonessential amino acids, and 10% (v/v) heat-inactivated FCS. Jurkat A*0201/Kb, a human T cell leukemia/HLA-A2.1-negative cell line stably transfected with an HLA-A*0201/Kb chimeric gene, was provided by W. M. Kast (Loyola University, Maywood, IL). Cells were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (complete medium).

Peptides and HLA-A2 binding assays

Peptides were synthesized by a solid phase method using a multiple peptide synthesizer and were analyzed by reverse phase HPLC as previously described (18, 19).

HLA-A2.1 molecules were purified as previously described (20). Briefly, cell lysates from JY cells were first depleted of HLA-B and -C molecules by repeated passage over a B1.23.2 (anti-HLA-B and -C) col-
uma. Remaining HLA-A molecules were captured on a W6/32 (anti-
HLA-A, B, C) column. The protein content and purity of class I prepara-
tions were monitored by SDS-PAGE analysis.

Quantitative assays to measure the binding of peptides to soluble class I molecules are based on the inhibition of binding of a radiolabeled stan-
dard HLA-A2.1. These assays were performed as previously described (21). Briefly, 1–10 nM radiolabeled peptide was coincubated at room temperature with 1 μM to 1 nM purified MHC in the presence of 1 μM human β2-microglobulin (The Scripps Laboratories, San Diego, CA) and a mix-
ture of protease inhibitors. After a 2-day incubation, the percentage of
MHC bound radioactivity was determined by size exclusion gel filtration chromatography using a TSK 2000 column. Alternatively, the percentage of
MHC-bound radioactivity was determined by capturing HLA/peptide complexes on W6/32 Ab-coated Optiplates (Packard Instrument, Meriden, CT), and determining bound counts per minute using the TopCount (Pack-
ard Instrument) microscintillation counter.

The radiolabeled standard peptide used for HLA-A2.1 assays was an F371–Y analogue of the HBV core 18–27 epitope (sequence FLPSD'TY-
PSV). The average IC50 of this peptide for HLA-A2.1 was 5.0 nM. In the case of competitive assays, the concentration of peptide yielding 50% in-
hibition of the binding of the radiolabeled peptide was calculated. Peptides were initially tested at one or two high doses. The IC50 of peptides yielding positive inhibition were then determined in subsequent experiments, in which IC50 values were then determined in duplicate at six different concentra-
tions used. Under the conditions used, where [label] < [MHC] and IC50 ≫ [MHC], the measured IC50 values are reasonable approximations of the true Kd values. Each competitor peptide was tested in two to four independent experiments. As a positive control, the unlabeled version of the radiolabeled probe was also tested in each experiment.

To allow the performance of kinetic of association (Kd) experiments using radiolabeled peptides, analogues representing F371 to Y substitutions of Her2/neu peptides were designed, and peptide-MHC assays were set-up as described above, and then incubated for various times ranging between 0 and 60 min at either room temperature or 37°C. Further association between HLA-A2.1 and the radiolabeled ligand was stopped by dilution, and the fraction of peptide bound was determined as described above by size exclusion chromatography. The initial linear part of the binding iso-
therm was used to estimate Kd values. To determine the kinetics of disso-
ciation, large volumes of HLA-A2.1-peptide complexes were generated using the basic assay protocol described above, and using excess amounts of radiolabeled peptide and purified MHC. After a 2-day incubation, complexes were isolated using size exclusion chromatography and then incu-
Bated at 37°C for various times in the presence of a 1000-fold excess of unlabeled peptide (to prevent reassociation of radiolabeled peptide). The percentage of radiolabeled peptide bound at various time points was de-
termined using size exclusion chromatography. Kd values were calculated as the slope of the regression line of ln (% bound) vs time.

HLA typing

mAb recognizing HLA-A2 was derived from culture supernatants of American Type Culture Collection MA2.1 hybridomas. PBLS from healthy donors were incubated with either primary mAb or murine IgG of appropri-
tie class as a control for 30 min at 4°C and then stained with secondary FITC-conjugated rabbit anti-mouse Ig (DAKO, Glostrup, Denmark). Data acquisition was performed on FACSscan flow cytometer (BD Biosciences, Mountain View, CA). For data shown in Fig. 3, donors were class I typed serologically (One Lambda, Canoga Park, CA), and HLA-A2-positive indi-
viduals were A2.1 subtyped by standard PCR methods.

Peptide- and tumor-specific CTL lines

For data shown in Fig. 2, PBMCs were isolated by centrifugation of venous blood from HLA-A2.1-positive healthy donors on Ficoll-Paque (Amer-
phramacia Biotech, Uppsala, Sweden). PBMC were resuspended in X-VIVO (BioWhittaker, Walkersville, MD) medium supplemented with 1% autologous plasma (hereafter termed standard medium (SM)). Twenty million cells were incubated in six-well plates for 2 h at 37°C (20 × 106 cells/well), and the nonadherent cells were removed by gentle pipet-
ing. The nonadherent cells (responder cells) were cryopreserved in FCS supplemented by 10% DMSO (Sigma-Aldrich, St. Louis, MO).

The adherent cells (stimulator cells) were cultured in SM containing 800 U/ml GM-CSF (Schering-Plough, Stockholm, Sweden) and 500 U/ml IL-4 (Schering-Plough) added days 2 and 5 after the initiation of the cultures. TNF-α (Cetus, Emeryville, CA; 50 ng/ml) was added during the last 24 h of culture. Stimulator cells were harvested and resuspended in SM con-
taining 20 μg/ml peptide and 3 μg/ml β2-microglobulin (Sigma-Aldrich). After culturing for 4 h at 37°C, stimulator cells were irradiated with 25 Gy, cultured in SM containing 2000 U/ml IL-7 (R&D Systems, Abingdon, UK) and 0.01 U/ml of IL12 (R&D Systems), and mixed with the thawed nonadherent responder cells at a responder-to-stimulator ratio of 10:1. After 7 days of in vitro culture, IL-7 (R&D Systems) was added to a final concen-
tration of 2000 U/ml. Cultures were restimulated four times at weekly intervals with autologous adherent PBMC irradiated with 60 Gy and cul-
tured for 2 h in SM containing 20 μg/ml peptide and 3 μg/ml β2-
microglobulin (Sigma-Aldrich). IL-2 (Chiron, Emeryville, CA) at a concentra-
tion of 20 U/ml was added at regular intervals 2 days after each restimulation.

In Fig. 3, tumor-specific CTL lines from HLA-A2-positive healthy do-
nors were generated with some variations on the protocol given above as described previously (22).

Human IFN-γ in situ capture ELISA

Ninety-six-well ELISA plates (Costar, Corning, NY) were coated overnight at 4°C with 50 μl/well mouse mAb specific for human IFN-γ (clone NIB42; BD Pharmingen, San Diego, CA) at a concentration of 4 μg/ml in coating buffer (100 mM NaHCO3, pH 8.2). Plates were then washed and blocked for 2 h, after which the CTLs (100 μl/well) and targets (100 μl /well) were added to each well, leaving empty wells for the standards (re-
combinant human IFN-γ; BD Pharmingen) and blanks (medium only). The plates were incubated for 48 h at 37°C with 5% CO2.

The cells were removed by washing the plates with PBS/0.05% Tween 20, and the amount of IFN-γ that was secreted and captured by the bound clone NIB42 mAb was measured using a sandwich format ELISA. A bi-
otinylated mouse mAb specific for human (clone 4S.B3; BD Pharmingen) was used to detect the secreted IFN-γ. HRP-coupled streptavidin (Zymed Laboratories, South San Francisco, CA), 3.3',5.5'-tetramethylbenzidine, and H2O2 (ImmunoPure TMB Substrate Kit; Pierce, Rockford, IL) were used according to the manufacturer’s directions for color development. The absorbance was read at 450 nm on a Multiskan RC ELISA plate reader (Labsystems, Helsinki, Finland). A culture was considered positive if it measured at least 50 pg of IFN-γ/well above background and was twice the background level of expression.
**Human IFN-γ ELISPOT assay**

Ninety-six-well filtration plates (MAIPS45 10; Millipore, Bedford, MA) were coated overnight at 4°C with 75 µLwell of 2 µg/mL anti-human IFN-γ mAb. Wells were then washed six times with PBS and blocked with 100 µLwell RPMI 1640/1% human serum albumin for 2 h at 37°C. Stimulator cells were washed once, resuspended in RPMI 1640 at 25 x 10⁶ cells/mL, plated at 80 µLwell, then mixed with 20 µL of 0.5 µg/mL peptide solution. CTLs were added at the concentration of 2 x 10⁶ cells/well in 100 µL. After 4 h of incubation at 37°C, the plates were washed six times with PBS/0.05% Tween 20. Wells were incubated overnight at 4°C with 75 µL/well mouse biotinylated anti-human IFN-γ mAb (7-B6-1-biotin; Mabtech, Nacka, Sweden) at a concentration of 0.75 µg/mL in PBS, 1% BSA, and 0.02% NaN₃. After washing six times with PBS/0.05% Tween 20, 75 µL of streptavidin-alkaline phosphatase conjugate solution (Mabtech, Nacka, Sweden) diluted 1/1000 in PBS (1% BSA/0.02% NaN₃) was added for 2 h at room temperature in the dark. After another washing step with PBS, 75 µL/well substrate solution was added to each well for 5–10 min. Color development was stopped by washing under running tap water. After drying at room temperature, IFN-γ-secreting T cells were counted using the automated image analysis system ELISPOT reader.

**Analysis of in vivo immunogenicity in HLA-A2.1/Kb transgenic mice**

HLA-A2.1/Kb mice (23) were coimmunized with varying doses of the HER-2 wild-type or analogue peptide and 50 µg/mouse of the PADRE Th epitope prepared in IFA. The dose of immunizing peptide ranged from 100 µg/mouse to 10 ng/mouse, in log intervals. As a control, mice were injected with an IFA emulsion without peptide. Eleven days after immunization, splenocytes from injected animals were cultured with 1 x 10⁶ cells/well in 100 µL of RPMI 1640/1% human serum albumin for 2 ha t room temperature in the dark. After another washing step, PBS/0.05% substrate solution was added to each well for 5–10 min. Color development was stopped by washing under running tap water. After drying at room temperature, IFN-γ-secreting T cells were counted using the automated image analysis system ELISPOT reader. The assays were performed according to standard protocols (24, 25).

**Results**

Fixed anchor analogues derived from the HER-2.369 epitope can sensitize target cells at low peptide concentrations for recognition by HER-2.369-specific CTLs

Three HER-2.369 analogue peptides, produced by modifying both main anchor positions by introducing L, V, or T at position 2 and V at the C terminus, were previously demonstrated to have improved HLA-A2.1 binding and/or supertype cross-reactive binding compared with the wild-type epitope (22). We first analyzed to what extent these HER-2/neu.369 (HER-2.369) variants would be able to sensitize HLA-A2-expressing T2 cells for recognition by HER-2.369 wild-type-specific CTLs, as measured in ELISPOT IFN-γ production assays. Both V2V9 (KVFGSLAFV) and L2V9 (KIFGSLAFV) analogues more efficiently sensitized T2 cells for HER-2.369-specific CTL lysis compared with the T2V9 (KIFGSLAFV) and wild-type (KIFGSLAFV) epitopes (Fig. 1). In the case of the L2V9 analogue, this result could be explained by the superior HLA-A2.1 binding compared with the T2V9 analogue or the wild-type 369 epitope. The V2V9 analogue, however, bound only marginally better to HLA-A2.1 compared with the wild-type HER-2.369 epitope (23 vs 37 nM), but nevertheless was associated with increased ability to induce HER-2.369-specific CTL recognition.

**The HER-2.369 V2V9 analogue can induce specific CTLs in vitro at lower concentrations than the wild-type epitope**

We next determined the capacity of the HER-2.369 peptide variants V2V9 and L2V9 to induce peptide-specific CTLs. A range of concentrations was used for each peptide to determine the minimal concentration required to elicit specific CD8⁺ T cell responses from HLA-A2-positive healthy donors stimulated with autologous DC cells pulsed with various concentrations of wild-type (Fig. 2A), V2V9 analogue (Fig. 2B), or L2V9 analogue (Fig. 2C). Seven days after the third stimulation, the CTL cultures were tested in IFN-γ release ELISPOT assays against T2 cells pulsed with 10 µg of wild-type peptide, V2V9 analogue, L2V9 analogue, or irrelevant peptide (Pol476–484, peptide from the reverse transcriptase of HIV-1). The wild-type HER-2 epitope was found to generate specific CTLs, but only at the concentration of 1 µg/mL, whereas no specific CTL responses were detected at the lower concentrations (10 or 1 ng/mL). In contrast, the variant peptide V2V9 was able to efficiently elicit CTL responses to the wild-type 369 epitope when cultures were stimulated with 1 µg/mL and 10 ng/mL peptide. Of particular interest, these CTLs raised by the V2V9 analogue were able to recognize not only the V2V9 analogue, but also the 369 wild-type epitope as well as the L2V9 analogue. The L2V9 variant peptide, which had the highest HLA-A2.1 binding affinity, did not generate CTL responses at any of the tested concentrations. Similar results were obtained with PBL from two other HLA-A2-positive donors (data not shown).

**CTLs induced by the HER-2.369 V2V9 analogue can recognize HER-2-expressing, HLA-A2.1-positive tumor cells**

The HER-2.369 epitope was previously demonstrated to be naturally processed and expressed on HER-2-expressing, HLA-A2-positive tumor targets (17, 26). The experiments described above showed that the CTLs raised against the V2V9 peptide were also able to recognize low concentrations of the wild-type HER-2 peptide, indicating that these CTLs may also recognize HER-2-expressing tumor targets. To test this hypothesis, microcultures of CTLs were induced by either the HER-2.369 V2V9 peptide or the wild-type control peptide (data not shown) from two healthy HLA-A2-positive donors. Nineteen of 26 V2V9-specific cultures from both donors were able to recognize the wild-type 369 epitope, as measured in IFN-γ release assays (data not shown). Of importance, five of the CTL cultures specific for the wild-type 369 epitope were able to recognize the SW403 HER-2-expressing, HLA-A2.1-positive tumor targets, but not HER-2-expressing, HLA-A2.1-negative HT29 tumor targets (Fig. 3). We therefore conclude that CTLs raised by the V2V9 peptides are capable of recognizing not only the wild-type HER-2.369 epitope, but also HER-2-expressing tumor targets.
IFN-γ/H9253 peptides were used at the indicated concentrations to stimulate PBMC from healthy HLA-A2-positive donors three times at weekly intervals according to the CTL induction protocol in Materials and Methods. Cultures were tested in IFN-γ ELISPOT assays using T2 cells prepulsed with the WT, V2V9, L2V9, or HIV control peptides. As mentioned above, the HER-2.369 V2V9 analogue possessed a markedly higher immunogenicity in vitro in HLA-A2.1/Kb transgenic mice (Fig. 4). Specifically, the HER-2.369 V2V9 analogue was able to induce a specific CTL response in HLA-A2.1/Kb mice immunized with 10 ng of peptide or more. In contrast, the wild-type HER-2.369 epitope was only able to do so at an immunizing dose of 100 ng of peptide, not at any of the lower doses. Comparable results were obtained with both the IFN-γ ELISA and the ELISPOT assay (Fig. 4). Taken together, the results demonstrate that the higher immunogenicity in vitro of the HER-2.369 V2V9 peptide is paralleled by a markedly increased immunogenicity in vivo of this analogue compared with the wild-type HER-2.369 epitope. Next, we further analyzed the mechanism behind the enhanced immunogenicity of the V2V9 peptide.

High stability of HER-2.369 V2V9 HLA-A2.1 complexes

Both the association and the dissociation rates contribute to the steady state binding equilibrium of peptide/MHC complexes and, ultimately, are also expected to affect that of the TCR/peptide/MHC complexes. To further characterize the mechanisms of the increased immunogenicity of the V2V9 analogue peptide in comparison with its wild-type counterpart, the kinetics of association and dissociation of these two epitopes were analyzed. The results are summarized in Table I and Fig. 5. Previous data compared the equilibrium binding capacity at room temperature of the F3 peptides are similar (within 2-fold) to the first column of Table I), the binding at room temperature binding affinity values have been found to correlate well with immunogenicity (27) and because the empty MHC molecules rapidly unfold at 37°C, making 37°C equilibrium competition analysis somewhat impractical and inaccurate. In our experience, to accurately estimate 37°C binding constants, it is most effective to separately radiolabel each peptide and to independently determine on-off rate parameters. We next investigated whether the HER-2.369 V2V9 analogue would be more efficient than the HER-2.369 wild-type epitope in inducing immunity in vivo. HLA-A2.1/Kb transgenic mice were immunized with varying doses of the HER-2 wild-type or analogue peptide coemulsified in IFA with the PADRE Th epitope. Eleven days after immunization, splenocytes were cultured with the immunizing peptide and APC for 5 days, and peptide-specific IFN-γ production was measured using an in situ capture ELISA or the ELISPOT assay. The results demonstrated that the HER-2.369 V2V9 analogue possessed a markedly higher immunogenicity in vivo in HLA-A2.1/Kb transgenic mice (Fig. 4). Specifically, the HER-2.369 V2V9 analogue can induce specific CTLs in vivo in HLA-A2.1/Kb transgenic mice at lower concentrations than the HER-2.369 wild-type epitope.

The HER-2.369 V2V9 analogue can induce specific CTLs in vivo in HLA-A2.1/Kb transgenic mice at lower concentrations than the HER-2.369 wild-type epitope

The HER-2.369 V2V9 analogue can induce specific CTLs in vivo in HLA-A2.1/Kb transgenic mice at lower concentrations than the HER-2.369 wild-type epitope.
F3 analogue (3.3 vs 1.9 × 10⁻⁸ M). The on rates of these two peptides (see second and third columns of Table I), measured after ¹²⁵I labeling, were essentially identical regardless of whether they were measured at room temperature or 37°C (3.4 vs 3.7 × 10³ s⁻¹ M⁻¹ at 23°C and 2.6 vs 2.4 × 10³ s⁻¹ M⁻¹ at 37°C). These values were substantially lower (~3-fold) than those obtained with the control HBV core 18–27 peptide.

Next, off-rate values at 37°C were determined (fourth column of Table I). Surprisingly, the F3 and V2F3V9 epitopes were remarkably more stable than the HBV core 18–27 F6*Y epitope (4 × 10⁻⁵ s⁻¹ for HBV core 18–27 F6*Y, 2 × 10⁻⁵ s⁻¹ for F3, and 7 × 10⁻⁶ for V2F3V9). Thus, when the 37°C K_d values were calculated as the ratio of K_d/K_a (fifth column of Table I), both the wild-type epitope and the analogue were associated with affinities similar to that of the HBV core 18–27 epitope (note that the K_a of the HBV core 18–27 did not change significantly with temperature, but that of the HER-2 epitopes did). The gain in affinity was particularly remarkable for the V2F3V9 peptide, which increased >10-fold (33 vs 2.9 nM), compared with the F3 analogue, which increased only 2- to 3-fold. Thus, these HER-2-derived epitopes achieved binding affinities similar to that of HBV core 18–27, but with a different thermodynamic profile (the HBV core is “fast on, fast off,” whereas the HER-2 peptides are associated with a “slow on, slow off” kinetic profile). It is reasonable to speculate that the higher affinity for A2.1 at 37°C of these peptides (both F3 wild type and V2F3V9 analogue) and their stability (especially the V2F3V9) could be linked to their high biological activity.

**Table I. Summary of thermodynamic parameters**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>K_d at RT</th>
<th>K_a at RT</th>
<th>K_d at 37°C</th>
<th>K_a at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV core 18–27 F6*Y</td>
<td>5 × 10⁻⁹</td>
<td>1.0 × 10³</td>
<td>7.1 × 10³</td>
<td>4 × 10⁻⁵</td>
</tr>
<tr>
<td>HER-2.369</td>
<td>3.6 × 10⁻⁸</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HER-2.369 F3&gt;V</td>
<td>1.9 × 10⁻⁸</td>
<td>3.4 × 10²</td>
<td>2.6 × 10¹</td>
<td>2 × 10⁻⁵</td>
</tr>
<tr>
<td>HER-2.369 I2&gt;V, L9&gt;V</td>
<td>2.3 × 10⁻⁸</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HER-2.369 I2&gt;V, F3&gt;V, L9&gt;V</td>
<td>3.3 × 10⁻⁸</td>
<td>3.7 × 10²</td>
<td>2.4 × 10¹</td>
<td>7 × 10⁻⁶</td>
</tr>
</tbody>
</table>

*a* K_d molar values from the inhibition assay.

*b* M⁻¹ s⁻¹ values calculated from data in Fig. 5.

*c* s⁻¹ values calculated from data in Fig. 5.

*d* K_d at 37°C molar values calculated as K_d/K_a ratios.
be immunogenic, no side-by-side comparison with the corresponding wild-type epitope with regard to immunogenicity was conducted. In this study we have compared the HER-2 analogues and the wild-type epitope using both human CTL induction protocols and the mouse HLA-A2.1/Kb transgenic model.

Several reports have demonstrated that amino acid modifications of CTL epitopes from tumor Ags associated with increased affinity of peptide/MHC binding can enhance their immunogenicity (8–11, 17, 29, 30). Terasawa et al. (30) designed a human agonist CTL epitope of the prostate-specific Ag (PSA). Compared with the corresponding native PSA epitope, this agonist epitope demonstrated enhanced binding to the HLA-A2.1 allele as well as enhanced stability of the peptide-MHC complex. In line with our data, this PSA agonist was shown to induce higher levels of T cell activation compared with the native PSA peptide in HLA-A2.1/Kb mice. Also, the gp100.209(2M) analogue, which is an MHC anchor residue modification of the antigenic peptide derived from the gp100.209–217 melanoma Ag, was demonstrated to be 100-fold more potent in activating naive T cells than its wild-type peptide (31). This approach has been applied to HER-2-derived epitopes, in which modifications of the p654–662 HER-2-derived epitope, which has a low MHC class I binding affinity, was found to improve induction of peptide- and tumor-specific CTLs (32). Baratin et al. (29) demonstrated that amino acid substitutions of a p53-derived murine CTL epitope associated with 10- to 100-fold enhanced MHC binding affinity could overcome its poor immunogenicity. Thus, especially in the case of low on rate epitopes, increased binding affinity can be associated with increased immunogenicity. In this study we analyzed the effect of substituting the main anchor positions of an epitope that is already classified as a good binder, as it binds HLA molecules with an affinity <50 nM. Accordingly, the affinity of the HER-2 V2V9 analogue described in this study was only marginally increased compared with wild-type epitope (23 vs 37 nM). Despite this modest increase in MHC class I binding, the V2V9 analogue was still associated with a strong increase in immunogenicity, which motivated our analysis of the kinetics of the association and dissociation of this peptide.

When the thermodynamic parameters for the HER-2.369 epitopes were established, the result, particularly for the V2V9 epitope, demonstrated an association with a slow on, slow off kinetic profile. The enhanced stability of the MHC binding of this epitope could be an important parameter explaining the stronger immunogenicity. Other studies using whole-cell, flow cytometry-based techniques and measuring the half-time of the peptide-MHC complex have found that high stability is associated with enhanced immunogenicity (30). Because of the indirect techniques used, these reports did not assess on/off rates of the peptide analogues. Restifo found that the dissociation rate of the gp100.209(2M) analogue was >100-fold slower than that of the wild-type peptide (N. Restifo, personal communication). Of particular interest, in a clinical trial where this anchor-modified gp100 peptide was used to immunize melanoma patients, a dramatic increase in tumor-reactive T cells was observed (4). One can speculate that peptides that posses enhanced stability may remain bound to MHC class I on the APCs for a longer period, allowing this peptide/MHC complex to reach the local lymph nodes and there activate a more potent CTL response. This consideration might be of particular relevance in immunization strategies when the antigenic epitope is exogenously provided, and epitope/MHC complexes cannot be replenished by synthesis of new epitope molecules from within the APC. It is also possible that the increased immunogenicity of the peptide analogue might be related to increased functional binding activity for T cells. This might be the indirect result of the increased stability of the complexes, allowing more avid TCR contact. Alternatively, this might also originate from subtle conformational alterations in the peptide residues interacting with the TCR induced by the alterations in the MHC anchor motifs. If this were the case, our analogue would be classified as a heteroclitic analogue, associated with increased TCR binding (13).

The risk that these substitution analogues may alter the conformation of the MHC class I/peptide complex and thereby induce a different set of CTLs that do not cross-react with the wild-type epitope must be considered. Results from melanoma patients vaccinated with the gp100 epitope, which had been modified at the second position (g9–209(2M)) to enhance MHC binding affinity, support this idea (33). Their data suggest that the T cell repertoire in the vaccinated patients had been altered by expanding an array of T cells with different fine specificities, some only of which recognized melanoma cells. Our results showed that a subset of the microcultures of CTLs induced by the analogue peptide that were able to recognize the wild-type peptide also were able to recognize the HLA-A2.1-positive SW403 HER-2-expressing tumor line. We conclude that this recognition was MHC class I-restricted, as a HER-2 expressing, but HLA-A2.1-negative, tumor was not recognized. In line with these findings, Kawashima et al. (34) were able to identify double-substitution analogues from CEA with strong tumor-specific immunogenicity in vitro, and others have found that...
double or single substitution of CTL epitopes is able to induce tumor-specific T cells (8–11, 17, 29, 30).

Taken together, our results demonstrate that the CTLs induced by the V2V9 analogue also recognize the processed wild-type epitope expressed on tumor cells, but do not rule out that the induced T cell repertoire may be altered compared with the repertoire induced by the wild-type epitope.

Our data also indicate that epitope analogues may be useful to develop more sensitive assays to monitor immune responses in patients in tumor vaccination trials based on wild-type recombinant proteins or epitopes. Thus, we demonstrate that a HER-2 specific CTL line generated against the wild-type epitope responded with a higher number of IFN-γ-producing cells at low peptide concentrations when stimulated with the V2V9 analogue peptide compared with the wild-type peptide (Fig. 2). In line with these data, T cells generated against a native epitope from a PSA showed higher levels of CTL activity against targets pulsed with the agonistic epitope compared with the natural PSA epitope (30).

Several clinical trials with HER-2-based vaccines have been conducted or are in progress in patients with HER-2-overexpressing tumors (35–37). The T cell responses in patients vaccinated with the HER-2.369 epitope in combination with GM-CSF were reported to be weak and short-lived (38). Our results therefore suggest that the HER-2.369 V2V9 analogue should be tested in clinical trials, which may lead to stronger and more persistent T cell responses, in analogy with our results observed in HLA-A2 transgenic mice. This analog could be used in a peptide-based format in the presence of an appropriate adjuvant, such as GM-CSF, or in a vaccine based on DC pulsed ex vivo with peptides (36, 37). Protective HER-2-based vaccines based on plasmid DNA vaccination have shown promising results in experimental animal models (39, 40). Therefore, the alternative strategy based on the construction of vectors that contain multiple epitope genes or the entire HER-2 gene, but with altered amino acid sequences of the V2V9 analog, could also be considered.

References
8. Bownds, S., P. Tong-On, S. A. Rosenberg, and M. Parkhurst. 2001. Induction of tumor-reactive cytotoxic T lymphocytes using a peptide from NY-ESO-1 modified at the carboxy-termi-
Changes in the fine specificity of gp100_{209–217}-reactive T cells in patients following vaccination with a peptide modified at an HLA-A2.1 anchor residue. J. Immunol. 162:1749.


