Competition Among Peptides in Melanoma Vaccines for Binding to MHC Molecules

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Abstract: The effectiveness of peptide-based cancer vaccines depends on the ability of peptides to bind to MHC molecules on the surface of antigen-presenting cells, where they reconstitute epitopes for cytotoxic T lymphocytes (CTLs). Multivalent vaccines have advantages over single-peptide vaccines; however, peptides may compete for binding to the same MHC molecules. In particular, it is possible that peptides with high affinity for MHC molecules prevent the binding of lower-affinity peptides. However, only small numbers of peptide/MHC complexes per cell are required for CTL recognition. Thus, the authors hypothesized that competition of peptides for MHC binding would not significantly reduce CTL recognition of individual peptides within a multiple-peptide mixture, and this hypothesis was tested by a series of experiments performed in vitro. In multiple experiments, two peptides with different affinities for HLA-A*0201 molecules were mixed at various concentrations and pulsed onto HLA-A2$^+$ cells, which were then evaluated for susceptibility to lysis by HLA-A*0201-restricted CTLs. CTL recognition of the melanoma peptides gp100$_{154\text{-}162}$ (KTWGQYWQV), gp100$_{280\text{-}288}$ (YLEPGPVTA), and tyrosinase$_{369\text{-}377D}$ (YMDGTMSQV) was maintained even when target cells were co-pulsed with equimolar concentrations of peptides with comparable or higher affinity for HLA-A2. In some cases, CTL recognition was maintained even when the higher-affinity peptide was present at concentrations several orders of magnitude higher than the target peptide. In addition, CTLs generated by in vitro stimulation with a peptide mixture developed reactivity to three different peptides, at a level comparable to that obtained by stimulation with each individual peptide separately. These data suggest that CTLs can respond to multiple peptides presented on the same antigen-presenting cells and justify further investigation, in clinical trials, of multiple-peptide cancer vaccines.

Key Words: peptides, melanoma, vaccines, MHC molecules

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Cytotoxic T lymphocytes (CTLs) recognize peptides presented on the surface of tumor cells in association with class I MHC molecules.[1] Vaccines using these peptides are being evaluated in the therapy for cancer, but optimal methods of peptide vaccination for cancer remain to be defined. Some tumor-associated peptides appear to be immunodominant, suggesting that vaccination against a single peptide epitope may be an effective vaccine strategy for select tumors.[2] However, tumors often contain antigen-loss variants that escape immune recognition by CTLs directed against a single epitope.[3–8] Also, a peptide that is generally immunodominant is not always recognized by CTLs from all individuals[9] (and data not shown). Thus, multivalent vaccines may be more effective at inducing immunity effective against a broad range of human tumors. For these reasons, vaccines incorporating multiple peptide epitopes have advantages over a vaccine using a single peptide.

Several ongoing vaccine trials use multiple peptides, but each peptide is administered separately and in different locations (ECOG 1696 and 4697) to avoid the possibility of competition among peptides. While this is feasible for vaccines with small numbers of peptides, dozens of peptide epitopes for melanoma reactive CTLs have now been defined.[10] Development of the next generation of multiple-peptide vaccines will depend on the ability to administer them simultaneously as complex mixtures. Peptide-based vaccines administered intradermally require that the peptides bind to MHC molecules on antigen-presenting cells (APCs) in the dermis. However, peptides administered simultaneously in one location may compete with each other for binding to MHC molecules.[11–14] A key question is whether, in vaccines containing multiple epitopes, higher-affinity peptides will prevent effective MHC presentation of lower-affinity peptides.

We hypothesize that competition of peptides for MHC binding will not significantly reduce CTL recognition when those peptides are presented at equimolar concentrations. Specifically, we performed in vitro experiments to examine whether one high-affinity peptide would inhibit reconstitution of a CTL epitope by a lower-affinity peptide or another

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high-affinity peptide. We also assessed the ability of a mixture of peptides to induce in vitro CTL responses.

MATERIALS AND METHODS

Peptides

Specific peptides were chosen for study because of their use in clinical trials for melanoma vaccines and/or because of their well-documented binding affinities to HLA-A*0201. These peptides were synthesized by the University of Virginia Biomolecular Core Facility using a Biosearch 9500 synthesizer followed by HPLC purification and sequence confirmation by mass spectrometry, or by Multiple Peptide Systems (San Diego, CA) under GMP conditions. From the lyophilized peptides, stock solutions were made in DMSO or water. These stock solutions were diluted to the appropriate concentration in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (Sigma-Aldrich), 2 mM L-glutamine (Gibco, Carlsbad, CA), 25 mM HEPES buffer (Gibco), and 100 U/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gibco) antibiotics (complete medium).

The HLA-A2-restricted peptides used in the present study include the following 9-mers: gp100 peptides KTWGQYWQV ($gp100_{154-162}$),[15,16] IMDQVPFSV ($gp100_{209-217-2M}$),[17,18] YLEPGPVTA ($gp100_{280-288}$),[19] and RLMKQDFSV ($gp100_{619-627}$)[20]; tyrosinase peptides MLLAYLYCL ($tyrosinase_{1-9}$)[21] and YMDGTMSQV ($tyrosinase_{369-377D}$)[22]; and GLYDGMEHL ($MAGE\text{-}A10_{254-262}$),[23] AAGIGILTV ($MART\text{-}1/MelanA_{27-35}$),[24] and GILGFVFTL (influenza matrix protein derived $M1_{58-66}$).[25] The peptides that were used as negative controls in these experiments were the HIV-1-derived GAG_{77-85} peptide ("GAG", SLYNTVATL)[26] and the malaria-derived $CSP_{334-342}$ epitope ("YLK", YLKKKIKNSL).[27]

Cell Lines

CTL lines were generated from lymphocytes of HLA-A*0201$^+$ melanoma patients. VMM77 CTLs were generated from a tumor-involved node of a patient previously vaccinated against the $gp100_{280-288}$ peptide (YLEPGPVTA), as described, and are specific for this peptide[28] but do not recognize the $tyrosinase_{368-376D}$ (YMDGTMSQV) epitope (unpublished result). The VMM5 CTL line was generated from the tumor-involved node lymphocytes of an unvaccinated, long-term survivor of metastatic melanoma.[29] This CTL line recognizes multiple HLA-A*0201-associated peptides, including $gp100_{280-288}$ (YLEPGPVTA), $gp100_{154-162}$ (KTWGQYWQV), and $MART\text{-}1/MelanA_{27-35}$ (AAGIGILTV), but does not recognize $tyrosinase_{369-377D}$ (YMDGTMSQV).[19,30] VMM204 CTLs were generated from a lymph node draining a site of vaccination in a patient enrolled in a vaccine trial using the $tyrosinase_{369-377D}$ peptide (YMDGTMSQV), as described, and are specific for this peptide[22] but are not reactive to the $M1_{58-66}$ epitope (unpublished result). JY is an EBV-transformed B lymphoblastoid line expressing HLA-A*0201.[31] T2 is an HLA-A*0201$^+$ human T-cell/B-cell fusion with an antigen processing defect, which was provided by Peter Cresswell.[32,33] C1R-A2 is an HLA-A*0201$^+$ human EBV-transformed B-cell line that lacks expression of class I MHC molecules other than HLA-A2.[34]

CTLs Generated In Vitro With Peptide Mixtures

Peripheral blood lymphocytes (PBLs) from an HLA-A2$^+$ melanoma patient, VMM5, were pulsed with an equimolar mixture of HLA-A2$^+$ peptides at 10 μM each, then washed after a 2-hour incubation at 37°C in 5% CO_2. These cells were maintained in complete medium containing 20 U/mL IL-2 (Chiron, Emeryville, CA) and restimulated on day 7 with the same peptide mixture. At day 14, the resultant T-cell cultures were evaluated by ELIspot assay for their recognition of target cells pulsed with each of the peptides in the mixture as described.[35]

Also, PBLs of an HLA-A2$^+$ melanoma patient who had been enrolled in a peptide vaccine trial, VMM484, were stimulated once in vitro with either a single melanoma peptide or an equimolar mixture of all four peptides (10 μg/mL each) to which the patient had been vaccinated. These included $gp100_{280-288}$ (YLEPGPVTA), $tyrosinase_{369-377D}$ (YMDGTMSQV), $gp100_{209-217-2M}$ (IMDQVPFSV), and $MAGE\text{-}A10_{254-262}$ (GLYDGMEHL). After 14 days, these short-term cultured lymphocytes were evaluated in quadruplicate for peptide-specific reactivity by IFN-γ ELIspot assay.

Quantitative HLA-A Class I Molecular Binding Assay

The HLA-A*0201 cell-free molecular binding assays were performed as described.[11–13] Briefly, an iodinated standard peptide and the test peptide were co-incubated with soluble HLA-A*0201 molecules. The concentration of test peptide that inhibited the binding of the standard iodinated peptide by 50% (IC_{50}) is reported as a measure of the affinity of the test peptide for the HLA-A*0201 molecules. The standard high-affinity peptide is an HLA A2.1-restricted analog of the hepatitis B virus core protein, HBc_{18-27} (FLPSDYFPSV).[13]

Peptide Competition Assays

Chromium release assays were used to evaluate whether peptide competition for MHC binding sites was sufficient to prevent CTL recognition of the test peptide. HLA-A*0201$^+$ cells (JY, C1R-A2, or T2) were labeled for approximately 2 hours with ^{51}Cr as described.[19] Solutions containing two different peptides at multiple concentrations were created in complete medium and added to target cells in a 96-well, V-bottom plate (Nunc International, Rochester, NY). Following a 1- to 2-hour incubation at 37°C in 5% CO_2, effector T cells were added to the peptide-pulsed targets at effector:target ratios ranging from 5:1 to 20:1. In each experiment, the CTLs were reactive to only one of the two peptides pulsed onto the APC. Thus, the peptide to which the CTLs were not reactive was used as the competitor or "blocking" peptide. After a 4-hour incubation at 37°C in 5% CO_2, the supernatant was collected and the specific ^{51}Cr release was measured.

Some assays employed an alternate approach that involved adding the peptide solutions to the target cells while they labeled with ^{51}Cr. The cells were then washed three times and aliquoted (1,000 per well) into wells of a 96-well plate before the addition of effector cells (CTLs). The plate was incubated for 4 hours at 37°C and the supernatant was harvested and measured as above.

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RESULTS

High-Affinity Peptides Fail to Inhibit Recognition of Lower-Affinity Peptides When Administered at Equimolar Concentrations

VMM204 T cells reactive for tyrosinase$_{369-377D}$ (YMDGTMSQV) target cells were incubated with that peptide at concentrations ranging from 100 nM to 10 μM (Fig. 1). The M1$_{58-66}$ influenza matrix protein (GILGFVFTL) peptide has a higher affinity for HLA-A2 molecules than tyrosinase$_{369-377D}$ (Table 1). However, when target cells were co-incubated with the tyrosinase$_{369-377D}$ peptide and the M1$_{58-66}$ peptide at equimolar concentrations (square boxes in Fig. 1), no inhibition of T-cell recognition of the tyrosinase$_{369-377D}$ peptide was apparent. At 10-fold higher concentrations of M1$_{58-66}$, recognition of tyrosinase$_{369-377D}$ persisted, but the percentage of specific lysis declined.

When evaluating VMM77 CTL reactive to the low-affinity gp100$_{280-288}$ peptide (YLEPGPVTA), co-pulsing target cells with a large excess (1,000-fold) of the higher-affinity tyrosinase$_{369-377D}$ peptide failed to inhibit CTL recognition and lysis of the target cell (Fig. 2).

Additional assays were performed with fewer dilutions and involved washing the peptide from the target cells before the 4-hour incubation with gp100$_{280-288}$ reactive VMM5 CTL. The gp100$_{280-288}$ and tyrosinase$_{369-377D}$ peptides were pulsed together onto ^{51}Cr-labeled T2 cells at varying concentrations. At each concentration, the ability of the intermediate-affinity tyrosinase$_{369-377D}$ peptide to block recognition of the lower-affinity gp100$_{280-288}$ peptide was evaluated. As seen in Figure 3, CTL reactivity for the gp100$_{280-288}$ peptide (YLEPGPVTA) was unaffected by equimolar concentrations of the tyrosinase$_{369-377D}$ peptide (YMDGTMSQV), and only at lower concentrations of gp100$_{280-288}$ (1 nM) was a slight inhibition of CTL reactivity observed (41% vs. 54% lysis) when there was a 100,000-fold excess of the tyrosinase$_{369-377D}$ peptide (100 μM vs. 1 nM). Although this decrease in lysis was statistically significant by *t*-test analysis ($P < 0.01$), complete blocking was not shown at any concentration, even with great excess of the blocking peptide.

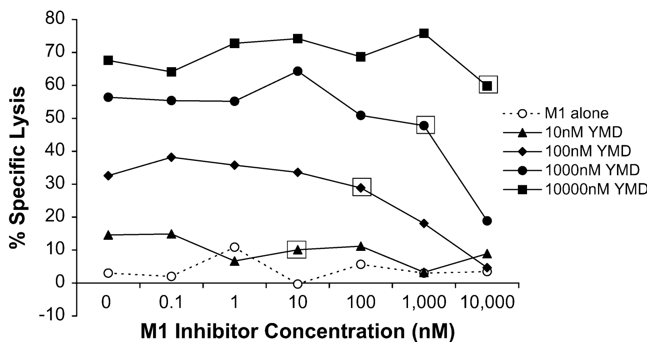

FIGURE 1. Recognition of tyrosinase$_{369-377D}$ was not inhibited by equimolar concentrations of the M1$_{58-66}$ peptide. T2 cells were co-incubated with the tyrosinase$_{369-377D}$ peptide (YMDGTMSQV, "YMD") and the M1$_{58-66}$ peptide (GILGFVFTL, "M1") at varied concentrations and evaluated for recognition and lysis by tyrosinase$_{369-377D}$-reactive VMM204 human cytotoxic T-cells in a 4-hour chromium release assay. Boxes are drawn around data points where the inhibitor ("M1") and the target antigen ("YMD") are at equimolar concentrations.

Similarly, varying concentrations of the melanoma-associated peptide MART-1$_{27-35}$ and the influenza M1$_{58-66}$ peptide were co-incubated with JY cells. Their susceptibility to lysis by the VMM5 CTL line that is reactive to the MART-1$_{27-35}$ peptide, but not reactive to the M1$_{58-66}$ peptide, was evaluated by ^{51}Cr release assay (Fig. 4). Although the MART-1$_{27-35}$ peptide has low affinity and the M1$_{58-66}$ peptide has high affinity for the HLA-A*0201 molecule, lysis of peptide pulsed target cells was diminished only when the M1$_{58-66}$ blocking peptide was added at 5,000-fold excess (50 μM) to the MART-1$_{27-35}$ peptide (10 nM)—though lysis even at this dilution was still at over 50% of that observed with the MART-1$_{27-35}$ peptide alone.

We also evaluated whether a peptide with high affinity for the MHC molecule HLA-A*0201 could inhibit the binding of another high-affinity peptide enough to block CTL recognition. We measured the binding affinity of gp100$_{154-162}$ (KTWGQYWQV) for HLA-A*0201 and found its IC$_{50}$ to be 20 nM. A comparable IC$_{50}$ of 12.4 nM was obtained for the influenza M1$_{58-66}$ peptide (see Table 1). Thus, both are considered to have high affinity for the HLA-A*0201 molecule. When these two high-affinity peptides were pulsed onto JY cells at varying concentrations, the M1$_{58-66}$ peptide did not block recognition of gp100$_{154-162}$ by melanoma reactive VMM5 CTLs at equimolar concentrations (Table 2, 1:1 μM and 100:100 μM). A 100-fold excess of M1$_{58-66}$ peptide was needed to block recognition of gp100$_{154-162}$, but even with this excess of 100 μM:1 μM, recognition was only partially blocked.

Generation of CTLs In Vitro With Peptide Mixtures

PBLs from the HLA-A2$^+$ melanoma patient VMM5 were stimulated in vitro with a mixture of seven HLA-A2$^+$ peptides at 10 μM each. These were tyrosinase peptides MLLAYLYCL (tyrosinase$_{1-9}$) and YMDGTMSQV (tyrosinase$_{369-377D}$); gp100 peptides KTWGQYWQV (gp100$_{154-162}$), IMDQVPFSV (gp100$_{209-217-2M}$), YLEPGPVTA (gp100$_{280-288}$), and RLMKQDFSV (gp100$_{619-627}$); and AAGIGILTV (MART-1$_{27-35}$). These PBLs were maintained in medium containing 20 U/mL of IL-2 and restimulated after 7 days with the same peptide mixture. At day 14, the resultant T-cell cultures were assayed by ELIspot assay for recognition of target cells pulsed with each of the peptides in the mixture. The lymphocytes stimulated with a mixture of low- to intermediate-affinity peptides developed reactivity to the MART-1$_{27-35}$ epitope. In a parallel culture, lymphocytes stimulated with those same seven peptides plus the high-affinity M1$_{58-66}$ peptide developed reactivity to the MART-1$_{27-35}$ peptide and to the M1$_{58-66}$ peptide. Reactivity to the MART-1$_{27-35}$ peptide was not diminished in the cultures with M1$_{58-66}$ added (Fig. 5).

We also stimulated VMM484 PBLs harvested from an HLA-A2$^+$ melanoma patient who had been enrolled in a peptide vaccine trial with a mixture of peptides included in the vaccine; two VMM484 PBL samples from two different time points after vaccination (PBL 05/07/2004 and 05/21/2004) were evaluated simultaneously. Those PBLs were stimulated

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TABLE 1. Binding Affinity of Peptides for HLA-A2

Peptide	Binding Affinity for HLA-A*0201	Binding Affinity for HLA-A*0201 (IC_{50})
GILGFVFTL influenza matrix protein $M1_{58-66}$	High	12.4 nM
KTWGQYWQV $gp100_{154-162}$	High	20 nM
YMDGTMSQV $tyrosinase_{369-377D}$	Intermediate	74 nM[19]
YLEPGPVTA $gp100_{280-288}$	Low	303 nM[18]
AAGIGILTV MelanA/$MART1_{27-35}$	Low[27]§	—[27]§

§The competitor peptide used in the assays for $M1_{58-66}$, $gp100_{154-162}$, $tyrosinase_{369-377D}$, and $gp100_{280-288}$ was a standard high-affinity peptide, HBc_{18-27} (FLPSDYFPSV), which is an A2.1-restricted analog of the hepatitis B virus core protein.[13] The competitor peptide used for the published result for $MART\text{-}1_{27-35}$ was the intermediate-affinity $tyrosinase_{369-377D}$ peptide: 50 nM of $MART\text{-}1_{27-35}$ peptide was required for the IC_{50}, competing with 1 nM $tyrosinase_{369-377D}$ peptide. Thus, the binding affinity of this peptide is lower than the affinity of the $tyrosinase_{369-377D}$ peptide.

once in vitro with each of four melanoma peptides, individually, or with an equimolar mixture of all four peptides, at 10 μg/mL each. These were $gp100_{209-217-2M}$ (IMDQVPFSV), $gp100_{280-288}$ (YLEPGPVTA), $MAGE\text{-}A10_{154-162}$ (GLYDGMEHL), and $tyrosinase_{369-377D}$ (YMDGTMSQV). After 14 days, specific reactivity to $tyrosinase_{369-377D}$, $gp100_{209-217-2M}$, and $MAGE\text{-}A10_{154-162}$ from cultures stimulated with each of those peptides was detected in an IFN-γ ELIspot assay. The culture stimulated with $gp100_{280-288}$ did not develop reactivity to this epitope. Relative to the cells stimulated with single peptides, the PBLs cultured with the mixture of all four peptides had reactivity against the same three antigens without significant change. Representative data from one set of PBLs are shown in Figure 6, and summary data are presented in Table 3.

DISCUSSION

We know from peptide/MHC binding affinity assays that competition for MHC binding occurs between peptides. However, in murine and human systems, multivalent peptide preparations, created by acid stripping of cell surface MHC molecules pulsed onto dendritic cells (DCs), have resulted in effective anti-tumor immune responses.[36–38] Thus, vaccines containing multiple peptides may be effective immunogens. However, direct assessment of competition among individual peptides in this setting has not been previously reported, and the tumor immunology community is concerned that peptide competition may interfere with the efficacy of vaccination with peptide mixtures. The results of the present study suggest that peptide competition will not sufficiently reduce the level of peptide presentation required for recognition by CTLs. Specifically, the data suggest that the individual HLA-A*0201 peptides making up a multiple-peptide melanoma vaccine will retain their immunogenicity and will not undergo significant competitive inhibition when those peptides are presented in equimolar quantities.

More generally, the results suggest that if peptides are pulsed onto APCs at equimolar concentrations, one peptide is not likely to block presentation of another peptide below the threshold for CTL recognition. In fact, at least 10- to 1,000-fold excess was required to observe any reduction in CTL recognition. Even recognition of the lower-affinity peptides could not be blocked unless extreme excesses of blocking peptides were used. These data support the administration of a peptide-based vaccine that consists of a mixture of multiple

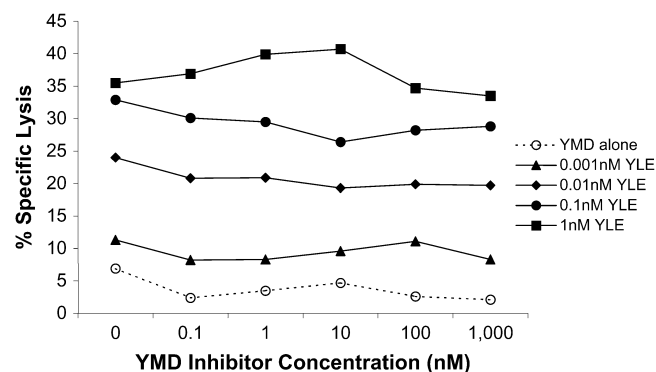

FIGURE 2. Recognition of $gp100_{280-288}$ peptide was not inhibited by excess $tyrosinase_{369-377D}$ peptide. C1R-A2 target cells were co-incubated with the $gp100_{280-288}$ peptide (YLEPGPVTA, ''YLE'') and the $tyrosinase_{369-377D}$ peptide (YMDGTMSQV, ''YMD'') at varied concentrations and evaluated for recognition and lysis by VMM77 human cytotoxic T-cells reactive to the $gp100_{280-288}$ peptide in a 4-hour chromium release assay.

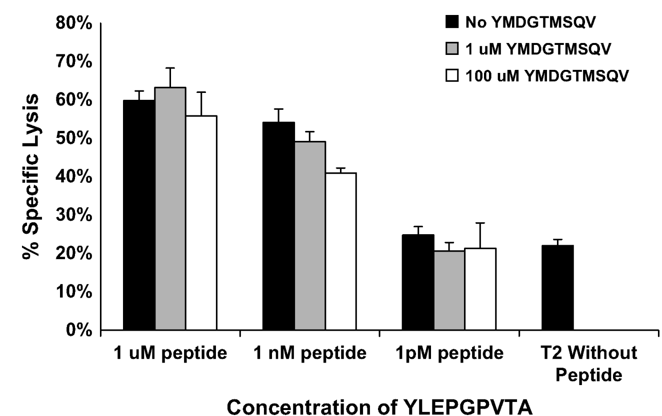

FIGURE 3. Recognition of $gp100_{280-288}$ peptide was not inhibited by excess $tyrosinase_{369-377D}$ peptide, including high peptide concentrations. T2 cells were pulsed with one of three concentrations of $gp100_{280-288}$ peptide (''YLE'';1 μM, 1 nM, or 1 pM) plus 0 μM, 1 μM, or 100 μM $tyrosinase_{369-377D}$ competitor (''YMD'') before adding VMM5 $gp100_{280-288}$-reactive CTLs. Lymphocyte reactivity was assessed by lysis in a 4-hour chromium release assay.

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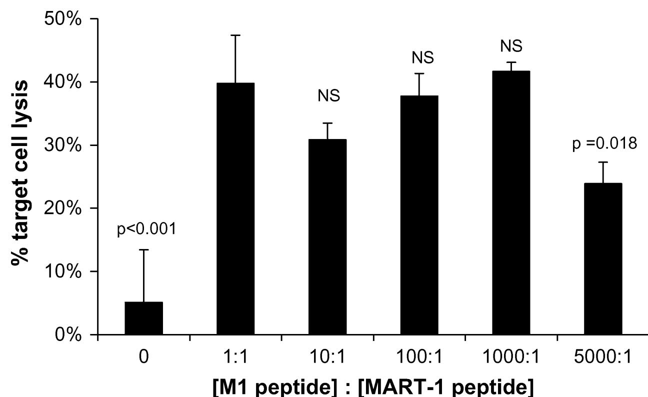

FIGURE 4. Recognition of MART-$1_{27\text{-}35}$ peptide maintained even in the presence of excess $M1_{58\text{-}66}$ peptide. T2 cells were pulsed with 10 nM MART-$1_{27\text{-}35}$ target peptide and a range of concentrations of $M1_{58\text{-}66}$ competitor peptide (10 nM–50 μM) and evaluated for recognition and lysis by MART-$1_{27\text{-}35}$ reactive VMM5 human cytotoxic T lymphocytes (effector:target ratio 20:1) in a 4-hour chromium release assay. *P* values were assessed by *t*-test analysis, NS = not significant.

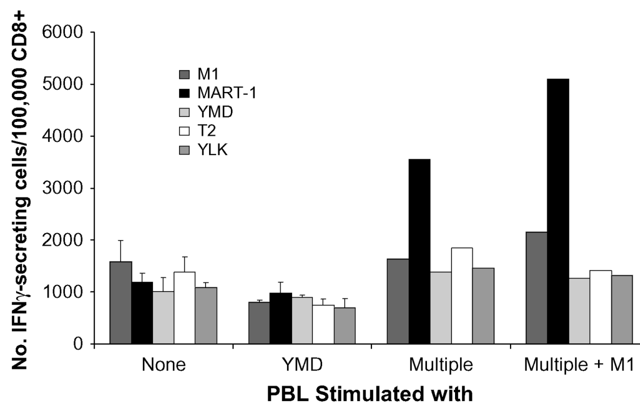

FIGURE 5. Reactivity of VMM5 T cells stimulated with individual or multiple peptides. VMM5 PBLs were stimulated twice, weekly, with (a) no peptides ''None,'' (b) $tyrosinase_{369\text{-}377D}$ ''YMD,'' (c) multiple HLA-A2 binding peptides: tyrosinase peptides MLLAYLYCL ($tyrosinase_{1\text{-}9}$) and YMDGTMSQV ($tyrosinase_{369\text{-}377D}$); gp100 peptides KTWGQYWQV ($gp100_{154\text{-}162}$), IMDQVPFSV ($gp100_{209\text{-}217\text{-}2M}$), YLEPGPVTA ($gp100_{280\text{-}288}$), and RLMKQDFSV ($gp100_{619\text{-}627}$); and AAGIGILTV (MART-$1_{27\text{-}35}$) ''Multiple,'' or (d) multiple HLA-A2 binding peptides + the flu $M1_{58\text{-}66}$ peptide. PBL were assayed by ELIspot on day 14 against a panel of target peptides. YLKKKIKNSL ($CSP_{334\text{-}342}$, ''YLK'') is a HLA-A2-restricted malarial peptide used as a negative control in the ELIspot assay.

peptides at near equimolar concentrations. As the number of identified peptide epitopes for melanoma-reactive CTLs has grown, it may be feasible to make vaccines containing dozens of peptides.

The number of peptide/MHC complexes required for recognition by peptide-specific CTLs may be less than the number required for initiation of a T-cell response. However, we have also found that CTLs can be generated in vitro by stimulation with peptide mixtures. Reactivity to the low-affinity MART-$1_{27\text{-}35}$ peptide was generated even in the presence of multiple peptides with higher affinity for HLA-A2. This is evidence that addition of these peptides does not prevent effective presentation of the MART-$1_{27\text{-}35}$ peptide on APCs in vitro. Furthermore, a parallel culture was performed with addition of equimolar quantities of the $M1_{58\text{-}66}$ peptide, which has very high affinity for HLA-A2.1 molecules. Reactivity to MART-$1_{27\text{-}35}$ was preserved, even in the presence of this very-high-affinity peptide (see Fig. 5). Mixtures of peptides may not induce CTL responses to all peptides in the mixture due to variations in the T-cell repertoire of the patient; however, presenting peptides in mixtures does not appear to inhibit generation of CTL responses when the appropriate T-cell repertoire is present.

TABLE 2. Competition Between Two High-Affinity Peptides

	$gp100_{154\text{-}162}$ 100 μM	$gp100_{154\text{-}162}$ 1 μM	$gp100_{154\text{-}162}$ 0 μM
$M1_{58\text{-}66}$ 0μM	46.7%	46.3%	−0.7%
$M1_{58\text{-}66}$ 1μM	ND	47.1%	−2.7%
$M1_{58\text{-}66}$ 100μM	47.4%	31.3%	−1.5%

JY cells were pulsed with mixtures of $gp100_{154\text{-}162}$ (KTWGQYWQV) and a competitor peptide, $M1_{58\text{-}66}$ (GILGFVFTL), at concentrations ranging from 0 to 100 μM. Specific lysis of those target cells is stated for each of several mixtures. JY cells pulsed with 0.01 μM $gp100_{154\text{-}162}$ were not recognized.

Competition between peptides with different affinities for binding to the same MHC class I molecule may not necessarily render a low-affinity peptide non-immunogenic. First, in the case of short-term pulsing of cells, peptide/MHC complexes form independently of the binding affinity of the peptide.[39] In this situation, surface antigen densities are a function of the peptide on-rate, which is similar for peptides of the same size that are pulsed onto APCs at comparable concentrations.[39] Surface antigen density, however, declines once the exogenous source of peptide is removed. Unlike the formation of peptide/MHC complexes, the rate of decline in

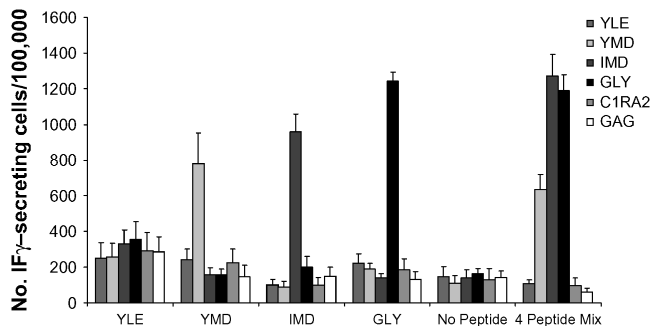

FIGURE 6. Reactivity of VMM484 PBLs stimulated with individual or multiple peptides. VMM484 PBLs were stimulated once in vitro with (a) $gp100_{280\text{-}288}$ ''YLE,'' (b) $tyrosinase_{369\text{-}377D}$ ''YMD,'' (c) $gp100_{209\text{-}217\text{-}2M}$ ''IMD,'' (d) MAGE-$A10_{254\text{-}262}$ ''GLY,'' (e) none, or (f) a mixture of all four peptides. Each culture (a–f) was assayed by IFN-γ ELIspot on day 14 against C1R-A2 cells that were pulsed with either each of the four peptides, unpulsed cells, or cells pulsed with a negative control peptide, ''GAG'' (HIV-1-derived $GAG_{77\text{-}85}$, SLYNTVATL).

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TABLE 3. Summary Data

	$gp100_{280-288}$	$Tyrosinase_{369-377D}$	$gp100_{209-217-2M}$	$MAGEA10_{254-262}$	Mean*
			No. responding T cells per 100,000		
Single peptide stimulations					730
Day A	0	1178	1311	929	
Day B	0	552	808	1057	
Stimulation with 4 peptides					621
Day A	9	504	722	921	
Day B	10	538	1174	1091	
			Ratio of response over background		
Single peptide stimulations					5.1
Day A	1	12.8	21.0	11.5	
Day B	1	3.4	6.4	6.7	
Stimulation with 4 peptides	–	–	–	–	4.9
Day A	1.07	4.9	6.6	8.1	
Day B	1.10	6.5	13.1	12.2	

Number of responding cells per 100,000 calculated by subtracting result from the maximum negative control. Negative values all set to zero. Similarly, the ratio of responding cells to the background is considered 1 or greater; values less than 1 are set to 1.

*Means of ratios calculated using ln ratios and back transforming.

antigen density depends on the affinity of the peptide for the class I molecule and is defined by the off-rate for each peptide. These findings strongly suggest, for example, that co-administration of four peptides with varied binding affinities at equimolar concentrations will result in a quarter reduction of each type of MHC/peptide complex, assuming the peptides are added in saturating quantities.

The concern that peptides may compete with each other for MHC binding during co-incubation with APCs may lead some investigators to divide DC preparations into multiple aliquots that are individually pulsed with each peptide antigen. We believe these additional preparative steps are not likely to be of benefit and will lead to a net decrease in the number of DCs presenting each peptide.

Similarly, current trials using multiple HLA-A2-restricted peptides specify that each peptide will be mixed with adjuvant separately and will be administered to a separate vaccine site. As the number of available peptides for use in vaccine trials greatly exceeds 50, the concept of applying each separately and administering each with a separate adjuvant becomes much less feasible. The present report supports administration of mixtures of multiple peptides as single vaccine preparations.

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