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(54) Title: HETEROCLITIC ANALOGS AND RELATED METHODS

(57) Abrégé/Abstract:
Heteroclitic analogs of Class I epitopes are prepared by providing conservative or semi-conservative amino acid substitutions at positions 3 and/or 5 and/or 7 of these epitopes. The analogs are useful in eliciting immune responses with respect to the corresponding wildtype epitopes.

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(54) Title: HETEROCLITIC ANALOGS OF CLASS I EPITODES

(57) Abstract: Heteroclitic analogs of Class I epitopes are prepared by providing conservative or semi-conservative amino acid substitutions at positions 3 and/or 5 and/or 7 of these epitopes. The analogs are useful in eliciting immune responses with respect to the corresponding wildtype epitopes.

HETEROCLITIC ANALOGS AND RELATED METHODS

Cross-Reference to Related Applications

The present application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application Serial No. 60/166,529, filed 18 November 1999, and Serial No. 60/239,008, filed 6 October 2000 which are incorporated by reference in their entireties.

Field of the Invention

The invention relates to methods for generating heteroclitic analogs of an original peptide which have increased stimulatory capacity for a given T cell.

Background of the Invention

Several studies suggest the cytotoxic T lymphocytes (CTLs) play a central role in the eradication of infectious disease and cancer by the immune system (Byrne, *et al., J. Immunol.* 51:682 (1984), McMichael, *et al., N. England J. Med.*, 309:13 (1983)). Since CTLs are stimulated by peptides comprising epitopes, considerable effort is ongoing in developing epitope-based vaccines that stimulate CTL responses. One class of epitopes, designated heteroclitic analogs, provides benefit as vaccine components since these analogs induce T cell responses stronger than those induced by the native epitope. Heteroclitic analogs are defined as peptides having increased stimulatory capacity or potency for a specific T cell, as measured by increased responses to a given dose, or by a requirement of lesser amounts to achieve the same response.

The advantages associated with using heteroclitic analogs in clinical applications are as follows. First, heteroclitic analogs have the ability to break/overcome tolerance by reversing a state of T cell anergy, activating non-tolerized cross-reactive clones of T cells, or by mediating "immune deviation," *i.e.*, the type of CTL produced, such as Th1 or Th2. Recent studies indicate that heteroclitic analogs are immunogenic (Zaremba, *et al., Cancer Research*, 57:4570 (1997); Rivoltoni, *et al., Cancer Research*, 59:301 (1999); Selby, *et al.*, 162(2):669 (1999)) in that they are capable of inducing CTLs that recognize endogenously processed epitope. This is confirmed by studies in different immunological systems

(Zugel, *et al.*, *J. Immunol.,* 161:1705 (1998), Wang, *et al.*, *J. Exp. Med.*, 190:983 (1999), Men, *et al., J. Immunol.*, 162:3566, (1999)). For example, studies by Zugel *et al.* (Zugel, *et al.*, *supra*) have shown that T cell tolerance to an immunodominant T cell epitope in adult mice can be overcome by immunization with heteroclitic cross-reactive peptide analogs of that peptide.

This is particularly significant in the field of cancer vaccines, where most of the CTL epitopes are derived from self antigens. Due to the fact that cancer related antigens are often self-antigens there is a corresponding phenomenon that there may be preexisting tolerance to these antigens, whereby generation of a T cell response to such epitopes is a challenge. Breaking of tolerance by heteroclitic analogs has been shown in a recent study in a murine Class II system (Wang, *et al., J. Exp. Med.* 190:983 (1999)). In this study, the mechanism involved in breaking of tolerance was the stimulation of nontolerized, low affinity clones, rather than reversal of anergy. The heteroclicity demonstrated herein is associated with the induction of high avidity CTL, this represents an important difference.

Second, peptide analogs have been demonstrated to modulate cytokine production from T cells (Pfeiffer, *et al.*, *J. Exp. Med.,* 181:1569 (1995), Tao, *et al., J. Immunol.,* 158:4237 (1997), Salazar, *et al., Int. J. Cancer* 85(6):829-38 (2000), Nicholson, *et al., Int. Immunol.* 12(2):205-13 (2000)). The immune deviation induced by such analogs has implications in several disease states, where generation of a specific subset of Th cell responses correlate with tumor regression (Zitvogel, *et al.*, *J. Exp. Med.*, 183:87 (1996), Celluzzi, *et al., J. Exp. Med.* 183:283 (1996)) or affected the clinical outcome of autoimmune or infectious disease (Romagnani, *et al., Annu. Rev. Immunol.,* 12:227-57 (1994)). Thus, immunization with heteroclitic analogs offers the capacity to modulate cytokine production by induction of specific subsets of effector T cells, thereby altering the course of disease.

Third, heteroclitic analogs offer an advantage in drug development since significantly smaller amounts of peptide are needed for treatment doses, due to their strong biological potency. This feature overcomes certain manufacturing and toxicity concerns. In this regard, it has been shown that a heteroclitic analog of a MART-1 peptide (Rivoltini, *et al., Cancer Research* 59:301 (1999)), which generated antigen specific T cells in melanoma patients, was active at much lower concentrations than the native epitope. Similar results were reported by Schlom and colleagues (Zaremba,

et al., Cancer Research 57:4570 (1997)) regarding heteroclitic analog of the CEA derived CAP1 epitope. However, a side-by-side precursor frequency analysis or a TCR avidity analysis against wildtype peptide was not performed.

Accordingly, because of their biological relevance, it would be extremely useful to predict amino acid substitutions that render heteroclitic activity to a given epitope. However, prior to the present disclosure there has been no easy method for predicting such substitutions. Indeed, in previous studies (Selby, *et al., J. Immunol.*, 162(2):669 (1999), Skipper, *et al., J. Exp. Med.* 183:527 (1996)), heteroclitic epitopes were fortuitously identified by eluting naturally occurring mutant peptides from melanoma cells, or by systematically screening a large number of analogs consisting of substitutions at almost every position in the epitope (Zaremba, *et al., Cancer Research*, 57:4570 (1997), Loftus, *et al., Cancer Research* 58:2433 (1998), Blake, *et al., J. Exp. Med.* 18:121 (1996)). Alternatively, heteroclitic analogs were identified by screening random combinatorial peptide libraries which also has required the arduous synthesis and screening of large numbers of peptides (Pinilla, *et al., Current Opinion in Immunology* 11:193-202 (1999)). Genetic approaches, such as screening of DNA expression libraries, have provided another method for generating CTL epitopes and analogs (Boon, *et al., Annu. Rev. Immunol.* 12:337-65 (1994), Gavin, *et al., Eur. J. Immunol.* 24(9):2124-33 (1994)). However, this approach may be problematic given the potentially small quantities and complexity of epitopes generated.

Disclosure of the Invention

The invention provides methods to prepare peptides containing epitopes which have enhanced ability to effect an immune response with respect to corresponding analogous wildtype epitopes. The resulting "heteroclitic analogs" are useful in immunological compositions for treatment of viral diseases, cancer, and other conditions which are characterized by displayed antigens on target cells.

Thus, in one aspect, the invention is directed to a method to enhance the immunogenicity of a peptide containing an epitope, the method comprising

i) providing a peptide comprising a first Class I epitope wherein said epitope consists essentially of an amino acid sequence having an N-terminus and a C-terminus and at least one primary anchor residue, wherein amino acid residues of the epitope are

numbered consecutively and the primary anchor residue nearest the N-terminus of the epitope is at position 2 or position 3; and ii) introducing one or more one conservative or semi-conservative substitution between the N-terminus and the C-terminus of the epitope at position 3 and/or 5 and/or 7 which position does not contain a primary anchor residue, thereby constructing a peptide comprising a second Class I epitope which exhibits enhanced immunogenicity compared to the first Class I epitope.

The second Class I epitope described above is generically referred to as a "heteroclitic analog."

In a preferred embodiment, the heteroclitic analog exhibits at least about 50% increased potency for a specific T-cell compared to the corresponding wildtype Class I epitope. The analog may contain only one substitution, or may contain two or three, and the substitution may be conservative or semi-conservative. The heteroclitic analog may induce both Th1 and Th2 cytokines when bound by an HLA Class I molecule and contacted with the relevant cytotoxic T-cell. Preferably, the Class I epitope comprises a supermotif selected from the group consisting of A1, A2, A3, A24, B7, B27, B44, B58 and B62, more preferably, the Class I epitope comprises an A2 supermotif, most preferably, an A2.1 motif.

The invention also provides methods of inducing a human cytotoxic T cell response against a preselected Class I peptide epitope, the method comprising providing the heteroclitic analog described above; and contacting a human CTL with the heteroclitic analog.

In some aspects, the step of contacting is carried out *in vitro*. In some aspects, the step of contacting is carried out by administering to a subject a nucleic acid molecule comprising a sequence encoding the heteroclitic analog peptide epitope.

The invention is also directed to peptides comprising the heteroclitic analog epitopes which are obtainable by the method described above. In particular, and preferably, such peptides include those where the epitope consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19 and SEQ ID NO: 20. The peptide may contain 9-20 amino acids, preferably 9-16, more preferably 9-15, but may also contain only a total of 9, 10, 11, 12, 13 or 14 amino acids. The defined heteroclitic analog epitopes may be included in a longer

polypeptide or protein which is a homopolymer of the same epitope or a heteropolymer which contains a variety of such epitopes or the heteroclitic analog epitope in combination with wildtype epitopes. These peptides and proteins may be included in compositions which are designed for pharmaceutical use.

The peptides or heteropolymers or homopolymers containing the heteroclitic analog epitopes may be combined with other components to enhance further or modulate their activity in eliciting an immune response. These additional varieties may be covalently bound or non-covalently included in a mixture. Thus, the heteroclitic analog epitope may be admixed or joined to a CTL epitope, or to an HTL epitope, especially where HTL epitope is a pan-DR binding molecule. A composition containing the heteroclitic analog epitope may further comprise a liposome, wherein the epitope is on or within the liposome, or the epitope may be joined to a lipid. The heteroclitic epitope may be bound to an HLA heavy chain, β2-microglobulin, and strepavidin complex, whereby a tetramer is formed. In addition, the heteroclitic epitope may be modified in a composition which comprises an antigen presenting cell, wherein the epitope is on or within the antigen presenting cell, wherein the epitope is bound to an HLA molecule on the antigen presenting cell. Thus, when a cytotoxic lymphocyte (CTL) that is restricted to the HLA molecule is present, a receptor of the CTL binds to a complex of the HLA molecule and the epitope. The antigen presenting cell may be a dendritic cell. The composition may also simply comprise an HLA molecule, wherein the peptide containing the epitope is bound by the HLA molecule. The composition may also comprise a label - *e.g.*, biotin, a fluorescent moiety, a non-mammalian sugar, a radiolabel or a small molecule to which a monoclonal antibody binds.

The compositions described are useful in eliciting an immune response against the corresponding wildtype epitope. Typically, the heteroclitic analog is included in such compositions which will further contain suitable excipients. The active component heteroclitic epitopes may be present in unit dosage form. Compositions useful in treating subjects may also comprise nucleic acid molecules that encode the peptides described above optionally including control sequences for their expression.

Brief Description of the Drawings

Figures 1A and 1B represent the results of testing a panel of analogs of CEA.691 and MAGE3.112 respectively for ability to induce IFNγ production in the corresponding CTL. Figures 1C and 1D are the corresponding dose response curves for CEA.691 and MAGE3.112 heteroclitic analogs respectively.

Figures 2A, 2B and 2C show the results of testing panels of analogs of MAGE2.157, HIVPol.476, and HBVPol.455 epitope analogs with respect to the ability of these analogs to induce IFNγ production in the corresponding CTLs. Figure 2D is the relevant dose response curve for the successful HIVPol.476 analogs.

Figures 3A and 3B show dose response curves of heteroclitic analogs of MAGE2.157 in comparison to wildtype with regard to their ability to induce IFNγ production or IL10 production from the appropriate CTLs.

Figures 4A and 4B are the dose response curves for wildtype and a heteroclitic analog of HIVPol.476 to produce IFNγ and IL10 in appropriate CTLs.

Figure 5 shows the results of testing a panel of potential heteroclitic analogs of the epitope p53.149M2 with respect to IFNγ production from appropriate CTLs.

Figures 6A and 6B are the corresponding dose response curves for production of IFNγ and IL10 by successful heteroclitic analogs of p53.149M2.

Figure 7 shows the results of testing a panel of potential analogs of p53.Mu184 epitope for IFNγ production in CTLs.

Figure 8 shows the dose response curve for wildtype and two successful heteroclitic analogs of p53.Mu184 with respect to IFNγ production.

Figures 9A-D show the cross-reactivity of heteroclitic analogs with regard to the corresponding wildtype epitope. In Figures 9A and 9B, IFNγ production is plotted as a function of concentration using stimulation by the immunizing peptide. Figures 9C and 9D show the corresponding results when wildtype epitope is used as the stimulant as opposed to the heteroclitic analog used for the initial induction of CTL.

Figure 10 shows the IFNγ release with respect to stimulation by p53.261 and its heteroclitic analogs.

Figure 11 shows Elispot results with respect to various heteroclitic analogs.

Figure 12 shows the results of stimulation of CTL activity against endogenous peptide using various heteroclitic analogs.

Modes of Carrying Out the Invention

1. Overview

The present invention relates to methods of designing heteroclitic analogs that bind to HLA Class I molecules. "Heteroclitic analogs," as described herein, are peptides comprising epitopes with increased potency for a specific T cell, as measured by increased responses to a given dose, or by a requirement of lesser amounts to achieve the same response as a homologous Class I peptide. The methods of the invention are useful to modify any Class I peptide, particularly those associated with human cancers and precancerous conditions, and from infectious agents such as viruses, bacteria, fungi, and protozoal parasites.

Importantly, the phenomenon of heteroclicity applies across HLA moelcues that bind a particular Class I peptide. For example, a heteroclitic analog peptide bearing the A2 supermotif is heteroclitic (*i.e.*, has higher potency) across all HLA molecules in the HLA-supertype (*e.g.*, A*0201, A*0202, A*0203, A*0204, A*0205, A*0206, A*0207, etc. Similarly, a heteroclitic analog peptide bearing a different sequence motif (*e.g.*, A1, A3, A24, B7, B27, B44, B58, B62, etc.) is more potent immune response across all HLA molecules within their specific HLA-superfamily.

Applicants have found specific rules for designing heteroclitic analogs which enhance the immune response to the corresponding wildtype epitope. These rules are applicable with respect to epitopes bearing motifs or supermotifs which bind to HLA molecules encoded by any Class I allele. By using these rules, it is possible to enhance the immunogenecity, therefore, of any "wildtype" or "native" Class I epitope.

Briefly, the rules state that the wildtype Class I epitope is modified by substituting a conservative or semi-conservative amino acid as position 3 and/or 5 and/or 7 of the epitope. The nature of the conservative or semi-conservative amino acid to be substituted is defined by the description in Preparation B hereinbelow, the results of which are summarized in Table 2. Thus, by consulting Table 2, one can determine suitable candidates for substitution at these positions. As shown in Table 2, each of the amino acids shown across the top of the table bears a numerically defined relationship to the remaining 19 genetically encoded amino acids. The lower the index, the higher the conservation; the same amino acid will have a similarity assignment of 1.0; maximally different amino acids will have similarity assignments

approaching 20. Using the method set forth in Preparation B, amino acids which are not gene-encoded can also be assigned similarity indices and can be classified with respect to any natively occurring amino acid as conservative or semi-conservative (or non-conservative).

Heteroclitic analog peptides of the invention are particularly useful to induce an immune response against antigens to which a subject's immune system has become tolerant. Human subjects are particularly preferred, but the methods can also be applied to other mammals such as laboratory mice, taking account of the corresponding HLA motifs with regard to these subjects. Tolerance refers to a specific immunologic nonresponsiveness induced by prior exposure to an antigen. Tolerance can be overcome by identifying a particular Class I peptide epitope to which a patient is tolerant, modifying the peptide epitope sequence according to the methods of the invention, and inducing an immune response that cross-reacts against the tolerized epitope (antigen). Overcoming tolerance is particularly desirable, for example, when the immune system of the subject is tolerant of a viral or tumor-associated antigen, the latter antigens being often over-expressed self-proteins as a consequence of cell transformation.

To determine rules for designing heteroclitics, several different CTL lines were screened for reactivity against panels of analogs. Modification of T cell stimulatory capacity was achieved with no alternation of the primary MHC anchors.

The wildtype epitopes include tumor epitopes derived from self antigens that are specifically up-regulated in epithelial cell cancers and have been shown to be immunogenic. Viral epitopes used, such as those from the polymerase genes of the HIV and HBV, have been shown to be immunogenic as well.

The rules described herein provide a basis to design heteroclitic analogs, drastically reducing the screening otherwise required and are extremely useful in designing epitope-based vaccines for cancer and infectious diseases.

In the examples set forth below, 17% of the total analogs screened (which fit the heteroclicity rules disclosed herein) were heteroclitic (16/95). This is significant for two reasons: first, the efficiency of detecting heteroclitics increased from 2.2% to 17% by employing analogs that follow the rules of heteroclitic substitution; second, the number of peptides which need to be synthesized is reduced dramatically from about a 100 analogs per epitope to about 15 analogs per epitope, making the process

cost effective and amenable to high throughput. Through the application of the heteroclitic substitution rules of the invention, the efficiency of generating heteroclitic analogs was increased nearly 100 to 1000-fold, from 0.2% (4 identified from screening of 233 CEA.691 and MAGE3.112 analogs) to 30% (3 identified by screening of 9 predicted analogs). The latter frequency may be a gross underestimate since only 4 of 6 analogs showing potential heteroclitic activity in initial assays were subjected to further analysis.

Previous studies showed modulation of T cell responses by heteroclitic analogs involved TCR contact residues (Byrne, *et al., J. Immunol.* 51:682 (1984), McMichael, *et al., N. England. J. Med.* 309:13 (1983), Zugel, *et al., J. Immunol.* 161:1705 (1998), Rivoltini, *et al., Cancer Research* 59:301 (1999), Parhurst, *et al., J. Immunol.* 157:2539 (1996)), but the present study did not find this. For example, for the CEA.691 epitope, the TCR contact residue is position 8, while heteroclicity was observed with analog substitutions at positions 3 and 5. While not intending to be bound by any theory, alteration of MHC binding may be a mechanism. Binding analyses performed on the analogs indicated that there is an alteration in MHC binding for the better or worse in a majority of cases (80%). Out of the 13 analogs which were tested for HLA-A2 binding, ten analogs had alteration in MHC binding, with six analogs binding better than wildtype peptides and four analogs that bound worse than wildtype, but still generated a substantially increased biological response. Some studies modify primary MHC anchor residues in order to increase MHC binding (this approach has been used by some groups to generate analogs (Pfeiffer, *et al., J. Exp. Med.* 181:1569 (1995), Valmori, *et al., J. Immunol.* 160:1750-1758 (1998)). Increased biological responses without changing primary TCR contact residues or primary MHC anchor residues was observed in this study. Since increased responses were mediated with alteration in MHC binding, it is postulated that the effect may be mediated by changing secondary anchor positions. More evidence supporting this comes from the finding that heteroclitic substitutions occur at odd numbered positions (3, 5, 7) in the middle of the peptide. All these positions 3, 5, and 7 have been shown to be secondary anchor positions for binding to the HLA-A2 molecule (Boon, *et al., Annu. Rev. Immunol.* 12:337-65 (1994), Ishioka, *et al., J. Immunol.* 162(7):3915-25 (1999)).

Two of these positions (3 and 7) have been shown to be secondary anchor positions for binding to HLA-A2.1 molecule by several groups (Ruppert, *et al., Cell* 74:929 (1993), Madden, *Annu. Rev. Immunol.* 13:587-622 (1995)). Alteration of such secondary anchor positions can translate into T cell recognition differences (Valmori, *et al., J. Immonol.* 160:1750 (1998); Davis, *et al., Annu. Rev. Immunol.* 16:523 (1998)), however in these studies T cell recognition differences were associated with changes in MHC binding and no rules were defined for the kinds of amino acid substitutions involved in obtaining heteroclicity. The mechanism by which such a translation from changing secondary anchors to change in T cell recognition takes place is currently unclear. However, some models suggest that changes in the way residues at secondary anchor positions engage the MHC may lead to alteration in the orientation or increased flexibility of TCR contact residues, resulting in enhancement of the binding of these analogs to the TCR (Kersh, *et al., J. Exp. Med.* 184:1259 (1996), Evavold, *et al., J. Immunol.* 148:347 (1992), Alam, *et al., Immunity* 10:227 (1999), Hampl, *et al., Immunity* 7:379-85 (1997)). Also, some previous studies implied that modulation of T cell responses by heteroclitic analogs directly involve main TCR contact residues (Zaremba, *et al., Cancer Research* 57:4570 (1997), Loftus, *et al., Cancer Research* 58:2433 (1998), Dressel, *et al., J. Immunol.* 159:4943 (1997)). This finding, however, is not corroborated by the current systematic analysis. The enhanced T cell recognition against analogs identified in the present study is not likely due to increases in MHC binding capacity, though increased binding is likely to play an important role in the case of analogs in which primary anchor positions have been optimized. The present study suggests that heteroclitic analogs are most likely generated by subtle alterations in conformation rather than by gross alterations of TCR or MHC binding capacity.

Differential regulation of production of Th1 or Th2 cytokines was not observed. Instead, the present data suggested that the heteroclitic analogs increased the production of both Th1 and Th2 responses, although the magnitude and kinetics of the increase may be different. In fact, some groups (Nicholson, *et al., Int. Immunol.* 12(2):205-13 (2000), Parhurst, *et al., J. Immunol.* 157:2539 (1996)) have recently reported such overall stimulation by peptide analogs. This is attributable to a stronger TCR signal induced by analogs, though the mechanism of such overall stimulation remains to be elucidated.

The efficacy of heteroclitic analogs *in vivo* using relevant tumor models or models in which tolerance to self antigens exists is evaluated. Accordingly, it is found that immunization with heteroclitic analogs is a more effective and efficient strategy for vaccination against tumors where raising effective CTLs has so far proved to be a challenge.

To summarize, applicants have identified heteroclitic analogs of a number of different HLA-A2.1-restricted CTL epitopes of cancer and viral origin. The relevant wildtype epitopes are shown in Table 1. All these epitopes have been shown to be immunogenic in our earlier reports (Kawashima, *et al., Human Immunology* 59:1-14 (1998), Ishioka, *et al., J. Immunol.* 162(7):3915-25 (1999)). In initial experiments, the antigenicity of 233 analogs of the CEA.691 and MAGE3.112 CTL epitopes was investigated. The nature of the four heteroclitic analogs identified suggested that heteroclitic substitutions involved conservative substitutions at positions 3, 5 and 7. This hypothesis, was tested in a subsequent study involving three additional epitopes MAGE2.157, HIVPol.476, and HBVPol.455. All of the heteroclitic analogs thus identified conformed to the rules proposed, namely that heteroclitic analogs were associated with conservative or semi-conservative substitutions at positions 3, 5 and/or 7.

To more closely mimic the clinical application of heteroclitic analogs in cancer immunotherapy, the murine epitope, p53.261 was also modified. A partial state of T cell tolerance has been reported for this epitope (Theobald, *et al., Proc. Natl. Acad. Sci.* 92:11993-11997 (1995), Theobald, *et al., J. Exp. Med.*, 185(5):833-841 (1997)). Four out of nine predicted p53.261 analogs were found to induce stronger analog-specific CTL responses *in vivo* compared to the CTL responses induced by the native peptide. More significantly, when the cross-reactivity of the CTL raised by immunization with heteroclitic analogs was analyzed, three p53.261 analogs induced CTL which responded vigorously against the native p53.261epitope. Finally, the relevance of these findings for human CTL was addressed by demonstrating that heteroclitic analogs of the MAGE3.112 epitope are immunogenic for human T cells *in vitro*. The resulting CTL can recognize wildtype naturally processed antigen in the form of tumor cell lines.

The studies presented herein demonstrate that heteroclicity is a global phenomenon, as heteroclitic analogs were identified for all the epitopes studied. In

addition, the present application shows that it is possible to detect heteroclitic analogs both in clonal T cell populations (as has been described earlier studies) as well as in bulk T cell populations following *in vivo* immunization. Moreover, it is demonstrated herein that heteroclicity (both in the HLA A2.1 system as well as for other Class I supermotifs) is associated with discrete structural features which allow rational prediction of heteroclicity.

It is demonstrated, further that p53.261 heteroclitic analogs induce CTLs with higher avidity and also induced these cells in greater numbers (precursor frequency) than those induced with wildtype peptide; heteroclitic CTL induction *in vivo*, and its application to breaking T cell tolerance is demonstrated.

The heteroclitic analogs were effective in raising bulk populations of specific T cells following *in vivo* immunization. Polyclonal responses that bear TCR from multiple TCR genes, are more efficacious in resolving disease states in a clinical setting. Finally, the ability to generate high precursor frequencies of CTL possessing strong cross-reactive avidity against wildtype epitope is important in instances where effective CTL responses against epitopes, normally tolerant to the immune system, are required.

2. Definitions

With regard to a particular amino acid sequence, an "epitope" is a set of amino acid residues which is involved in recognition by a particular immunoglobulin, or in the context of T cells, those residues necessary for recognition by T cell receptor proteins when presented in the context of an HLA encoded by the Major Histocompatibility Complex (MHC). In an immune system setting, *in vitro* or *in vivo*, an epitope is the collective features of a molecule, such as primary, secondary and tertiary peptide structure, and charge, that together form a site recognized by an immunoglobulin, T cell receptor or HLA molecule. Throughout this disclosure epitope and peptide are often used interchangeably. It is to be appreciated, however, that isolated or purified protein or peptide molecules larger than and comprising an epitope of the invention are still within the invention.

A "Class I epitope" refers to a peptide that binds to a Class I HLA molecule. As described herein, a Class I epitope is typically about 8 to about 13 amino acids in length. Binding to the HLA molecule is primarily controlled by two primary anchor

residues, one of which is at the C-terminus of the epitope and the other of which is at positions 2 or 3. Binding may be aided also by one or more secondary anchor residues. For the convenience of the reader, various primary HLA Class I binding anchors are set forth in Table 4. The pattern of anchors is referred to as a "motif." A "supermotif" is a peptide binding specificity shared by HLA molecules encoded by two or more HLA alleles. Preferably, a supermotif-bearing peptide is recognized with high or intermediate affinity (as defined herein) by two or more HLA antigens. Examples of Class I supermotifs include, *e.g.*, A1, A2, A3, A24, B7, B27, B44, B58 and B62.

Throughout this disclosure, "binding data" results are often expressed in terms of "IC_{50}'s." IC_{50} is the concentration of peptide in a binding assay at which 50% inhibition of binding of a reference peptide is observed. Given the conditions in which the assays are run (*i.e.*, limiting HLA proteins and labeled peptide concentrations), these values approximate Kd values. Assays for determining binding are described in detail, *e.g.*, in PCT publications WO 94/20127 and WO 94/03205, incorporated herein by reference. It should be noted that IC_{50} values can change, often dramatically, if the assay conditions are varied, and depending on the particular reagents used (*e.g.*, HLA preparation, etc.). For example, excessive concentrations of HLA molecules will increase the apparent measured IC_{50} of a given ligand.

Alternatively, binding is expressed relative to a reference peptide. Although as a particular assay becomes more, or less, sensitive, the IC_{50}'s of the peptides tested may change somewhat, the binding relative to the reference peptide will not significantly change. For example, in an assay run under conditions such that the IC_{50} of the reference peptide increases 10-fold, the IC_{50} values of the test peptides will also shift approximately 10-fold. Therefore, to avoid ambiguities, the assessment of whether a peptide is a good, intermediate, weak, or negative binder is generally based on its IC_{50}, relative to the IC_{50} of a standard peptide. Binding may also be determined using other assay systems known in the art.

The designation of a residue position in an epitope as the "carboxyl or C-terminus" refers to the residue position at the end of the epitope which is nearest to the carboxyl terminus of a peptide, which is designated using conventional nomenclature as defined below. The "C-terminus" of the epitope may or may not actually correspond to the end of the peptide or polypeptide.

The designation of a residue position in an epitope as "N-terminus" or "amino-terminal position" refers to the residue position at the end of the epitope which is nearest to the N-terminus of a peptide, which is designated using conventional nomenclature as defined below. The "N-terminus" of the epitope may or may not actually correspond to the end of the peptide or polypeptide.

A "computer" or "computer system" generally includes: a processor; at least one information storage/retrieval apparatus such as, for example, a hard drive, a disk drive or a tape drive; at least one input apparatus such as, for example, a keyboard, a mouse, a touch screen, or a microphone; and display structure. Additionally, the computer may include a communication channel in communication with a network. Such a computer may include more or less than what is listed above.

As used herein amino acids that are "conserved" or "conservative" and "semi-conserved" or "semi-conservative" are defined in accordance with Preparation B and set forth in Table 2.

As used herein, "high affinity" with respect to HLA Class I molecules is defined as binding with an IC_{50}, or K_D value, of 50 nM or less; "intermediate affinity" is binding with an IC_{50} or K_D value of between about 50 and about 500 nM. "High affinity" with respect to binding to HLA Class II molecules is defined as binding with an IC_{50} or K_D value of 100 nM or less; "intermediate affinity" is binding with an IC_{50} or K_D value of between about 100 and about 1000 nM.

An "immunogenic peptide" or "peptide epitope" is a peptide that comprises an allele-specific motif or supermotif such that the peptide will bind an HLA molecule and induce a CTL and/or HTL response. Thus, immunogenic peptides of the invention are capable of binding to an appropriate HLA molecule and thereafter inducing a cytotoxic T cell response, or a helper T cell response, to the antigen from which the immunogenic peptide is derived.

The phrases "isolated" or "biologically pure" refer to material that is substantially or essentially free from components which normally accompany the material as it is found in its native state. Thus, isolated peptides in accordance with the invention preferably do not contain materials normally associated with the peptides in their *in situ* environment.

A "PanDR binding peptide" is a member of a family of molecules that binds more that one HLA Class II DR molecule (*e.g.*, PADRE™ peptide, Epimmune Inc.,

San Diego, CA). The pattern that defines the PADRE™ family of molecules can be thought of as an HLA Class II supermotif. Peptides comprising the pattern found in PADRE™ molecules bind to most HLA-DR molecules and stimulate *in vitro* and *in vivo* human helper T lymphocyte (HTL) responses.

"Pharmaceutically acceptable" refers to a generally non-toxic, inert, and/or physiologically compatible composition.

3. Peptides of the Invention

Peptides in accordance with the invention can be prepared synthetically, by recombinant DNA technology or chemical synthesis, or from natural sources such as native tumors or pathogenic organisms. Peptide epitopes may be synthesized individually or as polyepitopic peptides. Although the peptide will preferably be substantially free of other naturally occurring host cell proteins and fragments thereof, in some embodiments the peptides may be synthetically conjugated to native fragments or particles.

HLA Class I peptides are well known in the art and are defined as peptides that bind to MHC Class I molecules. The peptides in accordance with the invention can be a variety of lengths, and either in their neutral (uncharged) forms or in forms which are salts. The peptides in accordance with the invention are either free of modifications such as glycosylation, side chain oxidation, or phosphorylation; or they contain these modifications, subject to the condition that modifications do not destroy the biological activity of the peptides as described herein.

Class I epitopes that serve as the corresponding "wildtype" can be derived from any proteinaceous source. For example, the Class I peptides can be derived from viral antigens, tumor-associated antigens, parasitic antigens, bacterial antigens or fungal antigens. In some preferred aspects of the invention, the Class I peptide(s) are derived from antigens for which a the immune system of a subject has developed a tolerance, *i.e.*, a specific immunologic nonresponsiveness induced by prior exposure to an antigen.

Thus, heteroclitic analogs based on a number of potential target epitopes can be used in the present invention. Examples of suitable tumor-associated antigens include prostate specific antigens (PSA), melanoma antigens MAGE 1, MAGE 2, MAGE 3, MAGE-11, MAGE-A10, as well as BAGE, GAGE, RAGE, MAGE-C1,

LAGE-1, CAG-3, DAM, MUC1, MUC2, MUC18, NY-ESO-1, MUM-1, CDK4, BRCA2, NY-LU-1, NY-LU-7, NY-LU-12, CASP8, RAS, KIAA-2-5, SCCs, p53, p73, CEA, Her 2/neu, Melan-A, gp100, tyrosinase, TRP2, gp75/TRP1, kallikrein, prostate-specific membrane antigen (PSM), prostatic acid phosphatase (PAP), prostate-specific antigen (PSA), PT1-1, B-catenin, PRAME, Telomerase, FAK, cyclin D1 protein, NOEY2, EGF-R, SART-1, CAPB, HPVE7, p15, Folate receptor CDC27, PAGE-1, and PAGE-4. Examples of suitable infectious disease-associated antigens include hepatitis B core and surface antigens (HBVc, HBVs), hepatitis C antigens, Epstein-Barr virus antigens, human immunodeficiency virus (HIV) antigens and human papilloma virus (HPV) antigens, *Mycobacterium tuberculosis* and *Chlamydia*. Examples of suitable fungal antigens include those derived from *Candida albicans, Cryptococcus neoformans, Coccidoides spp., Histoplasma spp,* and *Aspergillus fumigatis*. Examples of suitable protozoal parasitic antigens include those derived from *Plasmodium spp.*, including *P. falciparum*, *Trypanosoma spp., Schistosoma spp., Leishmania spp* and the like.

The epitopes that may be used as wildtype sequences to which the rules of the invention are applied to construct corresponding heteroclitic analogs can be found corresponding to any Class I epitope. For any desired antigen, such as those set forth above, the motif associated with a particular Class I allele can be used as a guide to determine the positions in the amino acid sequence of the antigen wherein such an epitope would reside. This determination can be done visually or, preferably, using computer technology and associated software. Thus, for example, by recognition of the A3 supermotif as containing, for example, valine in position 2 and arginine at the C-terminus, the amino acid sequence of any desired antigen can be surveyed for epitopes bearing this motif. That epitope can then be modified according to the rules set forth in the present invention to obtain the desired analogs.

When possible, it may be desirable to optimize HLA Class I binding epitopes of the invention, such as can be used in a polyepitopic construct, to a length of about 8 to about 13 amino acid residues, often 8 to 11, preferably 9 to 10. Preferably, the peptide epitopes are commensurate in size with endogenously processed pathogen-derived peptides or tumor cell peptides that are bound to the relevant HLA molecules, however, the identification and preparation of peptides that comprise epitopes of the invention can also be carried out using the techniques described herein.

In alternative embodiments, epitopes of the invention can be linked as a polyepitopic peptide, or as a minigene that encodes a polyepitopic peptide.

In another embodiment, it is preferred to identify native peptide regions that contain a high concentration of Class I epitopes and/or Class II epitopes. Such a sequence is generally selected on the basis that it contains the greatest number of epitopes per amino acid length. It is to be appreciated that epitopes can be present in a nested or overlapping manner, *e.g.*, a 10 amino acid long peptide could contain two 9 amino acid long epitopes and one 10 amino acid long epitope; upon intracellular processing, each epitope can be exposed and bound by an HLA molecule upon administration of such a peptide. This larger, preferably multi-epitopic, peptide can be generated synthetically, recombinantly, or via cleavage from the native source.

The peptides of the invention can be prepared in a wide variety of ways. For the preferred relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. (*See*, for example, Stewart & Young, SOLID PHASE PEPTIDE SYNTHESIS, 2D. ED., Pierce Chemical Co., 1984). Further, individual peptide epitopes can be joined using chemical ligation to produce larger peptides that are still within the bounds of the invention.

Alternatively, recombinant DNA technology can be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook, *et al.*, MOLECULAR CLONING, A LABORATORY MANUAL, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989). Thus, recombinant polypeptides which comprise one or more peptide sequences of the invention can be used to present the appropriate T cell epitope.

The nucleotide coding sequence for peptide epitopes of the preferred lengths contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, *et al., J. Am. Chem. Soc.* 103:3185 (1981). Peptide analogs can be made simply by substituting the appropriate and desired nucleic acid base(s) for those that encode the native peptide sequence; exemplary nucleic acid substitutions are those that encode an amino acid defined by the

motifs/supermotifs herein. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast, insect or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

Analogs of the present invention may include peptides containing substitutions to modify the physical property (*e.g.*, stability or solubility) of the resulting peptide. For example, peptides may be modified by the substitution of a cysteine (C) with α-amino butyric acid. Due to its chemical nature, cysteine has the propensity to form disulfide bridges and sufficiently alter the peptide structurally so as to reduce binding capacity. Substituting α-amino butyric acid for C not only alleviates this problem, but actually improves binding and crossbinding capability in certain instances. Substitution of cysteine with α-amino butyric acid may occur at any residue of a peptide epitope, *i.e.* at either anchor or non-anchor positions.

Modified peptides that have various amino acid mimetics or unnatural amino acids are particularly useful, as they tend to manifest increased stability *in vivo*. Such analogs may also possess improved shelf-life or manufacturing properties. More specifically, non-critical amino acids need not be limited to those naturally occurring in proteins, such as L-α-amino acids, or their D-isomers, but may include non-natural amino acids as well, such as amino acids mimetics, *e.g.* D- or L- naphylalanine; D- or L- phenylglycine; D- or L-2-thieneylalanine; D- or L-1, -2, 3-, or 4- pyreneylalanine; D- or L-3 thieneylalanine; D- or L-(2-pyridinyl)-alanine; D- or L-(3-pyridinyl)-alanine; D- or L-(2-pyrazinyl)-alanine; D- or L-(4-isopropyl)-phenylglycine; D-(trifluoromethyl)-phenylglycine; D-(trifluoromethyl)-phenylalanine; D-ρ-fluorophenylalanine; D- or L-ρ-biphenylphenylalanine; D- or L- ρ-methoxybiphenylphenylalanine; D- or L-2-indole(alkyl)alanines; and, D- or L-alkylalanines, where the alkyl group can be a

substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, or a non-acidic amino acids. Aromatic rings of a nonnatural amino acid include, *e.g.*, thiazolyl, thiophenyl, pyrazolyl, benzimidazolyl, naphthyl, furanyl, pyrrolyl, and pyridyl aromatic rings.

Peptide stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. *See, e.g.*, Verhoef, *et al.*, *Eur. J. Drug Metab. Pharmacokinetics* **11**:291 (1986). Half life of the peptides of the present invention is conveniently determined using a 25% human serum (v/v) assay. The protocol is generally as follows: Pooled human serum (Type AB, non-heat inactivated) is delipidated by centrifugation before use. The serum is then diluted to 25% with RPMI-1640 or another suitable tissue culture medium. At predetermined time intervals, a small amount of reaction solution is removed and added to either 6% aqueous trichloroacetic acid (TCA) or ethanol. The cloudy reaction sample is cooled (4°C) for 15 minutes and then spun to pellet the precipitated serum proteins. The presence of the peptides is then determined by reversed-phase HPLC using stability-specific chromatography conditions.

4. Class I motifs

In the past few years, evidence has accumulated to demonstrate that a large fraction of HLA Class I molecules can be classified into a relatively few supertypes, each characterized by largely overlapping peptide binding repertoires, and consensus structures of the main peptide binding pockets. Thus, peptides of the present invention are identified by any one of several HLA-specific amino acid motifs (*see, e.g.*, Tables 3-4), or if the presence of the motif corresponds to the ability to bind several allele-specific HLA antigens, a supermotif. The HLA molecules that bind to peptides that possess a particular amino acid supermotif are collectively referred to as an HLA "supertype."

For the convenience of the reader, the peptide motifs and supermotifs described below, and summarized in Tables 3-4, provide guidance for the identification and use of peptide epitopes in accordance with the invention. This will permit identification of candidate wildtype epitopes corresponding to various Class I motifs different from those illustrated in the examples below or epitopes bearing those

illustrated below but in different antigens in order to apply the rules set forth herein to construct analogs.

Heteroclitic analogs can be designed according to the methods of the invention from a peptide, without regard to the motif or supermotif to which the peptide belongs. The primary anchor residues of the HLA Class I peptide epitope supermotifs and motifs delineated below are summarized in Table 3. The HLA Class I motifs set out in Table 3(a) are those most particularly relevant to the invention claimed here. Allele-specific HLA molecules that comprise HLA Class I supertype families are listed in Table 4. In some cases, peptide epitopes may be listed in both a motif and a supermotif. The relationship of a particular motif and respective supermotif is indicated in the description of the individual motifs.

i. HLA-A1 supermotif

The HLA-A1 supermotif is characterized by the presence in peptide ligands of a small (T or S) or hydrophobic (L, I, V, or M) primary anchor residue in position 2, and an aromatic (Y, F, or W) primary anchor residue at the C-terminal position of the epitope. The corresponding family of HLA molecules that bind to the A1 supermotif (*i.e.*, the HLA-A1 supertype) is comprised of at least A*0101, A*2601, A*2602, A*2501, and A*3201 (*see, e.g.*, DiBrino, M. *et al.*, *J. Immunol.* 151:5930, 1993; DiBrino, M. *et al.*, *J. Immunol.* 152:620, 1994; Kondo, A. *et al.*, *Immunogenetics* 45:249, 1997). Other allele-specific HLA molecules predicted to be members of the A1 superfamily are shown in Table 4.

ii. HLA-A2 supermotif

Primary anchor specificities for allele-specific HLA-A2.1 molecules (*see, e.g.*, Falk *et al.*, *Nature* 351:290-296, 1991; Hunt *et al.*, *Science* 255:1261-1263, 1992; Parker *et al.*, *J. Immunol.* 149:3580-3587, 1992; Ruppert *et al.*, *Cell* 74:929-937, 1993) and cross-reactive binding among HLA-A2 and -A28 molecules have been described. (*See, e.g.*, Fruci *et al.*, *Human Immunol.* 38:187-192, 1993; Tanigaki *et al.*, *Human Immunol.* 39:155-162, 1994; Del Guercio *et al.*, *J. Immunol.* 154:685-693, 1995; Kast *et al.*, *J. Immunol.* 152:3904-3912, 1994 for reviews of relevant data.) These primary anchor residues define the HLA-A2 supermotif; which presence in peptide ligands corresponds to the ability to bind several different HLA-A2 and -A28

molecules. The HLA-A2 supermotif comprises peptide ligands with L, I, V, M, A, T, or Q as a primary anchor residue at position 2 and L, I, V, M, A, or T as a primary anchor residue at the C-terminal position of the epitope.

The corresponding family of HLA molecules (*i.e.*, the HLA-A2 supertype that binds these peptides) is comprised of at least: A*0201, A*0202, A*0203, A*0204, A*0205, A*0206, A*0207, A*0209, A*0214, A*6802, and A*6901. Other allele-specific HLA molecules predicted to be members of the A2 superfamily are shown in Table 4.

iii. HLA-A3 supermotif

The HLA-A3 supermotif is characterized by the presence in peptide ligands of A, L, I, V, M, S, or, T as a primary anchor at position 2, and a positively charged residue, R or K, at the C-terminal position of the epitope, *e.g.*, in position 9 of 9-mers (*see, e.g.*, Sidney *et al.*, *Hum. Immunol.* 45:79, 1996). Exemplary members of the corresponding family of HLA molecules (the HLA-A3 supertype) that bind the A3 supermotif include at least A*0301, A*1101, A*3101, A*3301, and A*6801. Other allele-specific HLA molecules predicted to be members of the A3 supertype are shown in Table 3.

iv. HLA-A24 supermotif

The HLA-A24 supermotif is characterized by the presence in peptide ligands of an aromatic (F, W, or Y) or hydrophobic aliphatic (L, I, V, M, or T) residue as a primary anchor in position 2, and Y, F, W, L, I, or M as primary anchor at the C-terminal position of the epitope (*see, e.g.*, Sette and Sidney, *Immunogenetics*, in press, 1999). The corresponding family of HLA molecules that bind to the A24 supermotif (*i.e.*, the A24 supertype) includes at least A*2402, A*3001, and A*2301. Other allele-specific HLA molecules predicted to be members of the A24 supertype are shown in Table 3.

v. HLA-B7 supermotif

The HLA-B7 supermotif is characterized by peptides bearing proline in position 2 as a primary anchor, and a hydrophobic or aliphatic amino acid (L, I, V, M, A, F, W, or Y) as the primary anchor at the C-terminal position of the epitope. The corresponding family of HLA molecules that bind the B7 supermotif (*i.e.*, the

HLA-B7 supertype) is comprised of at least twenty six HLA-B proteins including: B*0702, B*0703, B*0704, B*0705, B*1508, B*3501, B*3502, B*3503, B*3504, B*3505, B*3506, B*3507, B*3508, B*5101, B*5102, B*5103, B*5104, B*5105, B*5301, B*5401, B*5501, B*5502, B*5601, B*5602, B*6701, and B*7801 (*see, e.g.,* Sidney, *et al., J. Immunol.* 154:247, 1995; Barber, *et al., Curr. Biol.* 5:179, 1995; Hill, *et al., Nature* 360:434, 1992; Rammensee, *et al., Immunogenetics* 41:178, 1995 for reviews of relevant data). Other allele-specific HLA molecules predicted to be members of the B7 supertype are shown in Table 3.

vi. HLA-B27 supermotif

The HLA-B27 supermotif is characterized by the presence in peptide ligands of a positively charged (R, H, or K) residue as a primary anchor at position 2, and a hydrophobic (F, Y, L, W, M, I, A, or V) residue as a primary anchor at the C-terminal position of the epitope (*see, e.g.*, Sidney and Sette, *Immunogenetics*, in press, 1999). Exemplary members of the corresponding family of HLA molecules that bind to the B27 supermotif (*i.e.*, the B27 supertype) include at least B*1401, B*1402, B*1509, B*2702, B*2703, B*2704, B*2705, B*2706, B*3801, B*3901, B*3902, and B*7301. Other allele-specific HLA molecules predicted to be members of the B27 supertype are shown in Table 3.

vii. HLA-B44 supermotif

The HLA-B44 supermotif is characterized by the presence in peptide ligands of negatively charged (D or E) residues as a primary anchor in position 2, and hydrophobic residues (F, W, Y, L, I, M, V, or A) as a primary anchor at the C-terminal position of the epitope (*see, e.g.*, Sidney *et al.*, *Immunol. Today* 17:261, 1996). Exemplary members of the corresponding family of HLA molecules that bind to the B44 supermotif (*i.e.*, the B44 supertype) include at least: B*1801, B*1802, B*3701, B*4001, B*4002, B*4006, B*4402, B*4403, and B*4006.

viii. HLA-B58 supermotif

The HLA-B58 supermotif is characterized by the presence in peptide ligands of a small aliphatic residue (A, S, or T) as a primary anchor residue at position 2, and an aromatic or hydrophobic residue (F, W, Y, L, I, V, M, or A) as a primary anchor residue at the C-terminal position of the epitope (*see, e.g.*, Sidney and Sette,

Immunogenetics, in press, 1999 for reviews of relevant data). Exemplary members of the corresponding family of HLA molecules that bind to the B58 supermotif (*i.e.*, the B58 supertype) include at least: B*1516, B*1517, B*5701, B*5702, and B*5801. Other allele-specific HLA molecules predicted to be members of the B58 supertype are shown in Table 3.

ix. HLA-B62 supermotif

The HLA-B62 supermotif is characterized by the presence in peptide ligands of the polar aliphatic residue Q or a hydrophobic aliphatic residue (L, V, M, I, or P) as a primary anchor in position 2, and a hydrophobic residue (F, W, Y, M, I, V, L, or A) as a primary anchor at the C-terminal position of the epitope (*see*, *e.g.*, Sidney and Sette, *Immunogenetics*, in press, 1999). Exemplary members of the corresponding family of HLA molecules that bind to the B62 supermotif (*i.e.*, the B62 supertype) include at least: B*1501, B*1502, B*1513, and B5201. Other allele-specific HLA molecules predicted to be members of the B62 supertype are shown in Table 3.

x. HLA-A1 motif

The HLA-A1 motif is characterized by the presence in peptide ligands of T, S, or M as a primary anchor residue at position 2 and the presence of Y as a primary anchor residue at the C-terminal position of the epitope. An alternative allele-specific A1 motif is characterized by a primary anchor residue at position 3 rather than position 2. This motif is characterized by the presence of D, E, A, or S as a primary anchor residue in position 3, and a Y as a primary anchor residue at the C-terminal position of the epitope (*see*, *e.g.*, DiBrino *et al.*, J. Immunol., 152:620, 1994; Kondo *et al.*, *Immunogenetics* 45:249, 1997; and Kubo *et al.*, *J. Immunol.* 152:3913, 1994 for reviews of relevant data).

*xi. HLA-A*0201 motif*

An HLA-A2*0201 motif was determined to be characterized by the presence in peptide ligands of L or M as a primary anchor residue in position 2, and L or V as a primary anchor residue at the C-terminal position of a 9-residue peptide (*see*, *e.g.*, Falk *et al.*, *Nature* 351:290-296, 1991) and was further found to comprise an I at position 2 and I or A at the C-terminal position of a nine amino acid peptide

(*see, e.g.*, Hunt *et al.*, Science 255:1261-1263, March 6, 1992; Parker *et al.*, *J. Immunol.* 149:3580-3587, 1992). The A*0201 allele-specific motif has also been defined by the present inventors to additionally comprise V, A, T, or Q as a primary anchor residue at position 2, and M or T as a primary anchor residue at the C-terminal position of the epitope (*see, e.g.*, Kast *et al.*, *J. Immunol.* 152:3904-3912, 1994). Thus, the HLA-A*0201 motif comprises peptide ligands with L, I, V, M, A, T, or Q as primary anchor residues at position 2 and L, I, V, M, A, or T as a primary anchor residue at the C-terminal position of the epitope. The preferred and tolerated residues that characterize the primary anchor positions of the HLA-A*0201 motif are identical to the residues describing the A2 supermotif.

xii. HLA-A3 motif

The HLA-A3 motif is characterized by the presence in peptide ligands of L, M, V, I, S, A, T, F, C, G, or D as a primary anchor residue at position 2, and the presence of K, Y, R, H, F, or A as a primary anchor residue at the C-terminal position of the epitope (*see, e.g.*, DiBrino *et al.*, *Proc. Natl. Acad. Sci USA* 90:1508, 1993; and Kubo *et al.*, *J. Immunol.* 152:3913-3924, 1994).

xiii. HLA-A11 motif

The HLA-A11 motif is characterized by the presence in peptide ligands of V, T, M, L, I, S, A, G, N, C, D, or F as a primary anchor residue in position 2, and K, R, Y, or H as a primary anchor residue at the C-terminal position of the epitope (*see, e.g.*, Zhang *et al.*, *Proc. Natl. Acad. Sci USA* 90:2217-2221, 1993; and Kubo *et al.*, *J. Immunol.* 152:3913-3924, 1994).

xiv. HLA-A24 motif

The HLA-A24 motif is characterized by the presence in peptide ligands of Y, F, W, or M as a primary anchor residue in position 2, and F, L, I, or W as a primary anchor residue at the C-terminal position of the epitope (*see, e.g.*, *Kondo et al.*, *J. Immunol.* 155:4307-4312, 1995; and Kubo *et al.*, *J. Immunol.* 152:3913-3924, 1994).

5. Assays to Detect T-Cell Responses

Once heteroclitic analogs of the invention are synthesized, they can be tested for the ability to elicit a T-cell response. The preparation and evaluation of motif-bearing peptides such as heteroclitic analogs are described in PCT publications WO 94/20127 and WO 94/03205. Briefly, peptides comprising epitopes from a particular antigen are synthesized and tested for their ability to bind to the appropriate HLA proteins. These assays may involve evaluating the binding of a peptide of the invention to purified HLA Class I molecules in relation to the binding of a radioiodinated reference peptide. Alternatively, cells expressing empty Class I molecules (*i.e.* lacking peptide therein) may be evaluated for peptide binding by immunofluorescent staining and flow microfluorimetry. Other assays that may be used to evaluate peptide binding include peptide-dependent Class I assembly assays and/or the inhibition of CTL recognition by peptide competition. Those peptides that bind to the Class I molecule, typically with an affinity of 500 nM or less, are further evaluated for their ability to serve as targets for CTLs derived from infected or immunized individuals, as well as for their capacity to induce primary *in vitro* or *in vivo* CTL responses that can give rise to CTL populations capable of reacting with selected target cells associated with a disease.

Conventional assays utilized to detect T cell responses include proliferation assays, lymphokine secretion assays, direct cytotoxicity assays, and limiting dilution assays. Such assays are useful in comparing the induction of immune responses by heteroclitic analog peptides to response induced by non-heteroclitic analogs Class I peptides (*e.g.*, from which the heterocloitic analog sequence was based). For example, antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce CTL responses in responder cell populations. Antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells. Alternatively, mutant non-human mammalian cell lines that are deficient in their ability to load Class I molecules with internally processed peptides and that have been transfected with the appropriate human Class I gene, may be used to test for the capacity of the peptide to induce *in vitro* primary CTL responses.

Peripheral blood mononuclear cells (PBMCs) may be used as the responder cell source of CTL precursors. The appropriate antigen-presenting cells are incubated

with peptide, after which the peptide-loaded antigen-presenting cells are then incubated with the responder cell population under optimized culture conditions. Positive CTL activation can be determined by assaying the culture for the presence of CTLs that kill radio-labeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed forms of the antigen from which the peptide sequence was derived.

Additionally, a method has been devised which allows direct quantification of antigen-specific T cells by staining with Fluorescein-labelled HLA tetrameric complexes (Altman, J. D. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:10330, 1993; Altman, J. D. *et al.*, *Science* 274:94, 1996). Other relatively recent technical developments include staining for intracellular lymphokines, and interferon-γ release assays or Elispot assays. Tetramer staining, intracellular lymphokine staining and Elispot assays all appear to be at least 10-fold more sensitive than more conventional assays (Lalvani, A. *et al.*, *J. Exp. Med.* 186:859, 1997; Dunbar, P. R. *et al.*, *Curr. Biol.* 8:413, 1998; Murali-Krishna, K. *et al.*, *Immunity* 8:177, 1998).

If desired, HTL activation may also be assessed using such techniques known to those in the art such as T cell proliferation and secretion of lymphokines, *e.g.* IL-2 (*see, e.g.* Alexander, *et al.*, *Immunity* 1:751-761, 1994).

Alternatively, immunization of HLA transgenic mice can be used to determine immunogenicity of peptide epitopes. Several transgenic mouse models including mice with human A2.1, A11 (which can additionally be used to analyze HLA-A3 epitopes), and B7 alleles have been characterized and others (*e.g.*, transgenic mice for HLA-A1 and A24) are being developed. HLA-DR1 and HLA-DR3 mouse models have also been developed. Additional transgenic mouse models with other HLA alleles may be generated as necessary. The mice may be immunized with peptides emulsified in Incomplete Freund's Adjuvant and the resulting T cells tested for their capacity to recognize peptide-pulsed target cells and target cells transfected with appropriate genes. CTL responses may be analyzed using cytotoxicity assays described above. Similarly, HTL responses may be analyzed using such assays as T cell proliferation or secretion of lymphokines.

Heteroclitic analogs of the invention often induce both Th1 and Th2 cytokine responses. Therefore, one method to compare a heteroclitic candidate with a preselected Class I peptide is to test the induction of Th1 and Th2 cytokines. The

preselected Class I peptide will typically be a peptide from which the heteroclitic analog is derived, or if such a peptide does not exist, a Class I peptide with the highest similarity to the candidate. Heteroclitic analogs of the invention typically induce both Th1 and Th2 cytokine responses, but at a level greatly enhanced compared to the Class I peptide from which the analog was derived. For example, a given heteroclitic analog will stimulate an equivalent level of Th1 or Th2 cytokine (50 to 100 pg/ml) at a 10-fold or lower dose compared to the wildtype peptide from which the analog was derived. Additionally, where the Class I peptide induces only, or mainly, either a Th1 or Th2 response, a heteroclitic analog may induce both Th1 and Th2 responses. Th1 cytokines include, *e.g.*, IFNγ, IL-2 and IL-3. Th2 cytokines include, *e.g.*, IL-4, IL-5, IL-6 and IL-10. Production of cytokines can be measured, for example, using ELISA or other immunological quantitation methods. *See*, *e.g.*, McKinney, *et al. Journal of Immunological Methods* 237:105-117 (2000).

6. Use of Peptide Epitopes as Diagnostic Agents and for Evaluating Immune Responses

In one embodiment of the invention, heteroclitic analog peptides as described herein are used as reagents to evaluate an immune response. The immune response to be evaluated is induced by using as an immunogen any agent that may result in the induction of antigen-specific CTLs or HTLs that recognize and bind to the peptide epitope(s) to be employed as the reagent. The peptide reagent need not be used as the immunogen. Assay systems that are used for such an analysis include relatively recent technical developments such as tetramers, staining for intracellular lymphokines and interferon release assays, or Elispot assays.

For example, peptides of the invention are used in tetramer staining assays to assess peripheral blood mononuclear cells for the presence of antigen-specific CTLs following exposure to a tumor cell antigen or an immunogen. The HLA-tetrameric complex is used to directly visualize antigen-specific CTLs (*see*, *e.g.*, Ogg *et al.*, *Science* 279:2103-2106, 1998; and Altman *et al.*, *Science* 174:94-96, 1996) and determine the frequency of the antigen-specific CTL population in a sample of peripheral blood mononuclear cells. A tetramer reagent using a peptide of the invention is generated as follows: A peptide that binds to an HLA molecule is refolded in the presence of the corresponding HLA heavy chain and β_2-microglobulin

to generate a trimolecular complex. The complex is biotinylated at the carboxyl terminal end of the heavy chain at a site that was previously engineered into the protein. Tetramer formation is then induced by the addition of streptavidin. By means of fluorescently labeled streptavidin, the tetramer can be used to stain antigen-specific cells. The cells can then be identified, for example, by flow cytometry. Such an analysis may be used for diagnostic or prognostic purposes. Cells identified by the procedure can also be used for therapeutic purposes.

Peptides of the invention are also used as reagents to evaluate immune recall responses (*see, e.g.*, Bertoni, *et al.*, *J. Clin. Invest.* 100:503-513, 1997 and Penna, *et al.*, *J. Exp. Med.* 174:1565-1570, 1991). For example, patient PBMC samples from individuals with cancer are analyzed for the presence of antigen-specific CTLs or HTLs using specific peptides. A blood sample containing mononuclear cells can be evaluated by cultivating the PBMCs and stimulating the cells with a peptide of the invention. After an appropriate cultivation period, the expanded cell population can be analyzed, for example, for CTL or for HTL activity.

The peptides are also used as reagents to evaluate the efficacy of a vaccine. PBMCs obtained from a patient vaccinated with an immunogen are analyzed using, for example, either of the methods described above. The patient is HLA typed, and peptide epitope reagents that recognize the allele-specific molecules present in that patient are selected for the analysis. The immunogenicity of the vaccine is indicated by the presence of epitope-specific CTLs and/or HTLs in the PBMC sample.

The peptides of the invention are also used to make antibodies, using techniques well known in the art (see, *e.g.* *CURRENT PROTOCOLS IN IMMUNOLOGY*, Wiley/Greene, NY; and *Antibodies A Laboratory Manual,* Harlow and Lane, Cold Spring Harbor Laboratory Press, 1989), which may be useful as reagents to diagnose or monitor cancer. Such antibodies include those that recognize a peptide in the context of an HLA molecule, *i.e.*, antibodies that bind to a peptide-MHC complex.

7. Vaccine Compositions

Vaccines and methods of preparing vaccines that contain an immunogenically effective amount of one or more peptides as described herein are further embodiments of the invention. Once appropriately immunogenic epitopes have been defined, they can be sorted and delivered by various means, herein referred to as "vaccine"

compositions. Such vaccine compositions can include, for example, lipopeptides (*e.g.*,Vitiello, A. *et al.*, *J. Clin. Invest.* 95:341, 1995), peptide compositions encapsulated in poly(DL-lactide-co-glycolide) ("PLG") microspheres (*see, e.g.*, Eldridge, *et al.*, *Molec. Immunol.* 28:287-294, 1991: Alonso *et al.*, *Vaccine* 12:299-306, 1994; Jones *et al.*, *Vaccine* 13:675-681, 1995), peptide compositions contained in immune stimulating complexes (ISCOMS) (*see*, *e.g.*, Takahashi *et al.*, *Nature* 344:873-875, 1990; Hu *et al.*, *Clin Exp Immunol.* 113:235-243, 1998), multiple antigen peptide systems (MAPs) (*see e.g.*, Tam, J. P., *Proc. Natl. Acad. Sci. U.S.A.* 85:5409-5413, 1988; Tam, J.P., *J. Immunol. Methods* 196:17-32, 1996), peptides formulated as multivalent peptides; peptides for use in ballistic delivery systems, typically crystallized peptides, viral delivery vectors (Perkus, M. E. *et al.*, In: *Concepts in vaccine development*, Kaufmann, S. H. E., ed., p. 379, 1996; Chakrabarti, S. *et al.*, *Nature* 320:535, 1986; Hu, S. L. *et al.*, *Nature* 320:537, 1986; Kieny, M.-P. *et al.*, *AIDS Bio/Technology* 4:790, 1986; Top, F. H. *et al.*, *J. Infect. Dis.* 124:148, 1971; Chanda, P. K. *et al.*, *Virology* 175:535, 1990), particles of viral or synthetic origin (*e.g.*, Kofler, N. *et al.*, *J. Immunol. Methods.* 192:25, 1996; Eldridge, J. H. *et al.*, *Sem. Hematol.* 30:16, 1993; Falo, L. D., Jr. *et al.*, *Nature Med.* 7:649, 1995), adjuvants (Warren, H. S., Vogel, F. R., and Chedid, L. A. *Annu. Rev. Immunol.* 4:369, 1986; Gupta, R. K. *et al.*, *Vaccine* 11:293, 1993), liposomes (Reddy, R. *et al.*, *J. Immunol.* 148:1585, 1992; Rock, K. L., *Immunol. Today* 17:131, 1996), or, naked or particle absorbed cDNA (Ulmer, J. B. *et al.*, *Science* 259:1745, 1993; Robinson, H. L., Hunt, L. A., and Webster, R. G., *Vaccine* 11:957, 1993; Shiver, J. W. *et al.*, In: *Concepts in vaccine development*, Kaufmann, S. H. E., ed., p. 423, 1996; Cease, K. B., and Berzofsky, J. A., *Annu. Rev. Immunol.* 12:923, 1994 and Eldridge, J. H. *et al.*, *Sem. Hematol.* 30:16, 1993). Toxin-targeted delivery technologies, also known as receptor mediated targeting, such as those of Avant Immunotherapeutics, Inc. (Needham, Massachusetts) may also be used.

Vaccines of the invention include nucleic acid-mediated modalities. DNA or RNA encoding one or more of the peptides of the invention can also be administered to a patient. This approach is described, for instance, in Wolff *et. al.*, *Science* 247:1465 (1990) as well as U.S. Patent Nos. 5,580,859; 5,589,466; 5,804,566; 5,739,118; 5,736,524; 5,679,647; WO 98/04720; and in more detail below. Examples of DNA-based delivery technologies include "naked DNA", facilitated (bupivicaine,

polymers, peptide-mediated) delivery, cationic lipid complexes, and particle-mediated ("gene gun") or pressure-mediated delivery (*see*, *e.g.*, U.S. Patent No. 5,922,687).

For therapeutic or prophylactic immunization purposes, the peptides of the invention can also be expressed by viral or bacterial vectors. Examples of expression vectors include attenuated viral hosts, such as vaccinia or fowlpox. As an example of this approach, vaccinia virus is used as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into a host bearing a tumor, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host CTL and/or HTL response. Vaccinia vectors and methods useful in immunization protocols are described in, *e.g.*, U.S. Patent No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover *et al.*, *Nature* 351:456-460 (1991). A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, *e.g.* adeno and adeno-associated virus vectors, retroviral vectors, *Salmonella typhi* vectors, detoxified anthrax toxin vectors, and the like, will be apparent to those skilled in the art from the description herein.

Furthermore, vaccines in accordance with the invention encompass compositions of one or more of the claimed peptides. A peptide can be present in a vaccine individually. Alternatively, the peptide can exist as a homopolymer comprising multiple copies of the same peptide, or as a heteropolymer of various peptides. Polymers have the advantage of increased immunological reaction and, where different peptide epitopes are used to make up the polymer, the additional ability to induce antibodies and/or CTLs that react with different antigenic determinants of the pathogenic organism or tumor-related peptide targeted for an immune response. The composition can be a naturally occurring region of an antigen or can be prepared, *e.g.*, recombinantly or by chemical synthesis.

Carriers that can be used with vaccines of the invention are well known in the art, and include, *e.g.,* thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly L-lysine, poly L-glutamic acid, influenza, hepatitis B virus core protein, and the like. The vaccines can contain a physiologically tolerable (*i.e.*, acceptable) diluent such as water, or saline, preferably phosphate buffered saline. The vaccines also typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum

hydroxide, or alum are examples of materials well known in the art. Additionally, as disclosed herein, CTL responses can be primed by conjugating peptides of the invention to lipids, such as tripalmitoyl-S-glycerylcysteinlyseryl- serine (P_3CSS).

Upon immunization with a peptide composition in accordance with the invention, via injection, aerosol, oral, transdermal, transmucosal, intrapleural, intrathecal, or other suitable routes, the immune system of the host responds to the vaccine by producing large amounts of CTLs and/or HTLs specific for the desired antigen. Consequently, the host becomes at least partially immune to later infection, or at least partially resistant to developing an ongoing chronic infection, or derives at least some therapeutic benefit when the antigen was tumor-associated.

In some embodiments, it may be desirable to combine the heteroclitic analog peptides of the invention with components that induce or facilitate neutralizing antibody and or helper T cell responses to the target antigen of interest. A preferred embodiment of such a composition comprises Class I and Class II epitopes in accordance with the invention. An alternative embodiment of such a composition comprises a Class I and/or Class II epitope in accordance with the invention, along with a pan-DR binding peptide such as PADRE™ (Epimmune, San Diego, CA) molecule (described, for example, in U.S. Patent Number 5,736,142).

A vaccine of the invention can also include antigen-presenting cells (APC), such as dendritic cells (DC), as a vehicle to present peptides of the invention. Vaccine compositions can be created *in vitro*, following dendritic cell mobilization and harvesting, whereby loading of dendritic cells occurs *in vitro*. For example, dendritic cells are transfected, *e.g.*, with a minigene in accordance with the invention, or are pulsed with peptides. The dendritic cell can then be administered to a patient to elicit immune responses *in vivo*.

Vaccine compositions, either DNA- or peptide-based, can also be administered *in vivo* in combination with dendritic cell mobilization whereby loading of dendritic cells occurs *in vivo*.

Antigenic peptides are used to elicit a CTL and/or HTL response *ex vivo*, as well. The resulting CTL or HTL cells, can be used to treat tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a therapeutic vaccine peptide or nucleic acid in accordance with the invention. *Ex vivo* CTL or HTL responses to a particular tumor-associated antigen are induced by

incubating in tissue culture the patient's, or genetically compatible, CTL or HTL precursor cells together with a source of antigen-presenting cells, such as dendritic cells, and the appropriate immunogenic peptide. After an appropriate incubation time (typically about 7-28 days), in which the precursor cells are activated and expanded into effector cells, the cells are infused back into the patient, where they will destroy (CTL) or facilitate destruction (HTL) of their specific target cell (an infected cell or a tumor cell). Transfected dendritic cells may also be used as antigen presenting cells.

The vaccine compositions of the invention can also be used in combination with other treatments used for cancer, including use in combination with immune adjuvants such as IL-2, IL-12, GM-CSF, and the like.

Preferably, the following principles are utilized when selecting an array of epitopes for inclusion in a polyepitopic composition for use in a vaccine, or for selecting discrete epitopes to be included in a vaccine and/or to be encoded by nucleic acids such as a minigene. It is preferred that each of the following principles are balanced in order to make the selection. The multiple epitopes to be incorporated in a given vaccine composition may be, but need not be, contiguous in sequence in the native antigen from which the epitopes are derived.

1.) Epitopes are selected which, upon administration, mimic immune responses that have been observed to be correlated with tumor clearance. For HLA Class I, this includes 3-4 epitopes that come from at least one tumor-associated antigen (TAA). For HLA Class II, a similar rationale is employed; again 3-4 epitopes are selected from at least one TAA (*see e.g.*, Rosenberg *et al.*, *Science* 278:1447-1450). Epitopes from one TAA may be used in combination with epitopes from one or more additional TAAs to produce a vaccine that targets tumors with varying expression patterns of frequently-expressed TAAs.

2.) Epitopes are selected that have the requisite binding affinity established to be correlated with immunogenicity: for HLA Class I an IC_{50} of 500 nM or less, often 200 nM or less; and for Class II an IC_{50} of 1000 nM or less.

3.) Sufficient supermotif bearing-peptides, or a sufficient array of allele-specific motif-bearing peptides, are selected to give broad population coverage. For example, it is preferable to have at least 80% population coverage. A Monte Carlo analysis, a statistical evaluation known in the art, can be employed to assess the breadth, or redundancy of, population coverage.

4.) When selecting epitopes from cancer-related antigens it is often useful to select analogs because the patient may have developed tolerance to the native epitope. When selecting epitopes for infectious disease-related antigens, it is preferable to select either native or analoged epitopes.

5.) Of particular relevance are epitopes referred to as "nested epitopes." Nested epitopes occur where at least two epitopes overlap in a given peptide sequence. A nested peptide sequence can comprise both HLA Class I and HLA Class II epitopes. When providing nested epitopes, a general objective is to provide the greatest number of epitopes per sequence. Thus, an aspect is to avoid providing a peptide that is any longer than the amino terminus of the amino terminal epitope and the carboxyl terminus of the carboxyl terminal epitope in the peptide. When providing a multi-epitopic sequence, such as a sequence comprising nested epitopes, it is generally important to screen the sequence in order to insure that it does not have pathological or other deleterious biological properties.

6.) If a polyepitopic protein is created, or when creating a minigene, an objective is to generate the smallest peptide that encompasses the epitopes of interest. This principle is similar, if not the same as that employed when selecting a peptide comprising nested epitopes. However, with an artificial polyepitopic peptide, the size minimization objective is balanced against the need to integrate any spacer sequences between epitopes in the polyepitopic protein. Spacer amino acid residues can, for example, be introduced to avoid junctional epitopes (an epitope recognized by the immune system, not present in the target antigen, and only created by the man-made juxtaposition of epitopes), or to facilitate cleavage between epitopes and thereby enhance epitope presentation. Junctional epitopes are generally to be avoided because the recipient may generate an immune response to that non-native epitope. Of particular concern is a junctional epitope that is a "dominant epitope." A dominant epitope may lead to such a zealous response that immune responses to other epitopes are diminished or suppressed.

8. Minigene Vaccines

A number of different approaches are available which allow simultaneous delivery of multiple epitopes. Nucleic acids encoding the peptides of the invention are a particularly useful embodiment of the invention. Epitopes for inclusion in a

minigene are preferably selected according to the guidelines set forth in the previous section. A preferred means of administering nucleic acids encoding the peptides of the invention uses minigene constructs encoding a peptide comprising one or multiple epitopes of the invention.

The use of multi-epitope minigenes is described below and in, *e.g.*, co-pending application U.S.S.N. 09/311,784; Ishioka *et al.*, *J. Immunol.* 162:3915-3925, 1999; An, L. and Whitton, J. L., *J. Virol.* 71:2292, 1997; Thomson, S. A. *et al.*, *J. Immunol.* 157:822, 1996; Whitton, J. L. *et al.*, *J. Virol.* 67:348, 1993; Hanke, R. *et al.*, *Vaccine* 16:426, 1998. For example, a multi-epitope DNA plasmid encoding supermotif- and/or motif-bearing epitopes (*e.g.*, PSA, PSM, PAP, and hK2) derived from multiple regions of a TAA, a pan_DR binding peptide such as the PADRE™ universal helper T cell epitope, and an endoplasmic reticulum-translocating signal sequence can be engineered. A vaccine may also comprise epitopes that are derived from other TAAs.

The immunogenicity of a multi-epitopic minigene can be tested in transgenic mice to evaluate the magnitude of CTL induction responses against the epitopes tested. Further, the immunogenicity of DNA-encoded epitopes *in vivo* can be correlated with the *in vitro* responses of specific CTL lines against target cells transfected with the DNA plasmid. Thus, these experiments can show that the minigene serves to both: 1.) generate a CTL response and 2.) that the induced CTLs recognized cells expressing the encoded epitopes.

For example, to create a DNA sequence encoding the selected epitopes (minigene) for expression in human cells, the amino acid sequences of the epitopes may be reverse translated. A human codon usage table can be used to guide the codon choice for each amino acid. These epitope-encoding DNA sequences may be directly adjoined, so that when translated, a continuous polypeptide sequence is created. To optimize expression and/or immunogenicity, additional elements can be incorporated into the minigene design. Examples of amino acid sequences that can be reverse translated and included in the minigene sequence include: HLA Class I epitopes, HLA Class II epitopes, a ubiquitination signal sequence, and/or an endoplasmic reticulum targeting signal. In addition, HLA presentation of CTL and HTL epitopes may be improved by including synthetic (*e.g.* poly-alanine) or naturally-occurring flanking sequences adjacent to the CTL or HTL epitopes; these larger peptides comprising the epitope(s) are within the scope of the invention.

The minigene sequence may be converted to DNA by assembling oligonucleotides that encode the plus and minus strands of the minigene. Overlapping oligonucleotides (30-100 bases long) may be synthesized, phosphorylated, purified and annealed under appropriate conditions using well known techniques. The ends of the oligonucleotides can be joined, for example, using T4 DNA ligase. This synthetic minigene, encoding the epitope polypeptide, can then be cloned into a desired expression vector.

Standard regulatory sequences well known to those of skill in the art are preferably included in the vector to ensure expression in the target cells. Several vector elements are desirable: a promoter with a down-stream cloning site for minigene insertion; a polyadenylation signal for efficient transcription termination; an *E. coli* origin of replication; and an *E. coli* selectable marker (*e.g.* ampicillin or kanamycin resistance). Numerous promoters can be used for this purpose, *e.g.*, the human cytomegalovirus (hCMV) promoter. See, *e.g.*, U.S. Patent Nos. 5,580,859 and 5,589,466 for other suitable promoter sequences.

Additional vector modifications may be desired to optimize minigene expression and immunogenicity. In some cases, introns are required for efficient gene expression, and one or more synthetic or naturally-occurring introns could be incorporated into the transcribed region of the minigene. The inclusion of mRNA stabilization sequences and sequences for replication in mammalian cells may also be considered for increasing minigene expression.

Once an expression vector is selected, the minigene is cloned into the polylinker region downstream of the promoter. This plasmid is transformed into an appropriate *E. coli* strain, and DNA is prepared using standard techniques. The orientation and DNA sequence of the minigene, as well as all other elements included in the vector, are confirmed using restriction mapping and DNA sequence analysis. Bacterial cells harboring the correct plasmid can be stored as a master cell bank and a working cell bank.

In addition, immunostimulatory sequences (ISSs or CpGś) appear to play a role in the immunogenicity of DNA vaccines. These sequences may be included in the vector, outside the minigene coding sequence, if desired to enhance immunogenicity.

In some embodiments, a bi-cistronic expression vector which allows production of both the minigene-encoded epitopes and a second protein (included to enhance or decrease immunogenicity) can be used. Examples of proteins or polypeptides that could beneficially enhance the immune response if co-expressed include cytokines (*e.g.*, IL-2, IL-12, GM-CSF), cytokine-inducing molecules (*e.g.*, LeIF), costimulatory molecules, or for HTL responses, pan-DR binding proteins (*e.g.*, PADRE™, Epimmune, San Diego, CA). Helper (HTL) epitopes can be joined to intracellular targeting signals and expressed separately from expressed CTL epitopes; this allows direction of the HTL epitopes to a cell compartment different than that of the CTL epitopes. If required, this could facilitate more efficient entry of HTL epitopes into the HLA Class II pathway, thereby improving HTL induction. In contrast to HTL or CTL induction, specifically decreasing the immune response by co-expression of immunosuppressive molecules (*e.g.* TGF-β) may be beneficial in certain diseases.

Therapeutic quantities of plasmid DNA can be produced for example, by fermentation in *E. coli*, followed by purification. Aliquots from the working cell bank are used to inoculate growth medium, and grown to saturation in shaker flasks or a bioreactor according to well known techniques. Plasmid DNA can be purified using standard bioseparation technologies such as solid phase anion-exchange resins supplied by QIAGEN, Inc. (Valencia, California). If required, supercoiled DNA can be isolated from the open circular and linear forms using gel electrophoresis or other methods.

Purified plasmid DNA can be prepared for injection using a variety of formulations. The simplest of these is reconstitution of lyophilized DNA in sterile phosphate-buffered saline (PBS). This approach, known as "naked DNA," is currently being used for intramuscular (IM) administration in clinical trials. To maximize the immunotherapeutic effects of minigene DNA vaccines, an alternative method for formulating purified plasmid DNA may be desirable. A variety of methods have been described, and new techniques may become available. Cationic lipids, glycolipids, and fusogenic liposomes can also be used in the formulation (see, *e.g.*, as described by WO 93/24640; Mannino & Gould-Fogerite, *BioTechniques* 6(7): 682 (1988); U.S. Pat No. 5,279,833; WO 91/06309; and Felgner, *et al., Proc. Nat'l Acad. Sci. USA* 84:7413 (1987). In addition, peptides and compounds referred to

collectively as protective, interactive, non-condensing compounds (PINC) could also be complexed to purified plasmid DNA to influence variables such as stability, intramuscular dispersion, or trafficking to specific organs or cell types.

Target cell sensitization can be used as a functional assay for expression and HLA Class I presentation of minigene-encoded CTL epitopes. For example, the plasmid DNA is introduced into a mammalian cell line that is suitable as a target for standard CTL chromium release assays. The transfection method used will be dependent on the final formulation. Electroporation can be used for "naked" DNA, whereas cationic lipids allow direct *in vitro* transfection. A plasmid expressing green fluorescent protein (GFP) can be co-transfected to allow enrichment of transfected cells using fluorescence activated cell sorting (FACS). These cells are then chromium-51 (^{51}Cr) labeled and used as target cells for epitope-specific CTL lines; cytolysis, detected by ^{51}Cr release, indicates both production of, and HLA presentation of, minigene-encoded CTL epitopes. Expression of HTL epitopes may be evaluated in an analogous manner using assays to assess HTL activity.

In vivo immunogenicity is a second approach for functional testing of minigene DNA formulations. Transgenic mice expressing appropriate human HLA proteins are immunized with the DNA product. The dose and route of administration can be formulation dependent (*e.g.*, IM for DNA in PBS, intraperitoneal (IP) for lipid-complexed DNA). Twenty-one days after immunization, splenocytes are harvested and restimulated for one week in the presence of peptides encoding each epitope being tested. Thereafter, for CTL effector cells, assays are conducted for cytolysis of peptide-loaded, ^{51}Cr-labeled target cells using standard techniques. Lysis of target cells that were sensitized by HLA loaded with peptide epitopes, corresponding to minigene-encoded epitopes, demonstrates DNA vaccine function for *in vivo* induction of CTLs. Immunogenicity of HTL epitopes is evaluated in transgenic mice in an analogous manner.

Alternatively, the nucleic acids can be administered intradermally, *e.g.* by injection or ballistic delivery as described, for instance, in U.S. Patent No. 5,204,253. Using this technique, particles comprised solely of DNA are administered. In a further alternative embodiment, DNA can be adhered to particles, such as gold particles.

Minigenes can also be delivered using other bacterial or viral delivery systems well known in the art, *e.g.*, an expression construct encoding epitopes of the invention can be incorporated into a viral vector such as vaccinia.

9. Combinations of CTL Peptides with Helper Peptides

Vaccine compositions comprising the peptides of the present invention can be modified to provide desired attributes, such as improved serum half-life, or to enhance immunogenicity.

For instance, the ability of a peptide to induce CTL activity can be enhanced by linking the peptide to a sequence which contains at least one epitope that is capable of inducing a T helper cell response. The use of T helper epitopes in conjunction with CTL epitopes to enhance immunogenicity is illustrated, for example, in the co-pending applications U.S.S.N. 08/820,360, U.S.S.N. 08/197,484, and U.S.S.N. 08/464,234.

Although a CTL peptide can be directly linked to a T helper peptide, often CTL epitope/HTL epitope conjugates are linked by a spacer molecule. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions. The spacers are typically selected from, *e.g.*, Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. When present, the spacer will usually be at least one or two residues, more usually three to six residues and sometimes 10 or more residues. The CTL peptide epitope can be linked to the T helper peptide epitope either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the immunogenic peptide or the T helper peptide may be acylated.

In certain embodiments, the T helper peptide is one that is recognized by T helper cells present in the majority of the population. This can be accomplished by selecting amino acid sequences that bind to many, most, or all of the HLA Class II molecules. These are known as "loosely HLA-restricted" or "promiscuous" T helper sequences. Examples of peptides that are promiscuous include sequences from antigens such as tetanus toxoid at positions 830-843 (QYIKANSKFIGITE),

Plasmodium falciparum circumsporozoite (CS) protein at positions 378-398 (DIEKKIAKMEKASSVFNVVNS), and *Streptococcus* 18kD protein at positions 116 (GAVDSILGGVATYGAA). Other examples include peptides bearing a DR 1-4-7 supermotif, or either of the DR3 motifs.

Alternatively, it is possible to prepare synthetic peptides capable of stimulating T helper lymphocytes, in a loosely HLA-restricted fashion, using amino acid sequences not found in nature (*see, e.g.*, PCT publication WO 95/07707). These synthetic compounds called Pan-DR-binding epitopes (*e.g.*, PADRE™, Epimmune, Inc., San Diego, CA) are designed to most preferrably bind most HLA-DR (human HLA Class II) molecules. For instance, a pan-DR-binding epitope peptide having the formula: aKXVAAWTLKAAa, where "X" is either cyclohexylalanine, phenylalanine, or tyrosine, and "a" is either D-alanine or L-alanine, has been found to bind to most HLA-DR alleles, and to stimulate the response of T helper lymphocytes from most individuals, regardless of their HLA type. An alternative of a pan-DR binding epitope comprises all "L" natural amino acids and can be provided in the form of nucleic acids that encode the epitope.

HTL peptide epitopes can also be modified to alter their biological properties. For example, they can be modified to include D-amino acids to increase their resistance to proteases and thus extend their serum half life, or they can be conjugated to other molecules such as lipids, proteins, carbohydrates, and the like to increase their biological activity. For example, a T helper peptide can be conjugated to one or more palmitic acid chains at either the amino or carboxyl termini.

10. Combinations of CTL Peptides with T Cell Priming Agents

In some embodiments it may be desirable to include in the pharmaceutical compositions of the invention at least one component which primes cytotoxic T lymphocytes. Lipids have been identified as agents capable of priming CTL *in vivo* against viral antigens. For example, palmitic acid residues can be attached to the ε- and α- amino groups of a lysine residue and then linked, *e.g.*, via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be administered either directly in a micelle or particle, incorporated into a liposome, or emulsified in an adjuvant, *e.g.*, incomplete Freund's adjuvant. A preferred immunogenic composition comprises palmitic acid attached to

ε- and α- amino groups of Lys, which is attached via linkage, *e.g.,* Ser-Ser, to the amino terminus of the immunogenic peptide.

As another example of lipid priming of CTL responses, *E. coli* lipoproteins, such as tripalmitoyl-S-glycerylcysteinlyseryl- serine (P_3CSS) can be used to prime virus specific CTL when covalently attached to an appropriate peptide (*see*, *e.g.*, Deres, *et al., Nature* 342:561, 1989). Peptides of the invention can be coupled to P_3CSS, for example, and the lipopeptide administered to an individual to specifically prime a CTL response to the target antigen. Moreover, because the induction of neutralizing antibodies can also be primed with P_3CSS-conjugated epitopes, two such compositions can be combined to more effectively elicit both humoral and cell-mediated responses.

CTL and/or HTL peptides can also be modified by the addition of amino acids to the termini of a peptide to provide for ease of linking peptides one to another, for coupling to a carrier support or larger peptide, for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide, particularly Class I peptides. However, it is to be noted that modification at the carboxyl terminus of a CTL epitope may, in some cases, alter binding characteristics of the peptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal-NH_2 acylation, *e.g.*, by alkanoyl (C_1-C_{20}) or thioglycolyl acetylation, terminal-carboxyl amidation, *e.g.,* ammonia, methylamine, *etc.* In some instances these modifications may provide sites for linking to a support or other molecule.

11. Vaccine Compositions Comprising DC Pulsed with CTL and/or HTL Peptides

An embodiment of a vaccine composition in accordance with the invention comprises *ex vivo* administration of a cocktail of epitope-bearing peptides to PBMC, or isolated DC therefrom, from the patient's blood. A pharmaceutical to facilitate harvesting of DC can be used, such as Progenipoietin™ (Monsanto, St. Louis, MO) or GM-CSF/IL-4. After pulsing the DC with peptides and prior to reinfusion into patients, the DC are washed to remove unbound peptides. In this embodiment, a vaccine comprises peptide-pulsed DCs that present the pulsed peptide epitopes complexed with HLA molecules on their surfaces.

The DC can be pulsed *ex vivo* with a cocktail of peptides, some of which stimulate CTL response to one or more antigens of interest. Optionally, a helper T cell peptide such as a PADRE™ family molecule, can be included to facilitate the CTL response.

12. Administration of Vaccines for Therapeutic or Prophylactic Purposes

The peptides of the present invention and pharmaceutical and vaccine compositions of the invention are typically used therapeutically to treat cancer. Vaccine compositions containing the peptides of the invention are typically administered to a cancer patient who has a malignancy associated with expression of one or more antigens. Alternatively, vaccine compositions can be administered to an individual susceptible to, or otherwise at risk for developing cancer.

In therapeutic applications, peptide and/or nucleic acid compositions are administered to a patient in an amount sufficient to elicit an effective CTL and/or HTL response to the tumor antigen and to cure or at least partially arrest or slow symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, *e.g.*, the particular composition administered, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician.

As noted above, peptides comprising CTL and/or HTL epitopes of the invention induce immune responses when presented by HLA molecules and contacted with a CTL or HTL specific for an epitope comprised by the peptide. The peptides (or DNA encoding them) can be administered individually or as fusions of one or more peptide sequences. The manner in which the peptide is contacted with the CTL or HTL is not critical to the invention. For instance, the peptide can be contacted with the CTL or HTL either *in vivo* or *in vitro*. If the contacting occurs *in vivo*, the peptide itself can be administered to the patient, or other vehicles, *e.g.*, DNA vectors encoding one or more peptides, viral vectors encoding the peptide(s), liposomes and the like, can be used, as described herein.

When the peptide is contacted *in vitro*, the vaccinating agent can comprise a population of cells, *e.g.*, peptide-pulsed dendritic cells, or TAA-specific CTLs, which have been induced by pulsing antigen-presenting cells *in vitro* with the peptide or by

transfecting antigen-presenting cells with a minigene of the invention. Such a cell population is subsequently administered to a patient in a therapeutically effective dose.

For therapeutic use, administration should generally begin at the first diagnosis of cancer. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. The embodiment of the vaccine composition (*i.e.*, including, but not limited to embodiments such as peptide cocktails, polyepitopic polypeptides, minigenes, or TAA-specific CTLs or pulsed dendritic cells) delivered to the patient may vary according to the stage of the disease or the patient's health status. For example, a vaccine comprising TAA-specific CTLs may be more efficacious in killing tumor cells in patients with advanced disease than alternative embodiments.

The vaccine compositions of the invention may also be used therapeutically in combination with treatments such as surgery. An example is a situation in which a patient has undergone surgery to remove a primary tumor and the vaccine is then used to slow or prevent recurrence and/or metastasis.

Where susceptible individuals, *e.g.*, individuals who may be diagnosed as being genetically pre-disposed to developing a prostate tumor, are identified prior to diagnosis of cancer, the composition can be targeted to them, thus minimizing the need for administration to a larger population.

The dosage for an initial therapeutic immunization generally occurs in a unit dosage range where the lower value is about 1, 5, 50, 500, or 1,000 μg and the higher value is about 10,000; 20,000; 30,000; or 50,000 μg. Dosage values for a human typically range from about 500 μg to about 50,000 μg per 70 kilogram patient. Initial doses followed by boosting doses at established intervals, *e.g.,* from four weeks to six months, may be required, possibly for a prolonged period of time to effectively treat a patient. Boosting dosages of between about 1.0 μg to about 50,000 μg of peptide pursuant to a boosting regimen over weeks to months may be administered depending upon the patient's response and condition as determined by measuring the specific activity of CTL and HTL obtained from the patient's blood.

Administration should continue until at least clinical symptoms or laboratory tests indicate that the tumor has been eliminated or that the tumor cell burden has been substantially reduced and for a period thereafter. The dosages, routes of

administration, and dose schedules are adjusted in accordance with methodologies known in the art.

In certain embodiments, peptides and compositions of the present invention are employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, as a result of the minimal amounts of extraneous substances and the relative nontoxic nature of the peptides in preferred compositions of the invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these peptide compositions relative to these stated dosage amounts.

The vaccine compositions of the invention can also be used as prophylactic agents. For example, the compositions can be administered to individuals at risk of developing prostate cancer. Generally the dosage for an initial prophylactic immunization generally occurs in a unit dosage range where the lower value is about 1, 5, 50, 500, or 1000 μg and the higher value is about 10,000; 20,000; 30,000; or 50,000 μg. Dosage values for a human typically range from about 500 μg to about 50,000 μg per 70 kilogram patient. This is followed by boosting dosages of between about 1.0 μg to about 50,000 μg of peptide administered at defined intervals from about four weeks to six months after the initial administration of vaccine. The immunogenicity of the vaccine may be assessed by measuring the specific activity of CTL and HTL obtained from a sample of the patient's blood.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral, intrathecal, or local administration. Preferably, the pharmaceutical compositions are administered parentally, *e.g.,* intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, *e.g.,* water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH-adjusting

and buffering agents, tonicity adjusting agents, wetting agents, preservatives, and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.*

The concentration of peptides of the invention in the pharmaceutical formulations can vary widely, *i.e.,* from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected.

A human unit dose form of the peptide composition is typically included in a pharmaceutical composition that comprises a human unit dose of an acceptable carrier, preferably an aqueous carrier, and is administered in a volume of fluid that is known by those of skill in the art to be used for administration of such compositions to humans (*see, e.g.*, Remington's Pharmaceutical Sciences, 17th Edition, A. Gennaro, Editor, Mack Publishing Co., Easton, Pennsylvania, 1985).

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, such as lymphoid tissue, or to target selectively to infected cells, as well as to increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations, the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the peptide compositions. Liposomes for use in accordance with the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, *e.g.,* liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.,* Szoka, *et al., Ann. Rev. Biophys. Bioeng.* 9:467 (1980), and U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

For targeting cells of the immune system, a ligand to be incorporated into the liposome can include, *e.g.*, antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered intravenously, locally, topically, *etc.*, in a dose which varies according to, *inter alia*, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, *e.g.*, lecithin for intranasal delivery.

13. Kits

The peptide and nucleic acid compositions of this invention can be provided in kit form together with instructions for vaccine administration. Typically the kit would include desired peptide compositions in a container, preferably in unit dosage form and instructions for administration. An alternative kit would include a minigene construct with desired nucleic acids of the invention in a container, preferably in unit dosage form together with instructions for administration. Lymphokines such as IL-2

or IL-12 may also be included in the kit. Other kit components that may also be desirable include, for example, a sterile syringe, booster dosages, and other desired excipients.

Epitopes in accordance with the present invention were successfully used to induce an immune response. Immune responses with these epitopes have been induced by administering the epitopes in various forms. The epitopes have been administered as peptides, as nucleic acids, and as viral vectors comprising nucleic acids that encode the epitope(s) of the invention. Upon administration of peptide-based epitope forms, immune responses have been induced by direct loading of an epitope onto an empty HLA molecule that is expressed on a cell, and via internalization of the epitope and processing via the HLA Class I pathway; in either event, the HLA molecule expressing the epitope was then able to interact with and induce a CTL response. Peptides can be delivered directly or using such agents as liposomes. They can additionally be delivered using ballistic delivery, in which the peptides are typically in a crystalline form. When DNA is used to induce an immune response, it is administered either as naked DNA, generally in a dose range of approximately 1-5 mg, or via the ballistic "gene gun" delivery, typically in a dose range of approximately 10-100 μg. The DNA can be delivered in a variety of conformations, *e.g.*, linear, circular *etc.* Various viral vectors have also successfully been used that comprise nucleic acids which encode epitopes in accordance with the invention.

Accordingly compositions in accordance with the invention exist in several forms. Embodiments of each of these composition forms in accordance with the invention have been successfully used to induce an immune response.

One composition in accordance with the invention comprises a plurality of peptides. This plurality or cocktail of peptides is generally admixed with one or more pharmaceutically acceptable excipients. The peptide cocktail can comprise multiple copies of the same peptide or can comprise a mixture of peptides. The peptides can be analogs of naturally occurring epitopes. The peptides can comprise artificial amino acids and/or chemical modifications such as addition of a surface active molecule, *e.g.*, lipidation; acetylation, glycosylation, biotinylation, phosphorylation etc. The peptides can be CTL or HTL epitopes. In a preferred embodiment the peptide cocktail comprises a plurality of different CTL epitopes and at least one HTL

epitope. The HTL epitope can be naturally or non-naturally (*e.g.*, PADRE®, Epimmune Inc., San Diego, CA). The number of distinct epitopes in an embodiment of the invention is generally a whole unit integer from one through one hundred fifty (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, …, 150).

An additional embodiment of a composition in accordance with the invention comprises a polypeptide multi-epitope construct, *i.e.*, a polyepitopic peptide. Polyepitopic peptides in accordance with the invention are prepared by use of technologies well-known in the art. By use of these known technologies, epitopes in accordance with the invention are connected one to another. The polyepitopic peptides can be linear or non-linear, *e.g.*, multivalent. These polyepitopic constructs can comprise artificial amino acids, spacing or spacer amino acids, flanking amino acids, or chemical modifications between adjacent epitope units. The polyepitopic construct can be a heteropolymer or a homopolymer. The polyepitopic constructs generally comprise epitopes in a quantity of any whole unit integer between 2-150 (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99,…, 150). The polyepitopic construct can comprise CTL and/or HTL epitopes. One or more of the epitopes in the construct can be modified, *e.g.*, by addition of a surface active material, *e.g.* a lipid, or chemically modified, *e.g.*, acetylation, *etc*. Moreover, bonds in the multiepitopic construct can be other than peptide bonds, *e.g.*, covalent bonds, ester or ether bonds, disulfide bonds, hydrogen bonds, ionic bonds, *etc.*

Alternatively, a composition in accordance with the invention comprises construct which comprises a series, sequence, stretch, *etc.*, of amino acids that have homology to (*i.e.*, corresponds to or is contiguous with) to a native sequence. This stretch of amino acids comprises at least one subsequence of amino acids that, if cleaved or isolated from the longer series of amino acids, functions as an HLA Class I

or HLA Class II epitope in accordance with the invention. In this embodiment, the peptide sequence is modified, so as to become a construct as defined herein, by use of any number of techniques known or to be provided in the art. The polyepitopic constructs can contain homology to a native sequence in any whole unit integer increment from 70-100% (*e.g.*, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or, 100 percent).

A further embodiment of a composition in accordance with the invention is an antigen presenting cell that comprises one or more epitopes in accordance with the invention. The antigen presenting cell can be a "professional" antigen presenting cell, such as a dendritic cell. The antigen presenting cell can comprise the epitope of the invention by any means known or to be determined in the art. Such means include pulsing of dendritic cells with one or more individual epitopes or with one or more peptides that comprise multiple epitopes, by nucleic acid administration such as ballistic nucleic acid delivery or by other techniques in the art for administration of nucleic acids, including vector-based, *e.g.* viral vector, delivery of nucleic acids.

Further embodiments of compositions in accordance with the invention comprise nucleic acids that encode one or more peptides of the invention, or nucleic acids which encode a polyepitopic peptide in accordance with the invention. As appreciated by one of ordinary skill in the art, various nucleic acids compositions will encode the same peptide due to the redundancy of the genetic code. Each of these nucleic acid compositions falls within the scope of the present invention. This embodiment of the invention comprises DNA or RNA, and in certain embodiments a combination of DNA and RNA. It is to be appreciated that any composition comprising nucleic acids that will encode a peptide in accordance with the invention or any other peptide based composition in accordance with the invention, falls within the scope of this invention.

EXAMPLES

Preparation A

Peptide synthesis and generation of peptide analogs

The peptides used in these examples are shown in Table 1. All of the wildtype human CTL epitopes derived from tumor-associated antigens, as well as the wildtype

viral epitopes derived from the polymerase genes of the HIV and hepatitis B virus (HBV^2), have shown immunogenicity in human and transgenic mouse systems (Kawashima, I., *et al., Human Immunol.* (1998) 59:1; Ishioka, G., *et al., J. Immunol.* (1999) 162:3915).

Peptides that were tested initially for heteroclitic activity were synthesized by Chiron Technologies (Victor, Australia). Peptides requiring further biological characterization were synthesized at Epimmune using methods (Ruppert, J., *et al., Cell* (1993) 74:929) and their purity was routinely >95%, as determined by analytical reverse-phase HPLC. The identity of the latter peptides was confirmed by mass spectral analysis.

Preparation B

Scheme for selection of single amino acid substitutions

Table 2 shows the similarity assignments between any given amino acid pair so that a given amino acid substitution could be characterized as being a conservative, semi-conservative, or non-conservative substitution.

The degree of similarity between amino acid pairs was quantified by averaging, for each amino acid pair, the rank coefficient scores for PAM250, hydrophobicity, and side chain volume as described below. Based on the average values of these composite rankings, the table shows each pair to be conserved, semi-conserved or non-conserved.

The Dayhoff PAM250 score (Dayhoff, M.O., *et al., Atlas of Protein Sequence and Structure*, Vol. 5, suppl.3. (1978) M.O. Dayhoff, ed. National Biomedical Research Foundation, Washington DC, p. 345; Creighton, T.E., *Proteins: structures and molecular properties* (1993) (2nd edition) W.H. Freeman and Company, NY; http://prowl.rockefeller.edu/aainfo/pam250. html) is a commonly utilized protein alignment scoring matrix which measures the percentage of acceptable point mutations (PAM) within a defined time frame. The frequencies of these mutations are different from what would be expected from the probability of random mutations, and presumably reflect a bias due to the degree of physical and chemical similarity of the amino acid pair involved in the substitution. To obtain a score of amino acid similarity that could be standardized with other measures of similarity, the PAM250

scores were converted to a rank value, where 1 indicates the highest probability of being an accepted mutation.

The most commonly utilized scales to represent the relative hydrophobicity of the 20 naturally occurring amino acids (Cornette, J., *et al., J. Mol. Biol.* (1987) 195:659) are those developed on the basis of experimental data by Kyte and Doolittle (Kyte, J. and R.F. Doolittle, *J. Mol. Biol.* (1982) 157:105), and by Fauchere and Pliska (Fauchere, J. and V. Pliska, *Eur. J. Med. Chem.* (1983) 18:369). The Kyte/Doolittle scale measures the H_2O/organic solvent partition of individual amino acids. Because it considers the position of amino acids in folded proteins, it may most accurately reflect native hydrophobicity in the context of proteins. The Fauchere/Pliska scale measures the octanol/H_2O partitioning of N-acetyl amino acid amides, and most accurately reflects hydrophobicity in the context of denatured proteins and/or small synthetic peptides. To obtain scores for hydrophobicity, each amino acid residue was ranked on both the Kyte/Doolittle and Fauchere/Pliska hydrophobicity scales. An average rank between the two scales was calculated and the average difference in hydrophobicity for each pair was calculated.

Finally, for calculating amino acid side-chain volume, the partial volume in solution obtained by noting the increase in volume of water after adding either one molecule or one gram of amino acid residue was considered (Zamyatnin, A.A., *Ann. Rev. Biophys. Bioeng.* (1984) 13:145; Zamyatnin, A.A., *Prog. Biophys. Mol. Biol.* (1972) 24:107). The absolute difference in the partial volume of each possible pairing of the 20 naturally occurring amino acids was calculated and ranked, where 1 indicated residues with the most similar volumes, and 20 the most dissimilar.

Preparation C

Materials for Assays

1. APC lines

Cell lines that present peptides in the context of HLA-A2.1 were prepared as follows:

The .221A2.1 cell line was generated by transfecting the HLA-A2.1 gene into the HLA-A, -B, -C-null mutant EBV-transformed human B-lymphoblastoid cell line 3A4-721.221 (Kawashima, I., *et al., Human Immunol.* (1998) 59:1).

Tumor cell lines were prepared by transfection of Meth A cells, a methylcholanthrene-induced sarcoma, and the Jurkat cell line with the HLA-A2.1 or HLA-A2.1/K^b transgene transfection was performed using methods described elsewhere (Vitiello, A., *et al., J. Exp. Med.* (1991) 173:1007). A combination of the HLA-typed melanoma cell lines 624mel ($A2.1^+$, $MAGE^+$) and 888mel ($A2.1^-$, $MAGE^-$), were kindly provided by Y. Kawakami and S. Rosenberg (National Cancer Institute), and were used to measure presentation of endogenously processed MAGE3 epitopes (Boon, T., *et al., Ann. Rev. Immunol.* (1994) 12:337). The melanoma cell lines were treated with 100 IU/ml human IFNγ (Genzyme, Cambridge, MA) for 48 h at 37°C before using as APC.

All cells in this study were grown in RPMI-1640 medium supplemented with antibiotics, sodium pyruvate, nonessential amino acids, and 10% (v/v) heat-inactivated FBS.

2. *In vitro* induction of CTL from human PBMC and derivation of human CTL lines

To generate peptide-specific CTL lines against the MAGE3.112 and a carcinoembryonic antigen (CEA) epitope, CEA.691, PBMC from normal subjects were stimulated repeatedly *in vitro* with peptide as described (Kawashima, I., *et al., Human Immunol.* (1998) 59:1). Briefly, peptide-pulsed dendritic cells (differentiated from adherent PBMC by culturing in GM-CSF and IL4) were co-cultured with autologous $CD8^+$ T cells, obtained by positive selection with antibody-coated beads (Dynal A.S., Oslo, Norway) in a 48-well plate. After 7 days of culture in the presence of IL2, IL7, and IL10, each PBMC culture (well) was restimulated *in vitro* with adherent PBMC pulsed with peptide. Cultures were then tested for CTL activity by measuring IFNγ production after stimulation with .221A2.1 tumor APC, in the presence or absence of peptide. CTL lines were expanded from PBMC cultures demonstrating peptide-specific IFNγ responses by additional *in vitro* stimulation with adherent peptide-pulsed PBMC.

3. Murine CTL lines

CTL lines against epitopes HBV Pol.455 and HIV Pol.476 peptides were generated in HLA-A2.1/K^{bxs} transgenic mice by DNA immunization as described

elsewhere (Ishioka, G., *et al., J. Immunol.* (1999) 162:3915). HLA-A2.1/K^{bxs} and HLA-A2.1/K^{bxd} transgenic mice were bred at Epimmune. These strains represent the F1 generation of a cross between an HLA-A2.1/K^{b} transgenic strain generated on the C57BL/6 background (Vitiello, A., *et al., J. Exp. Med.* (1991) 173:1007), and SJL or BALB/c mice (Jackson Laboratories, Bar Harbor, ME), respectively. A CTL line against the MAGE2.157 epitope was generated by immunizing 8-12 wk old HLA-A2.1/K^{bxs} mice s.c. at the tail base with 50 μg of peptide and 140 μg of the HBV Core.128 Th epitope, TPPAYRPPNAPIL (SEQ. ID. NO: ___), emulsified in IFA and restimulating primed splenocytes repeatedly *in vitro* with peptide.

Preparation D

Assay Methods

1. Measurement of peptide binding affinity for the HLA-A2.1 molecule

Binding of test peptides to HLA-A2.1 was measured by determining the level of competition induced by a given test peptide for binding of a radiolabeled standard peptide to HLA-A2.1. The percentage of MHC-bound radioactivity was determined by gel filtration and the concentration of test peptide that inhibited 50% of the binding of the labeled standard peptide (IC_{50}) was calculated (Ruppert, J., *et al., Cell* (1993) 74:929; Sette, A., *et al., Mol. Immunol.* (1994) 31:813). The standard peptide was the HBV Core.18 epitope (sequence FLPSDFFPSV).

2. Measurement of murine and human IFNγ, IL5, and IL10 production by CTL

An *in situ* capture ELISA was used for measuring IFNγ release from CTL (McKinney, D., *et al., J. Immunol. Methods* (2000) 237:105). Briefly, CTL were stimulated with APC and peptide in ELISA-grade 96-well flat bottom wells that were precoated with either an anti-mouse IFNγ (clone R4-6A2, Pharmingen, San Diego, CA) or anti-human IFNγ mAb (clone NIB42, Pharmingen). After culturing cells, wells are washed and developed by adding a biotinylated anti-mouse IFNγ (clone XMG1.2, Pharmingen) or anti-human IFNγ (clone 4S.B3, Pharmingen) mAb followed by enzyme-conjugated streptavidin (Zymed, South San Francisco, CA) and 3, 3', 5, 5'

tetramethylbenzidine substrate (ImmunoPure TMB substrate kit, Pierce, Rockford, IL). The absorbance of each well was measured at 450 nm on a Labsystems Multiskan RC ELISA plate reader. The level of IFNγ produced in each well was determined by extrapolation from a mouse or human IFNγ standard curve established in the same assay.

Murine and human IL5 and IL10 were measured in culture supernates using ELISA kits (R&D Biosystems, Minneapolis, MN). These assays, employing the quantitative sandwich ELISA technique, were performed according to the manufacturer's protocol.

3. Enzyme-liked immunospot (Elispot) assay for measuring ex vivo CTL responses

Elispot assays were performed according to standard protocols (Murali-Krishna, K., *et al., Immunity* (1998) 8:177; Lewis, J.J., *et al., Int. J. Cancer* (2000) 87:391). Briefly, flat bottom 96-well nitrocellulose plates (Immobilon-P membrane, Millipore, Bedford, MA) were coated with anti-IFNγ mAb (10 μg/ml, clone R4-6A2) and incubated overnight at 4°C. After washing with PBS, plates were blocked with RPMI medium containing 10% FBS for 1 h at 37°C. Four $x10^5$ splenic $CD8^+$ cells isolated by magnetic beads (Miltenyi, Auburn, CA) and $5x10^4$ Jurkat-A2.1/K^b cells pulsed with 10 μg/ml of peptide were added to each well and cells were incubated for 20 h in RPMI medium containing 10% FBS. After incubation, the plates were washed thoroughly with PBS/0.05% Tween and biotinylated anti-IFNγ mAb (2 μg/ml, clone XMG1.2) was added to each well and plates were incubated for 4 h at 37°C. Plates were then washed four times with PBS (containing 0.1% Tween-20) and Vectastain ABC peroxidase (Vectastain Elite kit; Vector Laboratories, Burlingame, CA). After incubating for 1 h at room temperature, plates were washed three times with 1x PBS/0.05% Tween followed by three additional washes with 1x PBS. One hundred μl of AEC solution (Sigma Chemical, St. Louis, MO) was added to develop the spots. The reaction was stopped after 4-6 min under running tap water. The spots were counted by computer-assisted image analysis (Zeiss KS Elispot Reader, Jena, Germany). The net number of spots/10^6 $CD8^+$ cells was calculated as follows: [(number of spots against relevant peptide) - (number of spots against irrelevant control peptide)] x 2.5.

Example 1

Screening of Peptide Analogs for Heteroclitic Activity

A. Identification of CEA.691 and MAGE3.112 Analogs Associated with Increased IFNγ Release

Prior to screening analogs, a peptide dose titration of IFNγ production from CTL lines was performed over a wide range of doses of wildtype peptide. .221A2.1 tumor cells were pulsed with varying doses of peptide then 10^5 peptide-loaded cells were cultured with an equivalent number of murine or human CTL. After 24 hr (murine) or 48 hr (human) incubation at 37°C, levels of IFNγ released by CTL were measured by the *in situ* capture ELISA assay. After determining a dose titration curve, a suboptimal peptide dose where activity against wildtype peptide was barely detectable was selected for screening the antigenicity of a panel of peptide analogs. For all of the murine and human CTL lines, this suboptimal dose ranged from 0.1-1 μg/ml. It should be noted that although murine CTL lines were generated in HLA-A2.1/K^{bxs} transgenic mice which express an HLA molecule with murine H-2 K^b sequences in the third domain, all responded to peptide presented on APC expressing the native HLA-A2.1 molecule.

For screening of peptide analogs, .221A2.1 cells were pulsed with each analog at the selected suboptimal dose and peptide-loaded APC were cultured with CTL as described above. Analogs inducing enhanced CTL responses relative to wildtype peptide were then selected for further characterization. These analogs were characterized by performing a peptide dose titration side-by-side with the wildtype epitope under identical conditions described above.

CTL lines specific for the HLA-A2.1-restricted CEA.691 and MAGE3.112 epitopes were derived by repeated *in vitro* restimulations of human PBMCs with peptide-loaded dendritic cells or adherent monocytes, as described in Preparation C.

A total of 117 CEA.691 and 116 MAGE3.112 analogs were generated by systematically replacing each residue with 17 different single amino acids. CEA.691 is IMIGVLVGV (SEQ. ID. NO: 1); MAGE3.122 is KVAELVHFL (SEQ. ID. NO: 5). The residues Cys, Trp and Met were in general avoided unless they corresponded to conservative changes. Substitutions were introduced at all positions in the peptide except at the main MHC anchor positions, position 2 and the C-terminus.

These analogs were then tested *in vitro* for their antigenicity. As described above, preliminary dose titration experiments for each CTL line were performed to define an antigen concentration at which IFNγ production in response to wildtype peptide was barely detectable. This suboptimal concentration was then used subsequently for all antigenicity analysis on analog peptides for each epitope, to identify analogs associated with increased T cell stimulatory capacity. Results of such antigenicity analysis are shown in Figure 1. As shown in Figure 1A, the suboptimal 100 ng/ml dose the wildtype CEA.691 peptide yielded only marginal IFNγ production (<50 pg/well). By contrast, at the same dose, several CEA.691 analogs (M3, L4, P4, H5, L5, H6, T6, and I7) induced detectable levels of IFNγ production, in the 150 to 350 pg/well range. As shown in Figure 1B, MAGE3.112-specific CTL line 100 ng/ml of wildtype peptide induced the release of 100 pg/ml of IFNγ, whereas two analogs (I5 and W7) were associated with inducing IFNγ levels of over 300 pg/well.

All analogs of CEA.691 and MAGE3.112 that stimulated IFNγ above 100 pg/well were chosen for further characterization and a complete dose titration was carried out to identify heteroclitic analogs. Heteroclitic analogs are those that stimulate significant IFNγ release (>100 pg/well) at 10-fold or lower peptide concentrations than wildtype peptide. For the CEA.691 epitope two different analogs, M3 (SEQ. ID. NO: 2) and H5 (SEQ. ID. NO: 3), were identified. As seen in Figure 1C, for epitope CEA.691, the wildtype peptide yielded a significant detectable IFNγ signal in the 1 to 100 μg/ml dose range, while the analogs M3 and H5 stimulated significant release with as little as 0.01 ng/ml of peptide. By these criteria, these two CEA.691 analogs are, on a molar basis, 100,000-fold more potent in terms of IFNγ release than their unmodified wildtype counterpart.

Similarly, for the MAGE3.112 epitope two heteroclitic analogs, I5 and W7, were identified. As shown in Figure 1D, 1 μg/ml of wildtype peptide concentration required for significant IFNγ release whereas 0.1 ng/ml of either I5 (SEQ. ID. NO: 6) or W7 (SEQ. ID. NO: 7) analogs was required to stimulate an equivalent response. This corresponds to a greater than 100,000-fold increase in biological activity compared to wildtype peptide.

In general, the heteroclitic analogs not only induced a dose response shift, but also stimulated CTL's to produce higher levels of IFNγ compared to wildtype peptide

so that the maximal dose response (plateau) reached in response to the analog was much higher than the response obtained in response to the unmodified antigen.

Example 2

Identification of Additional Heteroclitic Analogs

Three additional A2.1-restricted epitopes, the MAGE2.157 YLQLVFGIEV, SEQ. ID. NO: 7 tumor epitope, and two epitopes from viral antigens, HBV Pol.455, GLSRYVARL (SEQ. ID. NO: 16) and HIV Pol.476 ILKEPVHGF (SEQ. ID. NO: 18), were analyzed. All of these epitopes have previously been shown to be immunogenic for CTL.

A panel of 240 different analogs was synthesized which included five conservative and five non-conservative amino acid substitutions at epitope positions 3, 5, 7 in each of the three epitopes, as well as at epitope positions 1, 4, 6, using the amino acid conservancy assignments described in the Preparation B and in Table 2. These analogs were tested for heteroclicity using murine CTL lines generated in HLA-A2.1/K^{bxs} transgenic mice and following an experimental strategy similar to the one described in Example 1 for the CEA.691 and MAGE3.112 epitopes. Murine CTL lines derived from HLA transgenic mice were used instead of human CTL lines due to technical ease associated with generating and maintaining mouse lines.

The results are shown in Figure 2A (MAGE2.157), 2B (HBV Pol.455), and 2C (HIV Pol.476) with a corresponding dose titration profile for HIV Pol.476 in Figure 2D. (See Example 3 for MAGE2.157 and HIV Pol.455.)

Analysis of a total of 85 different analogs of the MAGE2.157 epitope tested resulted in identification of two heteroclitic analogs, I5 (SEQ. ID. NO: 8) and F5 (SEQ. ID. NO: 9), that stimulated IFNγ responses at 100- to 100,000-fold lower doses than wildtype peptide (Table 1); both of these analogs had substitutions that were conservative or semi-conservative in nature occurring at an odd-numbered position in the center of the peptide (position 5).

For the HIV Pol.476 epitope, out of 78 different analogs screened, two were identified as having heteroclitic activity (H3 (SEQ. ID. NO: 19) and L3 (SEQ. ID. NO: 20)) (Table 1); both analogs carried either a conservative or semi-conservative substitution at an odd-numbered position in the center of the peptide. one heteroclitic

analog of HIV Pol.455 epitope out of 77 tested was identified; this analog had a conservative substitution (P) at position 7 of the peptide (SEQ. ID. NO: 17) (Table 1).

Thus, data obtained from 240 analogs for three additional epitopes of tumor and viral origin (MAGE2.157, HIV Pol.476, and HBV Pol.455), were consistent with the analysis of the MAGE3.112 and CEA.691 epitopes as set forth in Example 1.

Heteroclicity analysis was also performed on two p53 epitopes. One epitope, p53.149M2, SMPPPGTRV (SEQ. ID. NO: 10) represents a fixed anchor analog of a human p53 epitope having a methionine residue substitution which enhances MHC binding . The second epitope, p53 Mu.184, GLAPPQHLIRV (SEQ. ID. NO: 13) has a sequence that is completely conserved between mice and humans (Theobald, *et al.*, 92(26):11993 (1995)).

Dose titration analysis performed on the p53.149M2 revealed optimal and suboptimal responses at 1 μg/ml and 0.1 μg/ml dose range. A panel of 76 analogs for p53.149M2 (five conservative and five non-conservative substitutions at each position) was screened and only two analogs, C1 (SEQ. ID. NO: 11) and P7 (SEQ. ID. NO: 12), were identified both giving IFNγ release of 100 pg/well at a suboptimal dose, Figure 5. On further analysis, both analogs induced significant IFNγ production at 10-fold lower concentrations than wildtype peptide. In addition, the C1 analog also induced significant IL10 levels at 100-fold lower peptide concentrations, Figure 6.

For the p53mu.184 epitope optimal and suboptimal levels of peptide were determined to be 500 ng/ml and 10 ng/ml respectively after performing a dose titration analysis. A panel of 63 conservative and semi-conservative substitution analogs were tested for immunogenicity. Two analogs with enhanced immunogenicity were found - T3 (SEQ. ID. NO: 14) and T3,E6 (SEQ. ID. NO: 15). See Figures 7 and 8.

Example 3

Lymphokine Profile Induced by Heteroclitic Analogs

Heteroclitic analogs have been shown previously to differentially activate cytokine production from T cells whereby some analogs specifically activate T cells to produce Th1 cytokines whereas others preferentially activate the production of Th2 cytokines. To investigate the pattern of lymphokine release associated with the heteroclitic analogs of the invention, the production of Th2 cytokines IL5 and/or IL10

from CTL lines was compared to the production of IFNγ. Representative data from two different epitopes are shown in Figures 3 and 4.

Figure 3 shows the lymphokine profile induced by MAGE2.157 analogs. IFNγ (A) and IL10 (B) produced by MAGE2.157-specific CTL's in response to .221A2.1 targets pulsed with analogs I5 or F5, or wildtype (WT) peptide was measured over several different doses. Dotted lines indicate significant levels of IFNγ (100 pg/well) or IL10 (50 pg/ml). As seen in Figure 3A, the F5 and I5 analogs of MAGE2.157 induced significant levels of IFNγ production at 100-fold or 10,000-fold lower concentrations than wildtype peptide respectively. Moreover, the same analogs also induced significant IL10 production at 10-fold or 100-fold lower peptide concentrations than wildtype peptide.

Data from another epitope, HBV Pol.455, depicting the same trend are shown in Figures 4A and 4B. IFNγ (A) or IL10 (B) released by HBV Pol.455 CTL's in response to analog P7 or wildtype (WT) peptide over several different peptide doses are shown. Once again, the P7 analog of HBV Pol.455 induced significant levels of IFNγ (Fig. 4A) and IL10 (Fig. 4B) at 100-fold lower peptide concentrations than wildtype peptide. Taken together the data summarizing all the heteroclitic analogs tested for induction of Th2 cytokines (Table 1) indicates that most heteroclitic analogs stimulate increased production of both of Th1 and Th2 cytokines.

Example 4

HLA-A2.1 Binding Affinity of Heteroclitic Analogs

To verify that the enhanced recognition by CTL lines observed was not due to a fortuitous increase in MHC binding capacity of the analog epitope, the MHC binding affinity of all heteroclitic analogs was measured *in vitro* utilizing purified HLA-A2.1 molecules, and compared to their unmodified wildtype counterparts as described in Preparation D.

As summarized in Table 1, three analogs (MAGE3.112W7, HIV Pol.476H3, and HIV Pol.476L3) bound to HLA-A2.1 with four-fold or higher affinity than wildtype peptide and two analogs bound with lower affinity (MAGE2.157I5, MAGE2.157F5). The four remaining heteroclitic analogs, MAGE3.112I5, CEA.691M3, CEA.691H5, and HBV Pol.455P7, were associated with little or no

change in HLA-A2.1 binding capacity. Collectively these data suggest a lack of correlation between increased binding and heteroclicity.

Example 5

Prediction and Immunogenicity of Analogs for the Murine p53.261 Epitope

To test for immunogenicity *in vivo*, the HLA-A2.1-restricted murine p53.261 epitope was used since CTL responses against this epitope have been shown to be partially tolerized in HLA-A2.1/K^b transgenic mice. This permits analysis of the capacity of predicted heteroclitic analogs to break T cell tolerance *in vivo*. Although heteroclitic analogs heretofore have been detected through *in vitro* screening with CTL lines raised against wildtype epitopes, we reasoned that analogs identified by the substitution rules could potentially induce CTL *in vivo* that were heteroclitic against the wildtype epitope, an application of interest for designing vaccines against tolerant tumor-associated epitopes.

Immunogenicity for the p53.261 predicted analogs were tested in HLA-A2.1/K^{bxd} transgenic mice by co-immunizing mice with 50 μg of the p53.261 epitope (LLGRDSFEV) or its predicted analogs and 140 μg of HBV Core.128 helper epitope in IFA. Eleven days later, primed spleen cells were harvested and cultured *in vitro* with irradiated syngeneic LPS-activated spleen cells that had been pulsed with 10 μg/ml of peptide. After 10 days of culture, CTL were restimulated with peptide-pulsed LPS blasts in the presence of Con A-conditioned media as a source of IL2 (Ishioka, G., *et al., J. Immunol.* (1999) 162:3915). Spleen cells from mice immunized with the predicted analogs were stimulated *in vitro* against both wildtype peptide (to determine the cross-reactivity, avidity and precursor frequency of CTL's that respond to wildtype antigen) and the respective immunizing analog (to determine avidity and precursor frequency of CTL's responding to the analog). All short-term, bulk populations of CTL were tested for peptide specificity by the IFNγ *in situ* ELISA assay 5 days after the second restimulation *in vitro*, using Jurkat-A2.1 tumor cells as APC. Alternatively, CTL responses were performed on freshly isolated spleen cells from immunized animals using the Elispot assay.

A panel of nine analogs of the p53.261 epitope consisting of three conservative or semi-conservative substitutions at positions 3, 5, and 7 of the 9-mer

peptide was tested for immunogenicity in HLA-A2.1/K^{bxd} transgenic mice. Immunization of mice with each of the nine analogs and *in vitro* expansion of primed splenocytes with the respective immunizing analog resulted in identification of six analogs (L7, D3, H7, H3, N5, G5) that gave CTL responses characterized by IFNγ production of 100 pg/well at much lower peptide concentrations compared to CTL induced *in vivo* and expanded *in vitro* with wildtype peptide.

Spleen cells from mice immunized with either WT peptide or the indicated analogs were stimulated *in vitro* with the corresponding immunizing peptide (Figures 9A, B) or with WT peptide (Figures 9C, D). IFNγ release by these CTL's was then measured over a dose range against targets pulsed with the immunizing peptide (Figures 9A, B) or with WT peptide (Figures 9C, D). IFNγ release at 100 pg/well is shown as a dotted line. These results indicate that a significant percentage of the analogs induce CTL of a higher avidity than those induced by wildtype peptide itself.

The cross-reactivity of CTL primed with these heteroclitic analogs against wildtype peptide is shown in Figure 9C and Figure 9D. While CTL's obtained from animals immunized and restimulated with wildtype peptide induced 100 pg/well IFNγ at peptide doses between 0.1-10 μg/ml, CTL's obtained from animals immunized with analogs L7, H3, and D3, and stimulated and tested *in vitro* with wildtype peptide, required 10-, 100-, or 1000-fold lower doses of wildtype peptide respectively, to induce 100 pg/well of IFNγ (Fig. 4C). This suggests that in three out of six cases the predicted heteroclitic analogs were 10-1000-fold more active/potent at inducing CTL's reactive to wildtype peptide in situations where partial CTL tolerance to wildtype antigen exists.

Example 6

Cross Reactivity with Wildtype

The cross-reactivity of CTL induced by the D3 and H3 analogs were also tested against the wildtype epitope naturally processed by a p53-expressing Meth A tumor cell clone transfected with HLA-A2.1/K^b; it was found that CTL generated by p53.261 analogs that are heteroclitic for wildtype epitope respond to endogenously-processed p53.261 epitope presented by Meth A/A2.1K^b tumor cells.

The CTL population (10^5/well) were cultured with $2.5x10^4$ Meth A tumor cells or with a Meth A clone transfected with HLA-A2.1/K^b and IFNγ release was measured by the *in situ* ELISA assay. As shown in Figure 10, CTL lines raised against both D3 and H3 analogs of the p53.261 epitope responded to the endogenous epitope expressed by a Meth A/A2.1K^b tumor cell clone but not to the parental HLA-A2.1-negative Meth A tumor cell line.

Example 7

Precursor Frequency Analysis Using Elispot Assays

To confirm that cross-reactive CTL against wildtype peptide are generated in mice immunized with analogs $CD8^+$ cells were isolated from spleen cells of mice immunized with analogs or wildtype peptide, without further CTL expansion *in vitro* and the precursor frequency of CTL reactive against either wildtype or analog was determined using an Elispot assay.

$CD8^+$ cells isolated from mice immunized with either WT peptide or the D3, H3, L7, and H7 analogs were analyzed for their ability to release IFNγ when stimulated in the Elispot assay with WT peptide. Figure 11 shows that while the precursor frequencies of wildtype peptide-reactive CTL were 1/66,000 (15 spots/10^6) in mice immunized with wildtype peptide, precursor frequencies of wildtype peptide-reactive cells in mice immunized with predicted analogs were approximately 1/15,000 for analogs D3, H3, and L7 (60-75 spots/10^6 cells), and 1/83,000 (12 spots/10^6) for analog H7. This indicates wildtype-reactive cells were present at a four-fold higher frequency in mice immunized with three out of the four analogs compared to mice immunized with the native peptide. This finding is significant since it implies that *in vivo* immunization with heteroclitic analogs does indeed induce a higher number of CTL reactive against wildtype peptide, using a more direct assay system where *in vitro* expansion of *in vivo*-primed CTL is avoided.

Example 8

Heteroclitic Analogs Induce Human CTL Capable of Recognizing Tumor Cells *In Vitro*

Immunogenicity of heteroclitic analogs of MAGE3.112 was also tested by inducing primary CTL from PBMC, as described in Preparation C, against either the MAGE3.112 peptide or the I5 and W7 analogs of this epitope. After two rounds of *in vitro* stimulation, PBMC cultures in 48-wells were scored positive for CTL induction if the net IFNγ production was >100 pg/well and production was at least two-fold above background, after stimulating with .221-A2.1 APC in the presence or absence of peptide.

To underline the physiologic relevance of our observations to human tumor antigens, we examined whether heteroclitic analogs of the MAGE3.112 epitope could induce human CTL's in a primary *in vitro* induction system. Fresh naïve human PBMC from normal donors were stimulated repetitively *in vitro* with either wildtype or analogs as described previously (Kawashima, I., *et al., Human Immunol.* (1998) 59:1). Peptide-specific CTL responses were detected in cultures stimulated with either wildtype peptide (Fig. 12A) or the I5 (Fig. 12B) and W7 analogs (Fig. 12C). Briefly, .221A2.1 cells were pulsed overnight with 10 μg/ml of WT peptide (Fig. 12A), the I5 (Fig. 12B) analog, or the W7 analog (Fig. 12C). IFNγ production by CTL's growing in individual wells from a 48-well plate were tested against .221A2.1 cells in the presence or absence of peptide, or against the endogenous epitope-negative 888mel and the endogenous epitope-positive 624mel tumor cell lines. Only wells showing a positive peptide-specific CTL response are shown.

More importantly cultures induced with these analogs recognized the 624mel tumor cell line that endogenously processes and presents the wildtype sequence. This demonstrates that heteroclitic analogs can induce physiologically relevant human CTL's that recognize endogenously-generated wildtype peptide presented by tumor cells and that the phenomenon is relevant in both human and in transgenic mouse systems.

Example 9

Synthesis and Analysis of Heteroclitic Analogs Derived from the HLA-A2.1 Supermotif on HLA A2 Superfamily Members

To further validate the heteroclitic substitution rules for other HLA molecules within the A2 superfamily, the panel of nine analogs of the p53.261 epitopes discussed above consisting of three conservative/semiconservative substitutions at positions 3, 5 and 7 are tested for *in vivo* immunogenicity in transgenic mice expressing one of the following human HLA molecules: A*0202, A*0203, A*0204, A*0205, A*0206, A*0207, A*0209, A*0214, A*6802 and A*6901.

CTLs from the mice immunized with the above-described analogs are tested for induction of at least 100 pg/well of IFNγ production. This IFNγ production typically occurs at much lower peptide concentrations than those induced and restimulated with wildtype peptide (*e.g.*, the p53.261 epitope). These results indicate that our predicted heteroclitic analogs are more potent at inducing higher avidity CTL against the native wildtype epitope than wildtype peptide itself.

Typically, CTLs obtained from animals immunized and restimulated with a wildtype peptide will induce 100 pg/well IFNγ at peptide doses of 5-10μg/ml, whereas CTLs obtained from animals immunized with the above-described analogs, and stimulated and tested *in vitro* with wildtype peptide, require 10-fold, 100-fold or even 1000-fold lower doses of wildtype peptide respectively, to induce 100pg/well of IFNγ.

Example 10

Synthesis and Analysis of Heteroclitic Analogs Derived from the HLA-B7 Supermotif on HLA B7 Superfamily Members

To further validate the heteroclitic substitution rules, additional studies are carried out with heteroclitic analogs derived from a peptide bearing a sequence within the HLA-B7 supermotif. For example, the analogs can be tested for *in vivo* immunogenicity.

For this study, the HLA-B7 supermotif bearing peptide, APRTLVYLL epitope is chosen and synthesized. A panel of analogs consisting of three conservative/semiconservative substitutions at positions 3, 5 and 7 of the 9-mer

peptide, are tested for immunogenicity in HLA-B*0702/K^b transgenic mice. The panel includes APETLVYLL, APRTWVYLL, and APRTLVPLL, corresponding to a semi-conservative change is the third, fifth and seventh position, respectively.

CTLs from the mice immunized with the above-described analogs are tested for induction of at least 100 pg/well of IFNγ production. This IFNγ production will typically occur at much lower peptide concentrations than those induced and restimulated with wildtype peptide (*e.g.*, APRTLVYLL). These results will indicate that our predicted heteroclitic analogs are more potent at inducing higher avidity CTL than wildtype peptide itself.

Typically, CTLs obtained from animals immunized and restimulated with a wildtype peptide will induce 100 pg/well IFNγ at peptide doses of 5-10μg/ml, whereas CTLs obtained from animals immunized with the above-described analogs, and stimulated and tested *in vitro* with wildtype peptide, require 10-fold, 100-fold or even 1000-fold lower doses of wildtype peptide respectively, to induce 100pg/well of IFNγ.

To further validate the heteroclitic substitution rules for other HLA molecules with the B7 superfamily, the peptides APETLVYLL, APRTWVYLL, and APRTLVPLL are tested for *in vivo* immunogenicity in transgenic mice expressing one of the following human HLA molecules: B*0702, B*0703, B*0704, B*0705, B*1508, B*3501, B*3502, B*3503, B*3503, B*3504, B*3505, B*3506, B*3507, B*3508, B*5101, B*5102, B*5103, B*5104, B*5105, B*5301, B*5401, B*5501, B*5502, B*5601, B*5602, B*6701 and B*7801.

CTLs from the mice immunized with the above-described analogs are tested for induction of at least 100 pg/well of IFNγ production. This IFNγ production will typically occur at much lower peptide concentrations than those induced and restimulated with wildtype peptide (*e.g.*, APRTLVYLL). These results will indicate that our predicted heteroclitic analogs are more potent at inducing higher avidity CTL than wildtype peptide itself.

Typically, CTLs obtained from animals immunized and restimulated with a wildtype peptide will induce 100 pg/well IFNγ at peptide doses of 5-10 μg/ml, whereas CTLs obtained from animals immunized with the above-described analogs, and stimulated and tested *in vitro* with wildtype peptide, require 10-fold, 100-fold or

even 1000-fold lower doses of wildtype peptide respectively, to induce 100 pg/well of IFNγ.

Precursor frequency analysis using Elispot assays

To confirm that cross-reactive CTL against wildtype peptide are generated in mice immunized with analogs, $CD8^+$ cells are isolated from spleens immunized with analogs or wildtype peptide without further CTL expansion *in vitro*. From this material, the precursor frequency of CTL reactive against either wildtype or analog using Elispot assay is determined. The precursor frequencies of wildtype peptide reactive CTLs are typically much lower than the precurser frequencoies of the analogs.

Heteroclitic analogs can induce human CTL capable of recognizing epitopes in vitro

Heteroclitic analogs can be analyzed for induction of CTLs in a primary *in vitro* induction system. Fresh naïve human PBMC from normal donors are stimulated repetitively *in vitro*, with either wildtype or analogs, in 48 well plates as described previously. Peptide specific CTL responses are then detected in cultures stimulated with either a wildtype peptide or a heteroclitic analog. Cultures induced with these analogs can recognize targets that are endogenously processed and present the wildtype sequence. This demonstrates that heteroclitic analogs can induce physiologically relevant human CTLs that recognize endogenously generated wildtype peptide expressed on cells and that the phenomenon is relevant in both human and in transgenic mouse systems.

Example 11

Synthesis and Analysis of Heteroclitic Analogs Derived from the HLA-A3 Supermotif on HLA A3 Superfamily Members

To further validate the heteroclitic substitution rules, additional studies are carried out with heteroclitic analogs derived from a peptide bearing a sequence within the HLA-A3 supermotif. For example, the analogs can be tested for *in vivo* immunogenicity.

For this study, the HLA-A3 supermotif bearing peptide, KVFPYALINK (SEQ ID NO:22) epitope is chosen and synthesized. A panel of analogs of SEQ ID NO:22

consisting of three conservative/semiconservative substitutions at positions 3, 5 and 7 of the 9-mer peptide, are tested for immunogenicity in HLA-A*3101/K^b transgenic mice. The panel includes KVHPYALINK, KVFPQALINK and KVFPYAKINK, corresponding to a semi-conservative change in the third, fifth and seventh position, respectively.

CTLs from the mice immunized with the above-described analogs are tested for induction of at least 100 pg/well of IFNγ production. This IFNγ production typically occurs at much lower peptide concentrations than those induced and restimulated with wildtype peptide (*e.g.*, KVFPYALINK). These results indicate that our predicted heteroclitic analogs are more potent at inducing higher avidity CTL against wildtype than wildtype peptide itself.

Typically, CTLs obtained from animals immunized and restimulated with a wildtype peptide induce 100 pg/well IFNγ at peptide doses of 5-10μg/ml, whereas CTLs obtained from animals immunized with the above-described analogs, and stimulated and tested *in vitro* with wildtype peptide, require 10-fold, 100-fold or even 1000-fold lower doses of wildtype peptide respectively, to induce 100pg/well of IFNγ.

To further validate the heteroclitic substitution rules for other HLA molecules with the A3 superfamily, the peptides KVHPYALINK, KVFPQALINK, and KVFPYAKINK are tested for *in vivo* immunogenicity in transgenic mice expressing one of the following human HLA molecules: A*0301, A*1101, A*3101, A*3301 and A*6801.

CTLs from the mice immunized with the above-described analogs are tested for induction of at least 100 pg/well of IFNγ production. This IFNγ production typically occurs at much lower peptide concentrations than those induced and restimulated with wildtype peptide (*e.g.*, KVFPYALINK). These results will indicate that our predicted heteroclitic analogs are more potent at inducing higher avidity CTL than wildtype peptide itself.

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To confirm that cross-reactive CTL against wildtype peptide are generated in mice immunized with analogs, $CD8^+$ cells are isolated from spleens immunized with analogs or wildtype peptide without further CTL expansion *in vitro*. From this material, the precursor frequency of CTL reactive against either wildtype or analog using Elispot assay is determined. The precursor frequencies of wildtype peptide reactive CTLs are typically much lower than the precurser frequencies of the analogs.

Heteroclitic analogs can induce human CTL capable of recognizing epitopes in vitro

Heteroclitic analogs are analyzed for induction of CTLs in a primary *in vitro* induction system. Fresh naïve human PBMC from normal donors are stimulated repetitively *in vitro*, with either wildtype or analogs, in 48 well plates as described previously. Peptide specific CTL responses are then detected in cultures stimulated with either a wildtype peptide or a heteroclitic analog. Cultures induced with these analogs recognize targets that are endogenously processed and present the wildtype sequence. This demonstrates that heteroclitic analogs induce physiologically relevant human CTLs that recognize endogenously generated wildtype peptide expressed on cells and that the phenomenon is relevant in both human and in transgenic mouse systems.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

Claims

1. A method to enhance the immunogenicity of a peptide containing an epitope, the method comprising

i) providing a peptide comprising a first Class I epitope wherein said epitope consists essentially of an amino acid sequence having an N-terminus and a C-terminus and at least one primary anchor residue, wherein amino acid residues are numbered consecutively and the primary anchor residue nearest the N-terminus of the epitope is at position 2 or position 3; and

ii) introducing one or more one conservative or semi-conservative substitution between the N-terminus and the C-terminus of the epitope at position 3 and/or 5 and/or 7 provided said position is not a primary anchor residue,

thereby constructing a peptide comprising a second Class I epitope which exhibits enhanced immunogenicity compared to the first Class I epitope.

2. The method of claim 1, wherein the second Class I epitope exhibits at least about 50% increased potency for a specific T-cell compared to the first Class I epitope.

3. The method of claim 1, wherein only one substitution is introduced.

4. The method of claim 1, wherein the substitution is a conservative substitution.

5. The method of claim 1, wherein the substitution is a semi-conservative substitution.

6. The method of claim 1, wherein the peptide comprising said second Class 1 epitope induces both Th1 and Th2 cytokines when said peptide is bound by an HLA Class I molecule and contacted with a cytotoxic T-cell.

7. The method of claim 1, wherein the first Class I epitope comprises a supermotif selected from the group consisting of A1, A2, A3, A24, B7, B27, B44, B58 and B62.

8. The method of claim 1, wherein the first Class I epitope is derived from a viral antigen, a tumor-associated antigen, a parasitic antigen, a bacterial antigen or a fungal antigen.

9. A peptide comprising the second Class I epitope prepared by the method of claim 1.

10. A method to elicit an immune response which method comprises contacting cytotoxic T lymphocytes (CTLs) with the peptide of claim 9.

11. The method of claim 10, wherein the step of contacting is carried out *in vitro* in the presence of an antigen presenting cell.

12. The method of claim 10, wherein said contacting is carried out by administering to a subject a nucleic acid molecule comprising a nucleotide sequence encoding said peptide.

13. A composition comprising at least one peptide, the peptide comprising a Class I epitope obtainable by the method of claim 1.

14. The composition of claim 13, wherein the peptide contains 9-15 amino acids.

15. The composition of claim 13, wherein the peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19 and SEQ ID NO:20.

16. A composition of claim 13, wherein the peptide is admixed or joined to a CTL epitope.

17. A composition of claim 13, wherein the peptide is admixed or joined to an HTL epitope.

18. A composition of claim 17, wherein the HTL epitope is a pan-DR binding molecule.

19. A composition of claim 13, further comprising a liposome.

20. A composition of claim 13, wherein the epitope is coupled to a lipid.

21. A composition of claim 13, wherein said epitope is included in a heteropolymer.

22. A composition of claim 13, wherein the epitope is included in a homoplymer.

23. A composition of claim 13, wherein the epitope is bound to an HLA heavy chain, β2-microglobulin, and strepavidin complex, whereby a tetramer is formed.

24. A composition of claim 13, further comprising an antigen presenting cell, wherein the epitope is on or within the antigen presenting cell.

25. A composition of claim 24, wherein the epitope is bound to an HLA molecule on the antigen presenting cell, whereby when a cytotoxic lymphocyte (CTL) that is restricted to the HLA molecule is present, a receptor of the CTL binds to a complex of the HLA molecule and the epitope.

26. A composition of claim 25, wherein the antigen presenting cell is a dendritic cell.

27. A composition of claim 13, further comprising an HLA molecule, wherein the peptide is bound by the HLA molecule.

28. A composition of claim 13, further comprising a label.

29. A composition of claim 28, wherein the label is biotin, a fluorescent moiety, a non-mammalian sugar, a radio label or a small molecule to which a monoclonal antibody binds.

30. The composition of claim 13 which is a vaccine containing:
a unit dosage of said peptide, and
a pharmaceutical excipient.

31. A nucleic acid molecule comprising a nucleotide sequence encoding a peptide of 9-15 amino acids which comprises a second Class I epitope obtainable by the method of claim 1.

32. The nucleic acid molecule of claim 31 wherein said peptide comprises an epitope consisting of an amino acid sequence selected from the group consisting of SEQ. ID. NO: 2, SEQ. ID. NO: 3, SEQ. ID. NO: 5, SEQ. ID. NO: 6, SEQ. ID. NO: 8, SEQ. ID. NO: 9, SEQ. ID. NO: 11, SEQ. ID. NO: 12, SEQ. ID. NO: 14, SEQ. ID. NO: 15, SEQ. ID. NO: 17, SEQ. ID. NO: 19 and SEQ. ID. NO: 20

33. The nucleic acid molecule of claim 32 which further comprises control sequences for expression of said nucleotide sequence.

34. A pharmaceutical composition which comprises as active ingredient the nucleic acid molecule of claim 31.

SEQ. ID NO:1	IMIGVLVGV	CEA.691
SEQ. ID NO:2	IMMGVLVGV	CEA.691 M3
SEQ. ID NO:3	IMIGHLVGV	CEA.691 H5
SEQ. ID NO:4	KVAELVHFL	MAGE3.112
SEQ. ID NO:5	KVAEIVHFL	MAGE3.112 I5
SEQ. ID NO:6	KVAELVWFL	MAGE3.112 W7
SEQ. ID NO:7	YLQLVFGIEV	MAGE2.157
SEQ. ID NO:8	YLQLIFGIEV	MAGE2.157 I5
SEQ. ID NO:9	YLQLFFGIEV	MAGE2.157 F5
SEQ. ID NO:10	SMPPPGTRV	p53.149M2
SEQ. ID NO:11	CMPPPGTRV	p53.149M2 C1
SEQ. ID NO:12	SMPPPGPRV	p53.149M2 P7
SEQ. ID NO:13	GLAPPQHLIRV	p53.Mu184
SEQ. ID NO:14	GLTPPQHLIRV	p53.Mu184 T3
SEQ. ID NO:15	GLTPPEHLIRV	p53.Mu184 T3, E6
SEQ. ID NO:16	GLSRYVARL	HBV Po1455
SEQ. ID NO:17	GLSRYVPRL	HBV Po1455 P7
SEQ. ID NO:18	ILKEPVHGV	HIV Po1476
SEQ. ID NO:19	ILHEPVHGV	HIV Po1476 H3
SEQ. ID NO:20	ILIEPVHGV	HIV Po1476L3
SEQ. ID NO:21	LLGRDSFEV	p53.261
SEQ. ID NO:22	LLDRDSFEV	p53.261 D3
SEQ. ID NO:23	LLHRDSFEV	p53.261 H3
SEQ. ID NO:24	LLGRDSLEV	p53.261 L7
SEQ. ID NO:25	LLGRDSHEV	p53.261 H7
SEQ. ID NO:26	LLGRNSFEV	p53.261 N5
SEQ. ID NO:27	LLGRGSFEV	p53.261 G5
SEQ. ID NO:28	APAAAAAAY	
SEQ. ID NO:29	KVFPYALINK	
SEQ. ID NO:30	TPPAYRPPNAPIL	(HBV core)
SEQ. ID NO:31	FLPSDFFDSV	(HBV core)

FIG. 1A

TABLE I (Cont.) Characterization of heteroclitic analogs identified from tumor and viral antigens

Antigen	Sequqnce	Heteroclitic substitution	Type of sustitution	Position of sustitution	Th1 cytokines[a]	Th2 cytokines[b]	A*0201 binding (IC50, nM)[d]
CEA.691	IMIGVLVGV	None (WT)	None		1	10	54
CEA.691M3	IMMGVLVGV	I → M	Conservative	3	10^{-4}	1	27
CEA.691H5	IMIGHLVGV	V → H	Semi-conservative	5	10^{-6}	10^{-1}	16
IMAGE3.112	KVAELVHFL	None (WT)	None		1	NS[c]	94
IMAGE3.112I5	KVAEIVHFL	L → I	Conservative	5	10^{-5}	NS	66
IMAGE3.112W7	KVAELVWFL	H → W	Semi-conservative	7	10^{-5}	NS	7
IMAGE2.157	YLQLVFGIEV	None (WT)	None		1	10	40
IMAGE2.157I5	YLQLIFGIEV	V → I	Conservative	5	10^{-4}	10^{-1}	476
IMAGE2.157F5	YLQLFFGIEV	V → F	Semi-conservative	5	10^{-2}	10^{-1}	212
HBV Pol.455	GLSRYVARL	None (WT)	None		10	10	83
HBV Pol.455P7	GLSRYVPRL	A → P	Conservative	7	10^{-2}	10^{-2}	267
HIV Pol.476	ILKEPVHGV	None (WT)			>10	>10	369
HIV Pol.476H3	ILHEPVHGV	K → H	Conservative	3	1	1	78
HIV Pol.476L3	ILIEPVHGV	K → L	Semi-conservative	3	10^{-1}	1	63

FIG. 1B

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CA 02386341 2002-03-27
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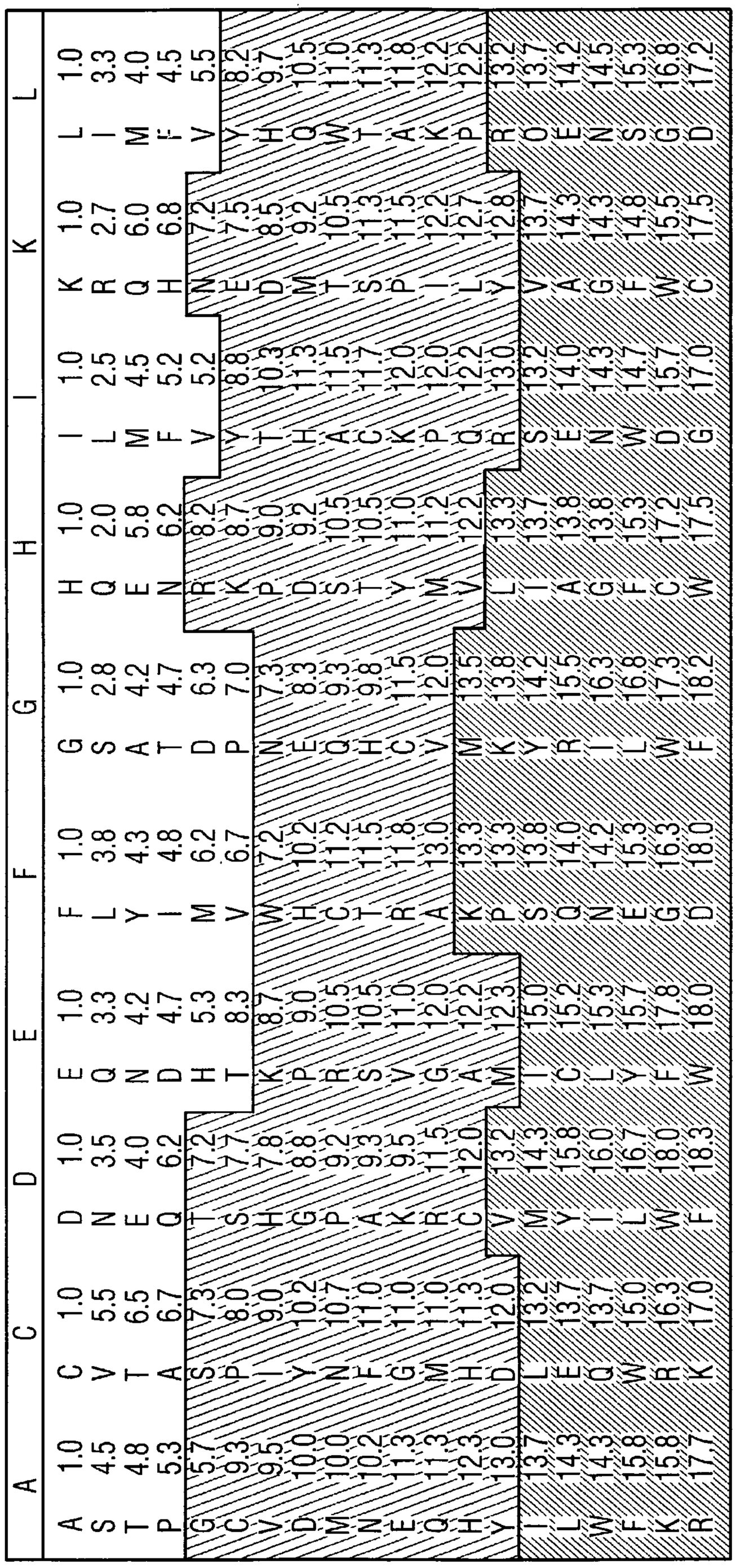
A C
A 1.0 C 1.0
S 4.5 V 5.5
T 4.8 T 6.5
P 5.3 A 6.7
5.7 7.3
9.3 8.0
9.5 9.0
10.0 10.2
10.0 10.7
10.2 11.0
11.3 11.0
11.3 11.3
12.3 11.3
13.0 12.0
G S
C P
V —
D Y
M N
N F
E G
Q M
H H
Y D
13.7 13.2
14.3 13.7
15.8 13.7
15.8 15.0
17.7 16.3
17.0
I L
L E
W Q
F W
K R
R K
A D
E 1.0 F 1.0
Q 3.3 L 3.8
N 4.2 Y 4.3
D 4.7 I 4.8
H 5.3 M 6.2
T 8.3 V 6.7
7.2 7.2
7.7 8.7
7.8 9.0
8.8 9.2
9.2 10.2
9.3 10.5
9.5 11.5
11.5 11.8
12.0 12.0
K W
P H
R C
S T
V R
G A
A K
M P
13.2 13.3
13.8 13.3
14.2 13.8
15.5 14.0
16.3 14.2
16.8 15.3
17.3 15.7
18.2 16.3
18.0
D 1.0 E 1.0
N 3.5 Q 3.3
E 4.0 N 4.2
Q 6.2 D 4.7
T 7.2 H 5.3
S 7.7 T 8.3
H 7.8 8.7
G 8.8 9.0
P 9.2 10.5
A 9.3 10.5
K 9.5 11.0
R 11.5 12.0
C 12.0 12.3
V 13.2 13.0
M 14.3 15.0
Y 15.8 15.2
I 16.0 15.3
L 16.7 15.7
W 18.0 17.8
F 18.3 18.0
G 1.0 H 1.0
S 2.8 Q 2.0
A 4.2 E 5.8
T 4.7 N 6.2
D 6.3 R 8.2
P 7.0 K 8.7
7.3 P 9.0
8.3 D 9.2
9.3 S 10.5
9.8 T 10.5
11.5 Y 11.0
12.0 M 11.2
12.2
N V
E L
Q I
H A
C G
V L
M I
K C
Y L
R W
I F
L K
W R
F D
G 1.0 I 1.0
S 2.5 L 2.5
A 4.5 M 4.5
T 5.2 F 5.2
D 5.2 V 5.2
P 8.8
10.3
11.3
11.5
12.0
12.0
12.2
13.0
Y H
T Q
H W
A T
C A
K K
P P
Q R
R 13.2
S O 13.7
T E 14.2
Y N 14.5
V S 15.3
A G 16.8
G D 17.2
F
W
C
K 1.0 L 1.0
R 2.7 I 3.3
Q 6.0 M 4.0
H 6.8 F 4.5
N 7.2 V 5.5
E 7.5 8.2
D 8.5 9.7
M 9.2 10.5
T 10.5 11.0
S 11.3 11.3
P 11.5 11.8
I 12.2 12.2
L 12.7 12.2
Y 12.8
V 13.7
A 14.3
G 14.3
F 14.8
W 15.5
C 17.5

FIG. 2A

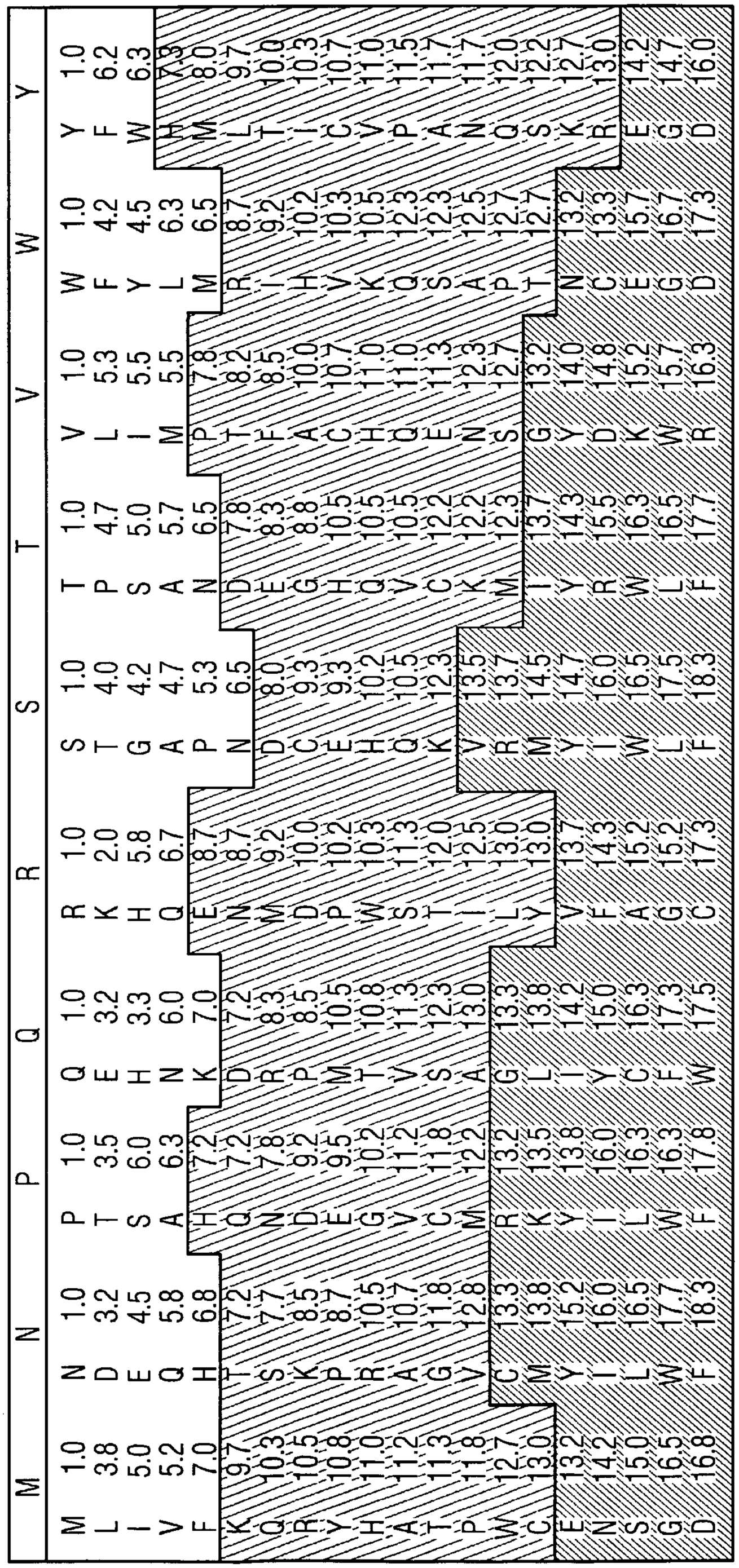
Y
W 1.0 Y 1.0
F 4.2 F 6.2
Y 4.5 W 6.3
L 6.3
M 6.5
W
F 1.0 T 1.0 V 1.0 R 1.0 Q 1.0 P 1.0 S 1.0
Y 4.2 P 4.7 L 5.3 K 2.0 E 3.2 T 3.5 T 4.0
L 4.5 S 5.0 I 5.5 H 3.3 H 6.0 S 6.0 G 4.2
M 6.3 A 5.7 M 5.5 Q 6.7 N 7.0 A 6.3 A 4.7
N 6.5 P 6.5 K 6.8 P 5.3
T
V
S
R
Q
P
N
M
L
I
V
F

FIG. 2B

SUPERMOTIFS	POSITION	POSITION	POSITION
	2 (Primary Anchor)	3 (Primary Anchor)	C Terminus (Primary Anchor)
A1	**T, I,** *L, V, M, S*		**F, W, Y**
A2	**L, I, V, M,** *A, T, Q*		**I, V,** *M, A, T, L*
A3	**V, S, M, A,** *T, L, I*		**R, K**
A24	**Y, F,** *W, I, V, L, M, T*		**F, I,** *Y, W, L, M,*
B7	**P**		**V, I, L, F,** *M, W, Y, A*
B27	**R, H, K**		**F, Y, L,** *W, M, I, V, A*
B44	**E,** *D*		**F, W, L, I, M, V, A**
B58	**A, T, S**		**F, W, Y,** *L, I, V, M, A*
B62	**Q, L,** *I, V, M, P*		**F, W, Y,** *M, I, V, L, A*
MOTIFS			
A1	**T, S, M**		**Y**
A1		**D, E,** *A, S*	**Y**
A2.1	**L, M,** *V, Q, I, A, T*		**V,** *L, I, M, A, T*
A3	**L, M, V, I, S, A, T, F,** *C, G, D*		**K, Y, R,** *H, F, A*
A11	**V, T, M, L, I, S, A, G, N,** *C, D, F*		**K,** *R, Y, H*
A24	**Y, F, W,** *M*		**F, L, I, W**
A*3101	**M, V, T,** *A, L, I, S*		**R,** *K*
A*3301	**M, V, A, L, F,** *I, S, T*		**R, K**
A*6801	**A, V, T,** *M, S, L, I*		**R, K**
B*0702	**P**		**L, M, F,** *W, Y, A, I, V*
B*3501	**P**		**L, M, F, W, Y,** *I, V, A*
B51	**P**		**L, I,V, F,** W, Y, *A, M,*
B*5301	**P**		**I, M, F, W, Y,** *A, L, V*
A*5401	**P**		**A, T, I, V,** *L, M, F, W, Y*

FIG. 3A

SUPERMOTIFS	*POSITION*	*POSITION*	*POSITION*
	2 (Primary Anchor)	*3 (Primary Anchor)*	*C Terminus (Primary Anchor)*
A1	**T, I,** *L, V, M, S*		**F, W, Y**
A2	**V,** *Q, A, T*		**I, V,** L, *M, A, T,*
A3	**V, S, M, A,** *T, L, I*		**R, K**
A24	**Y, F,** *W, I, V, L, M, T*		**F, I,** *Y, W, L, M,*
B7	**P**		**V, I, L, F,** *M, W, Y, A*
B27	**R, H, K**		**F, Y, L,** *W, M, I, V, A*
B58	**A, T, S**		**F, W, Y,** *L, I, V, M, A*
B62	**Q, L,** *I, V, M, P*		**F, W, Y,** *M, I, V, L, A*
MOTIFS			
A1	**T, S, M**		**Y**
A1		**D, E,** *A, S*	**Y**
A2.1	*V, Q, A, T**		**V,** *L, I, M, A, T*
A3.2	**L, M, V, I, S, A, T, F,** *C, G, D*		**K, Y, R,** *H, F, A*
A11	**V, T, M, L, I, S, A, G, N,** *C, D, F*		**K,** *R, H, Y*
A24	**Y, F, W,**		**F, L, I, W**

** if 2 is V, or Q, the C-term is not L*

FIG. 3B

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HLA-supertype	Allelle-specific HLA-supertype members: Verified[a]	Predicted[b]
A1	A*0101, A*2501, A*2601, A*2602, A*3201	A*0102, A*2604,A*3601, A*4301, A*8001
A2	A*0201, A*0202, A*0203, A*0204, A*0205, A*0206, A*0207 A*0209, A*0214, A*6802, A*6901	A*0208, A*0210, A*0211, A*0212, A*0213
A3	A*0301, A*1101, A*3101, A*3301, A*6801	A*0302, A*1102, A*2603, A*3302, A*3303, A*3401 A*3402, A*6601, A*6602, A*7401
A24	A*2301, A*2402, A*3001	A*2403, A*2404, A*3002, A*3003
B7	B*0702, B*0703, B*0704, B*0705, B*1508, B*3501, B*3502, B*3503 B*3503, B*3504, B*3505, B*3506, B*3507, B*3508, B*5101, B*5102 B*5103, B*5104, B*5105, B*5301, B*5401, B*5501, B*5502, B*5601 B*5602, B*6701, B*7801	B*1511, B*4201, B*5901
B27	B*1401, B*1402, B*1509, B*2702, B*2703, B*2704, B*2705, B*2706 B*3801, B*3901, B*3902, B*7301	B*2701, B*2707, B*2708, B*3802, B*3903, B*3904, B*3905, B*4801, B*4802, B*1510, B*1518, B*1503
B44	B*1801, B*1802, B*3701, B*4402, B*4403, B*4404, B*4001, B*4002 B*4006	B*4101, B*4501, B*4701, B*4901, B*5001
B58	B*5701, B*5702, B*5801, B*5802, B*1516, B*1517	
B62	B*1501, B*1502, B*1513, B*5201	B*1301, B*1302, B*1504, B*1505, B*1506, B*1507, B*1515, B*1520, B*1521, B*1512, B*1514, B*1510

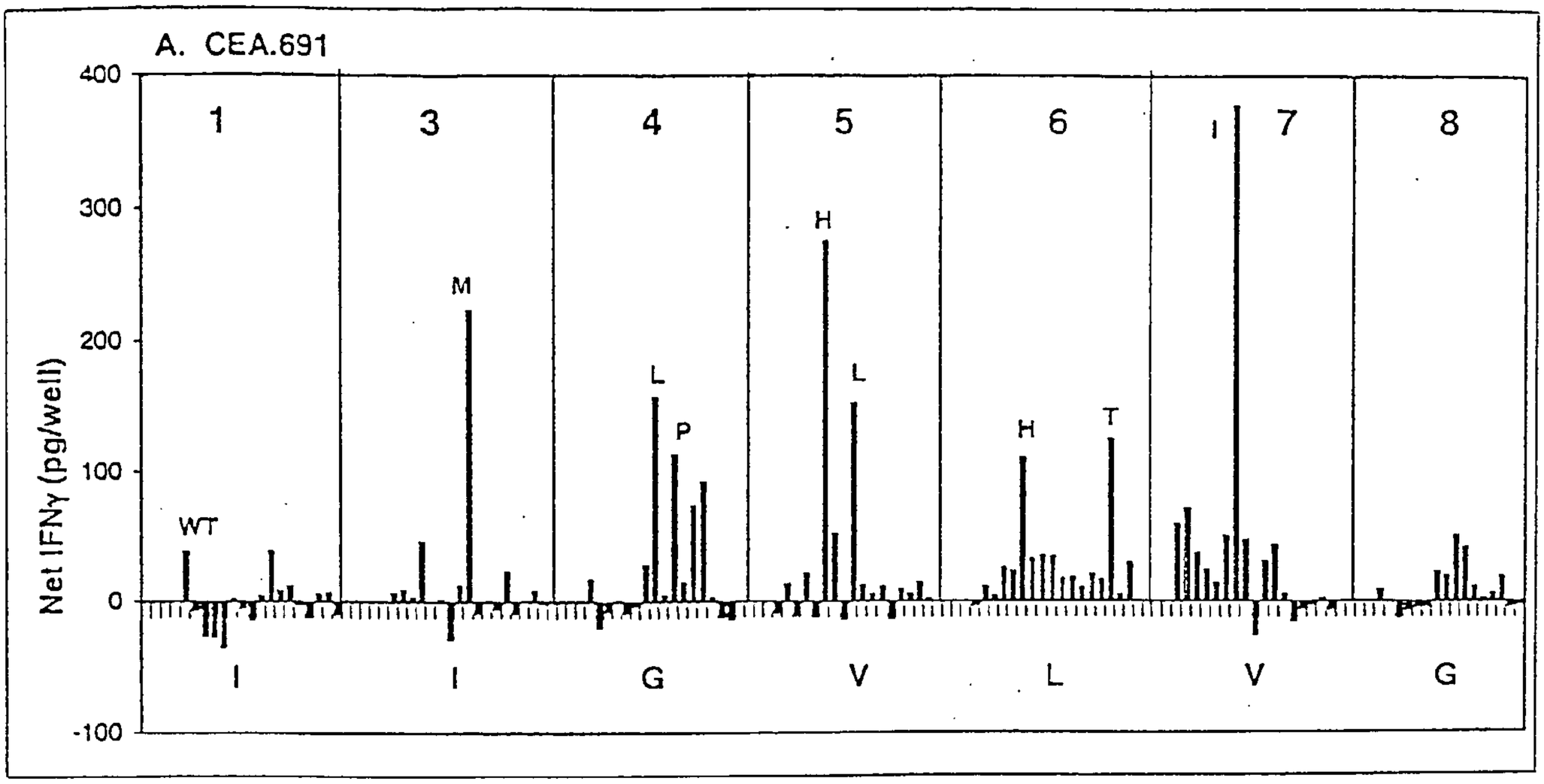
FIG. 4A

SUPERMOTIFS	*POSITION*	*POSITION*	*POSITION*
	2 (Primary Anchor)	*3 (Primary Anchor)*	*C Terminus (Primary Anchor)*
A1	**T, I,** *L, V, M, S*		**F, W, Y**
A2	**V,** *Q, A, T*		**I, V,L,** *M, A, T,*
A3	**V, S, M, A,** *T, L, I*		**R, K**
A24	**Y, F,** *W, I, V, L, M, T*		**F, I,** *Y, W, L, M,*
B7	**P**		**V, I, L, F,** *M, W, Y, A*
B27	**R, H, K**		**F, Y, L,** *W, M, I, V, A*
B58	**A, T, S**		**F, W, Y,** *L, I, V, M, A*
B62	**Q, L,** *I, V, M, P*		**F, W, Y,** *M, I, V, L, A*
MOTIFS			
A1	**T, S, M**		**Y**
A1		**D, E,** *A, S*	**Y**
A2.1	*V, Q, A, T**		**V,** *L, I, M, A, T*
A3.2	**L, M, V, I, S, A, T, F,** *C, G, D*		**K, Y, R,** *H, F, A*
A11	**V, T, M, L, I, S, A, G, N,** *C, D, F*		**K,** *R, H, Y*
A24	**Y, F, W,**		**F, L, I, W**

** if 2 is V, or Q, the C-term is not L*

FIG. 4B

Figure 1A and 1B



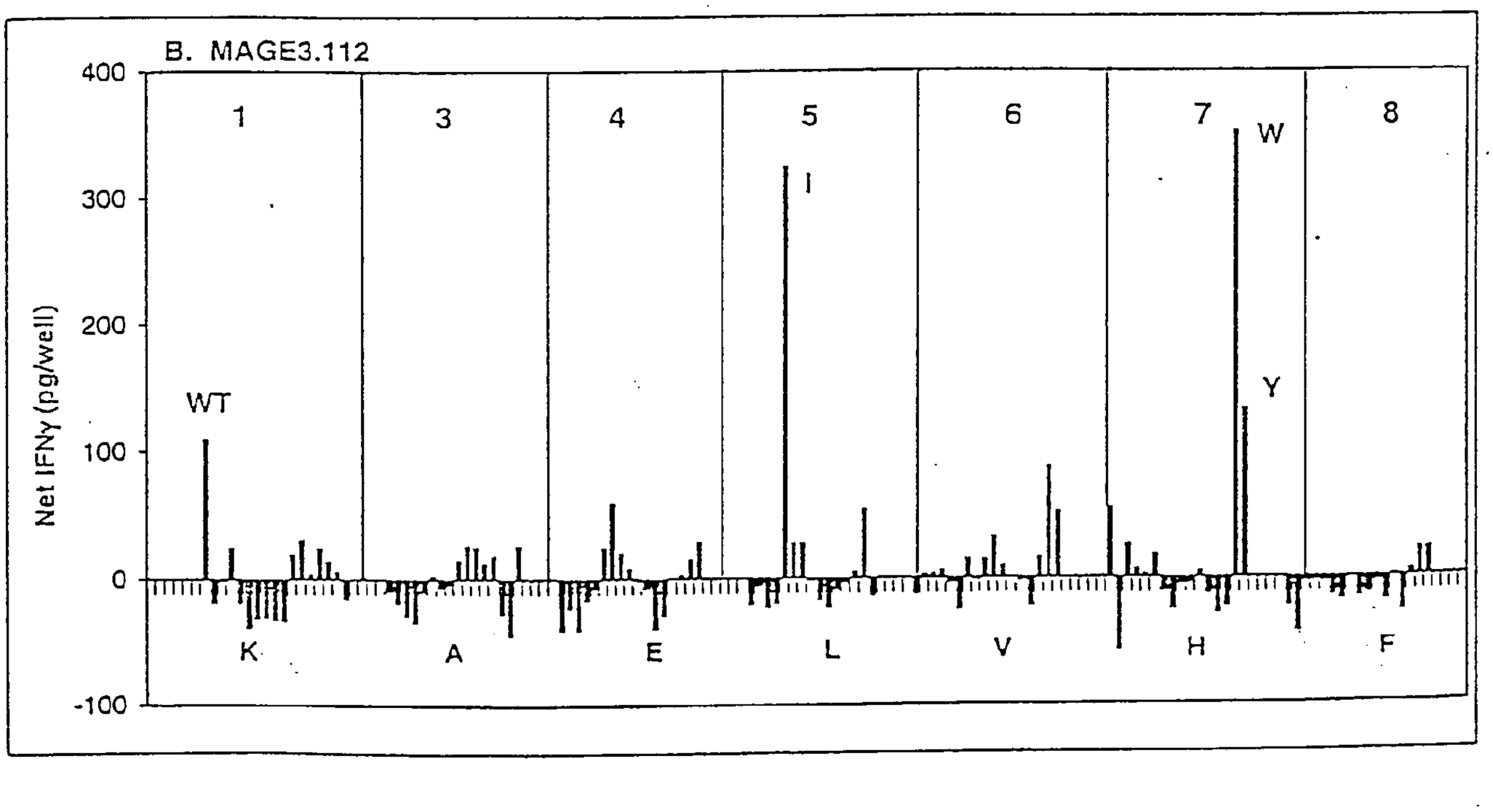
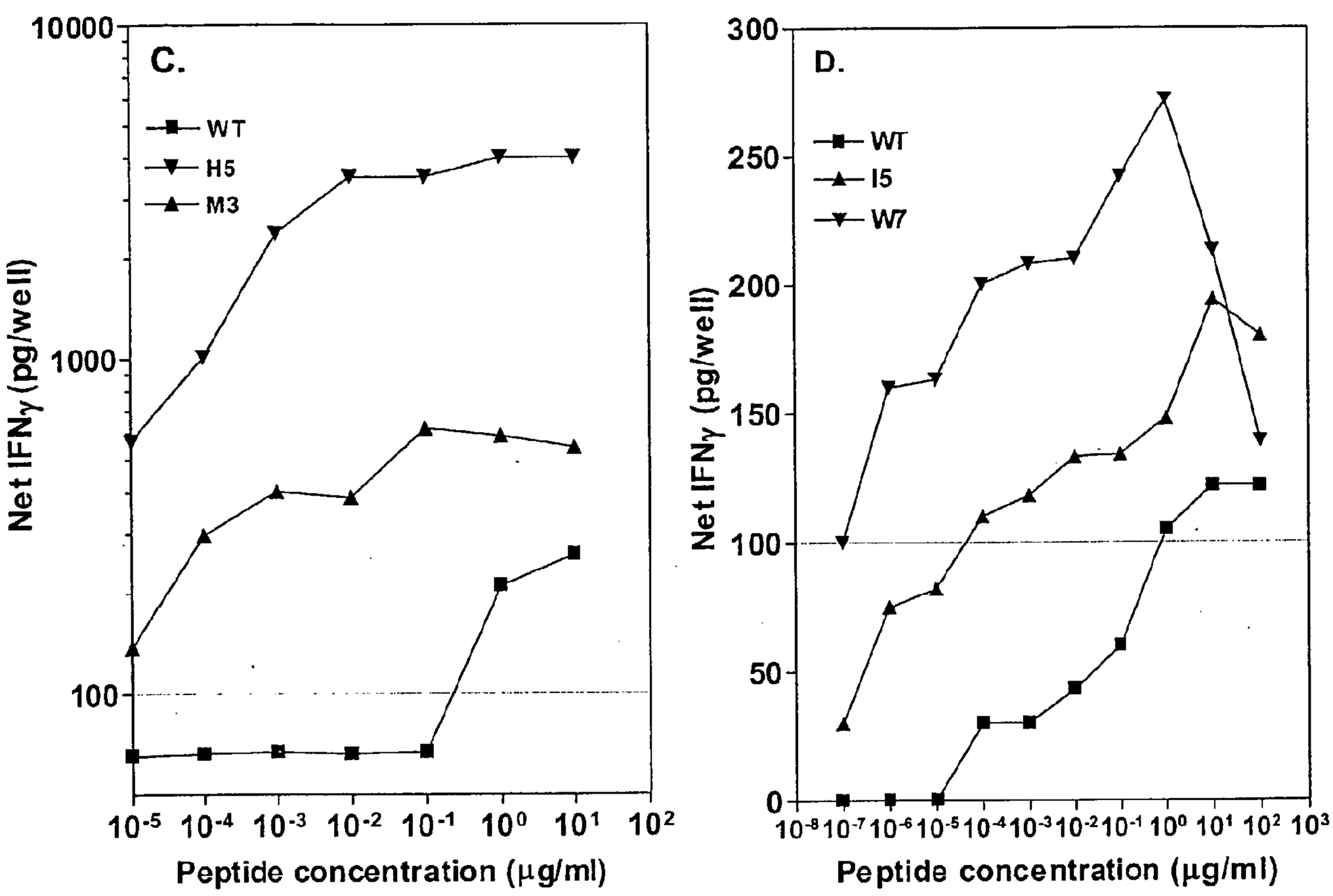


Figure 1C, D


C.
WT
H5
M3
Net IFNγ (pg/well)
10000
1000
100
10-5 10-4 10-3 10-2 10-1 100 101 102
Peptide concentration (μg/ml)
D.
WT
I5
W7
Net IFNγ (pg/well)
300
250
200
150
100
50
0
10-8 10-7 10-6 10-5 10-4 10-3 10-2 10-1 100 101 102 103
Peptide concentration (μg/ml)

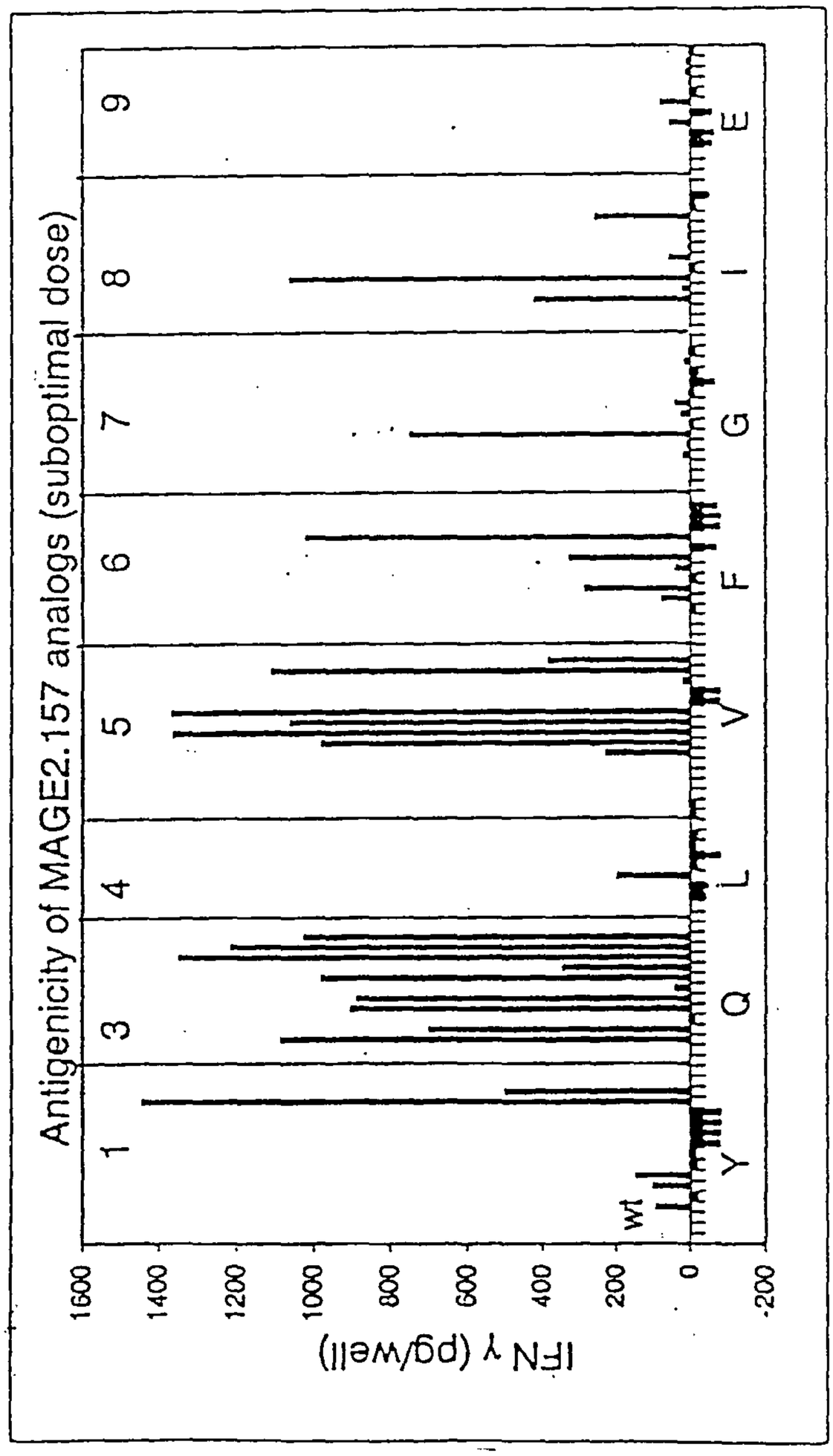

Antigenicity of MAGE2.157 analogs (suboptimal dose)
IFN γ (pg/well)
1600
1400
1200
1000
800
600
400
200
0
-200
wt
Y
1
3
Q
4
L
5
V
6
F
7
G
8
I
9
E

Figure 2A

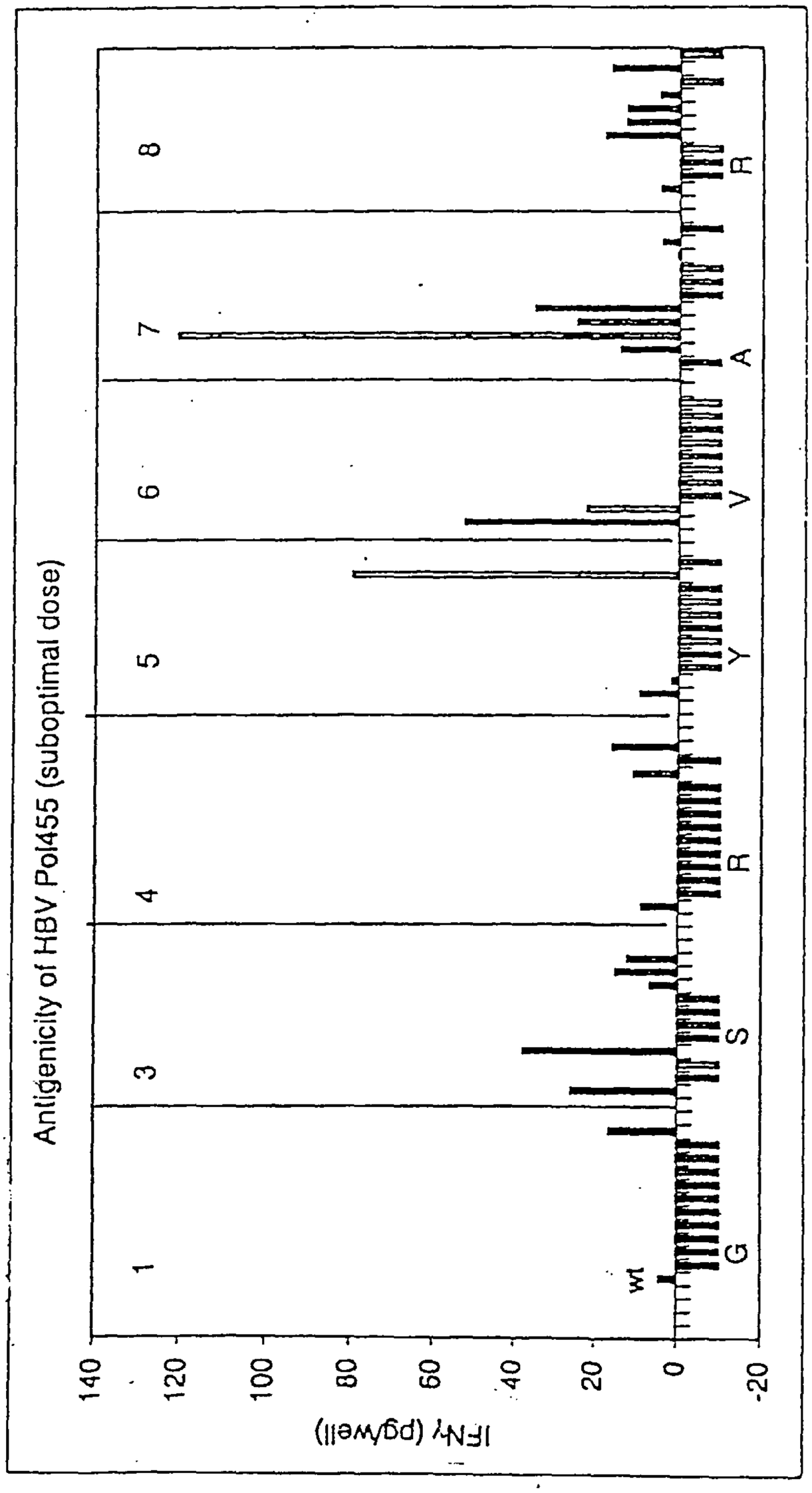

Antigenicity of HBV Pol455 (suboptimal dose)
IFNγ (pg/well)
140
120
100
80
60
40
20
0
-20
wt
1
3
4
5
6
7
8
G
S
R
Y
V
A
R

Figure 2B

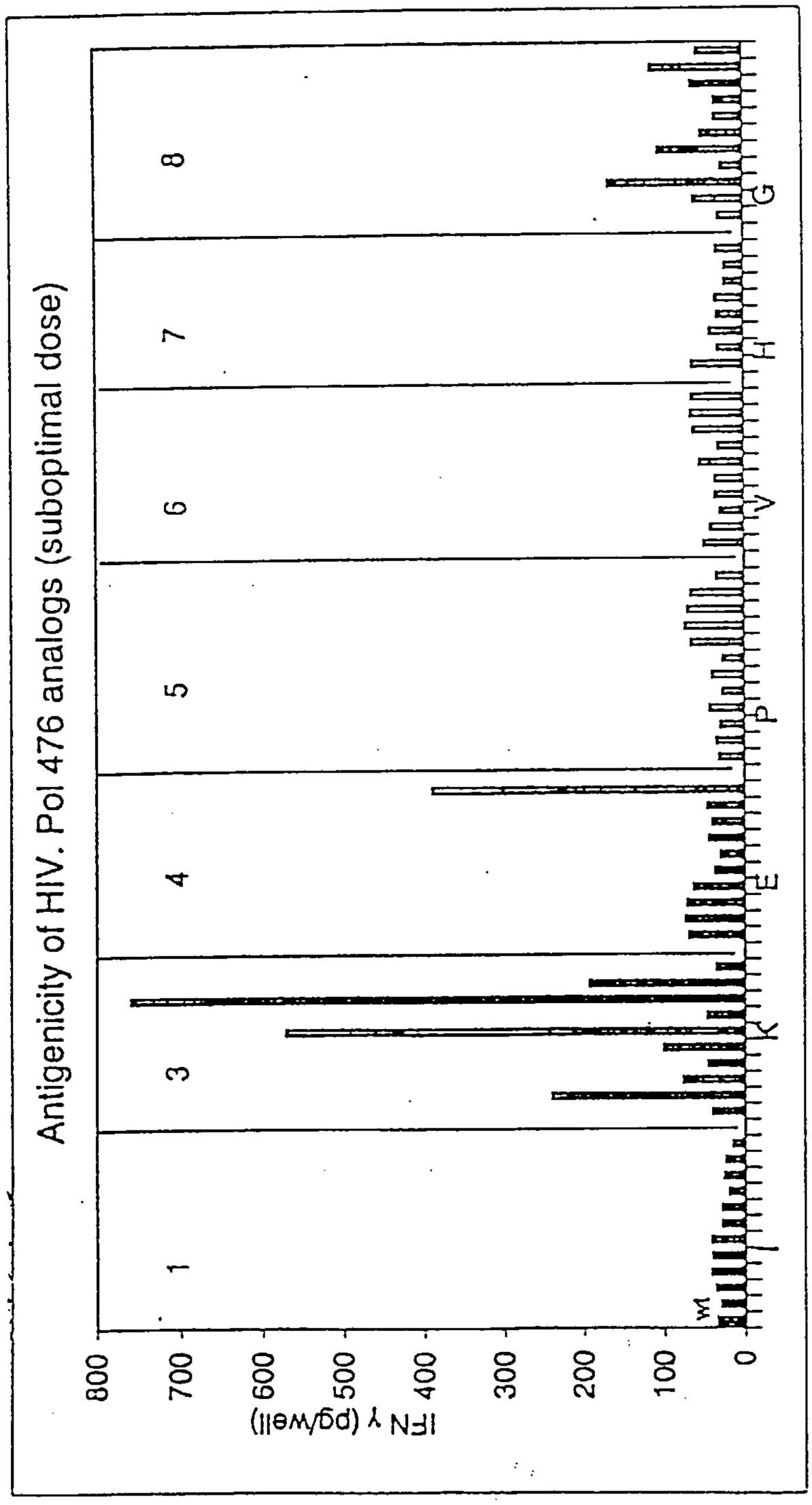

IFN γ (pg/well)
800
700
600
500
400
300
200
100
0
Antigenicity of HIV. Pol 476 analogs (suboptimal dose)
1
3
4
5
6
7
8
W
K
E
P
V
H
G

Figure 2C

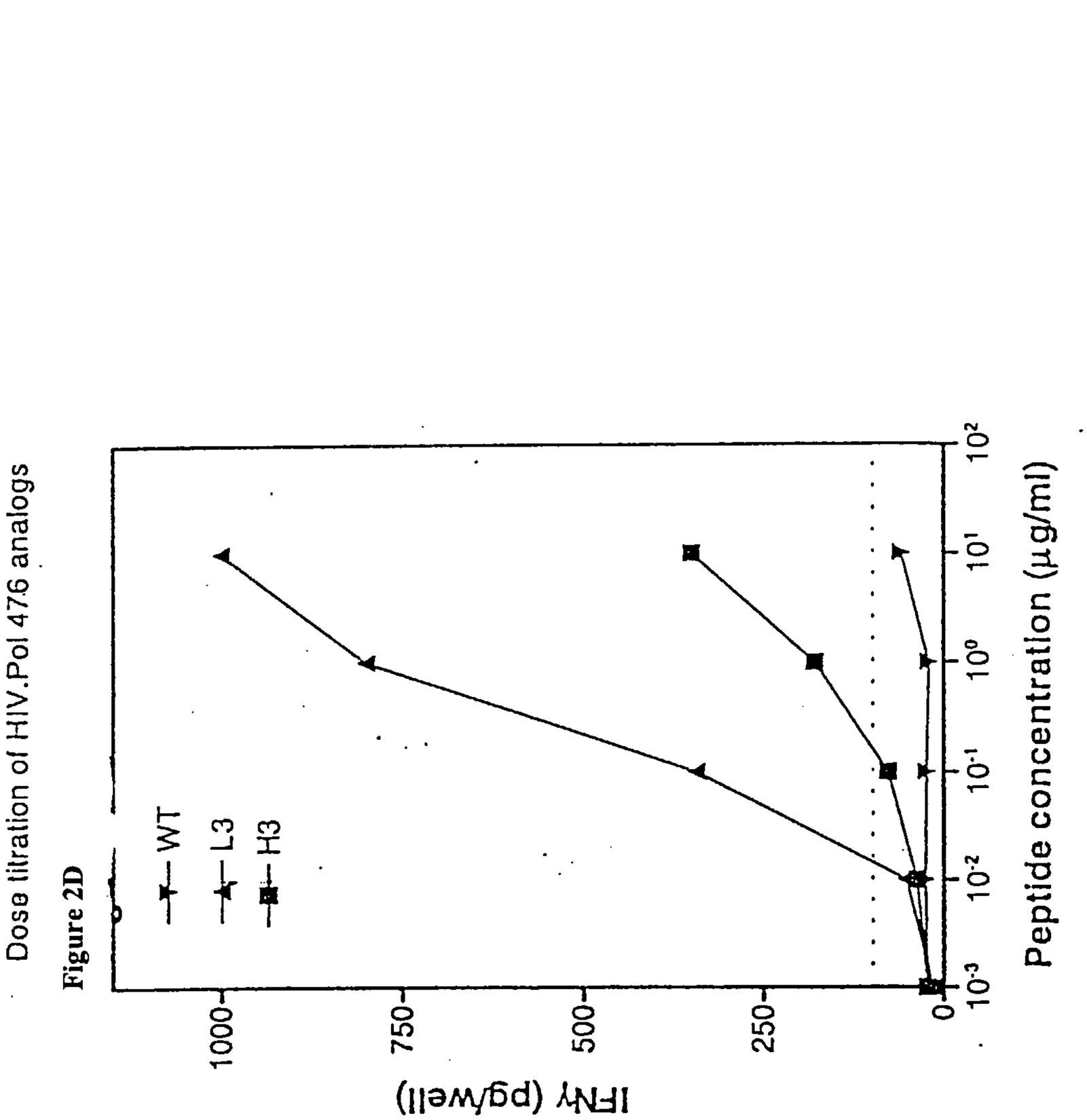

Dose titration of HIV.Pol 476 analogs
WT
L3
H3
IFNγ (pg/well)
1000
750
500
250
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
Peptide concentration (µg/ml)

Figure 2D

Figure 3A

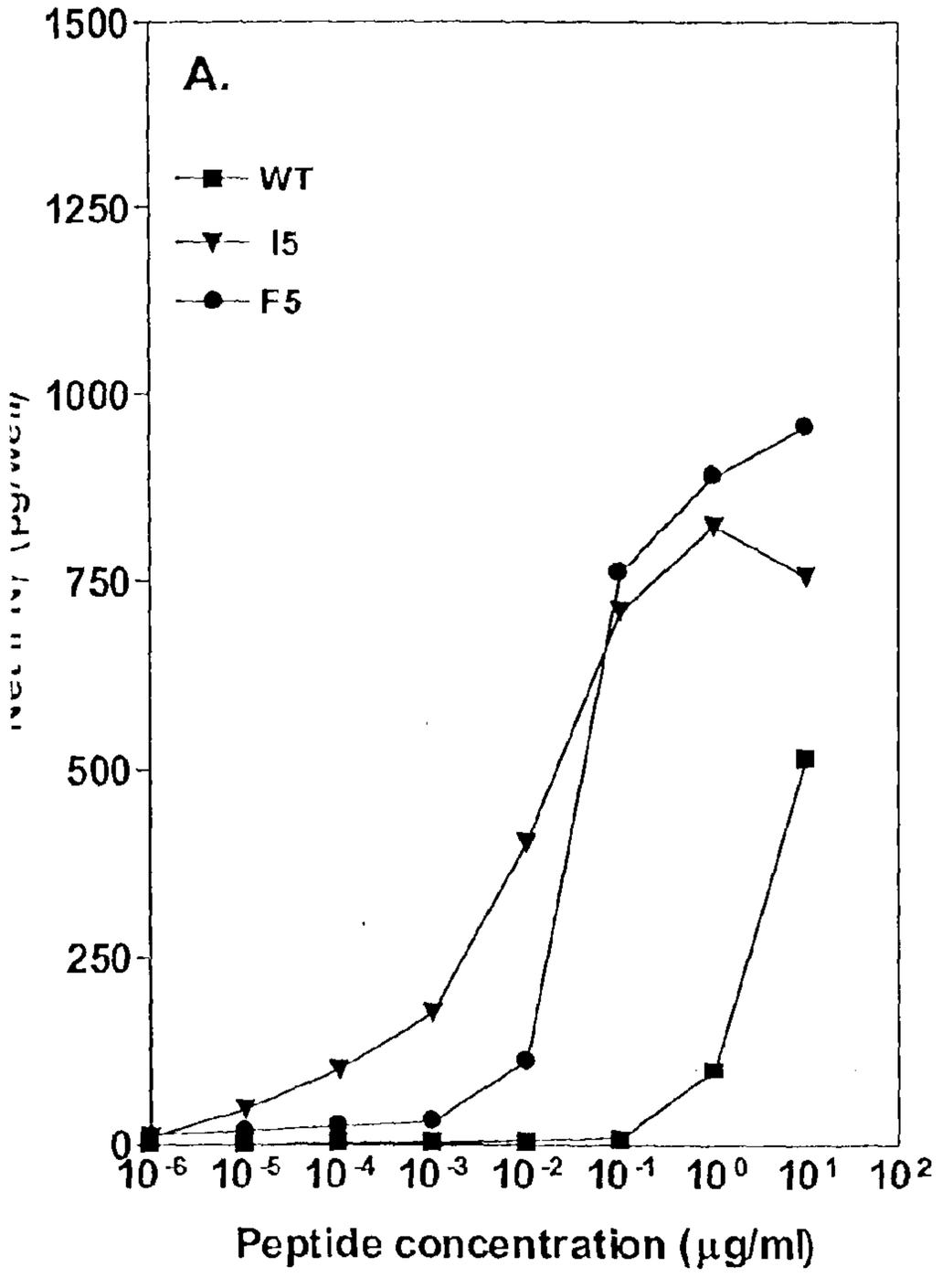
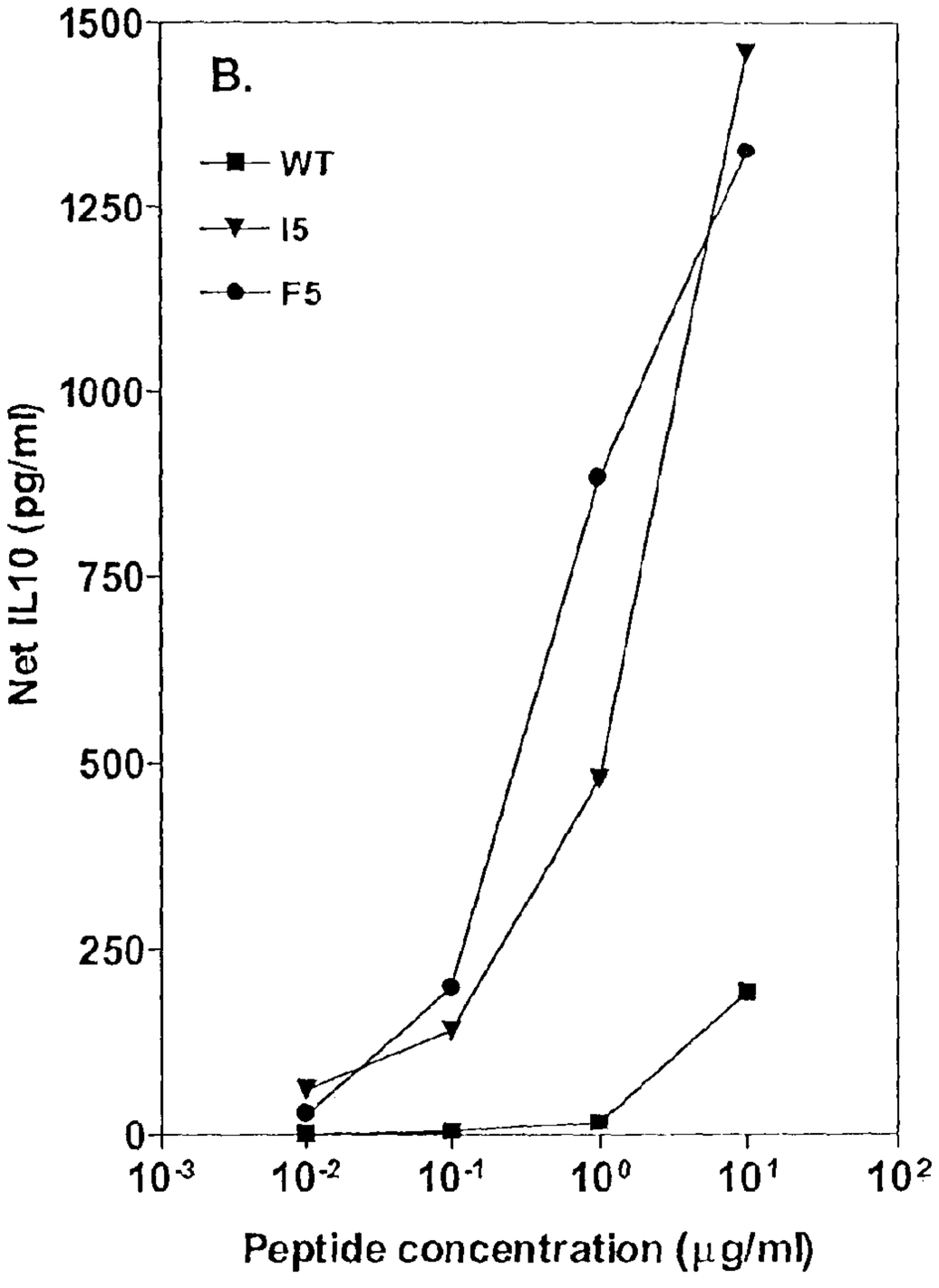
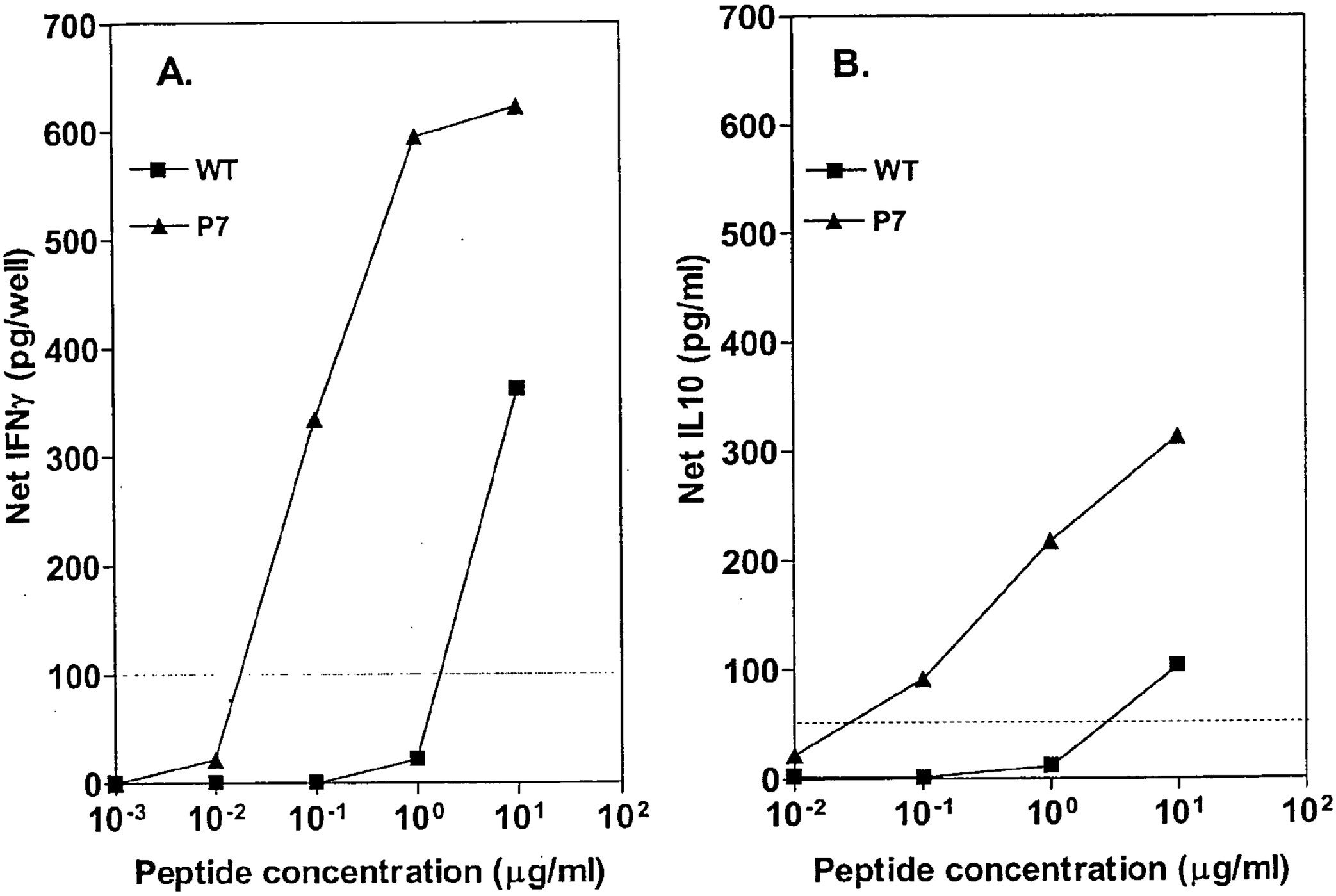


Figure 3B



MAGE 2.157 ANALOGS

Figure 4


A.
B.
WT
P7
Net IFNγ (pg/well)
Net IL10 (pg/ml)
700
600
500
400
300
200
100
0
10-3
10-2
10-1
100
101
102
Peptide concentration (μg/ml)

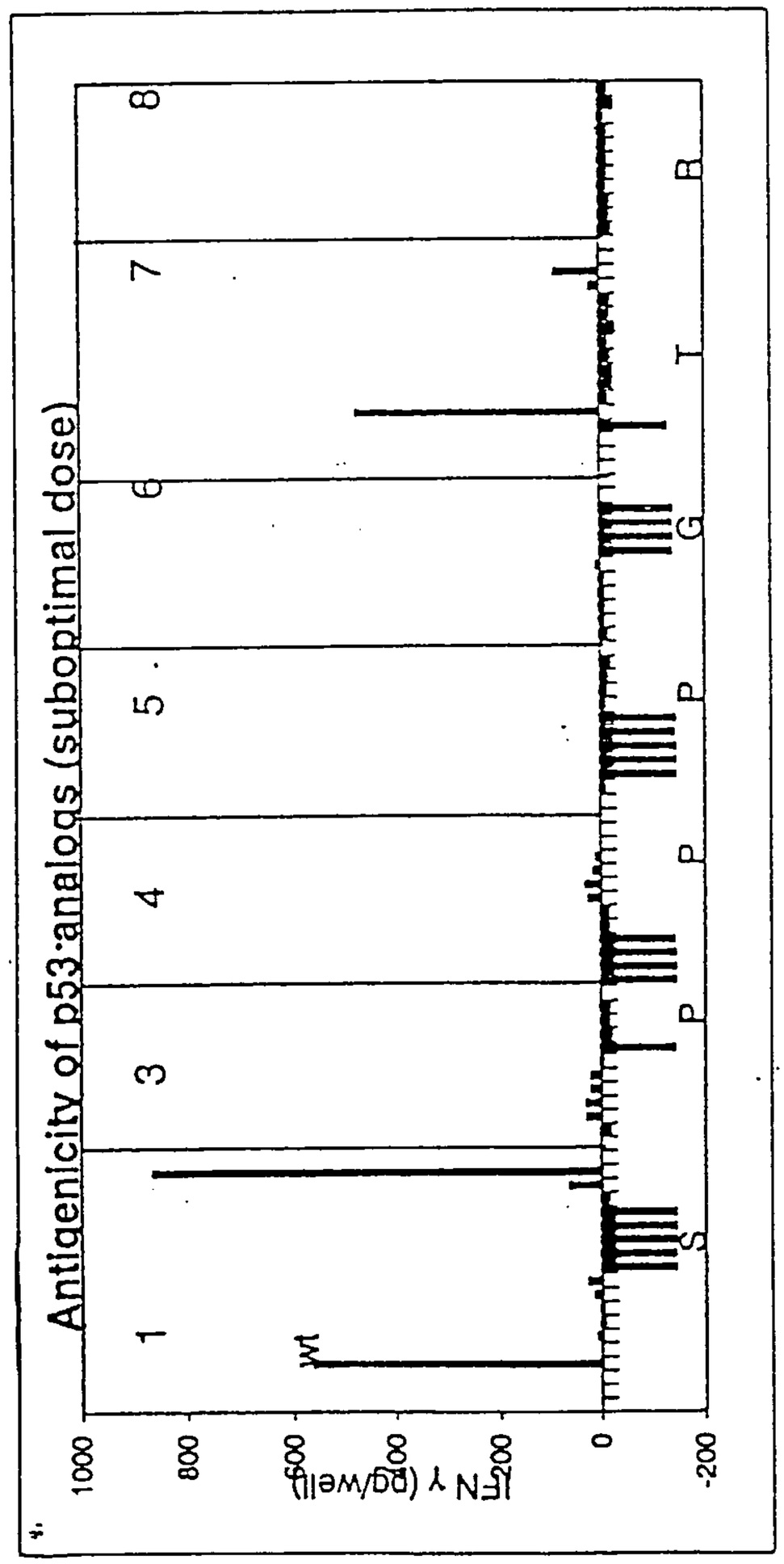

Antigenicity of p53 analogs (suboptimal dose)
IFN γ (pg/well)
1000
800
600
400
200
0
-200
1
2
3
4
5
6
7
8
WT
S
P
P
P
G
T
B

Figure 5

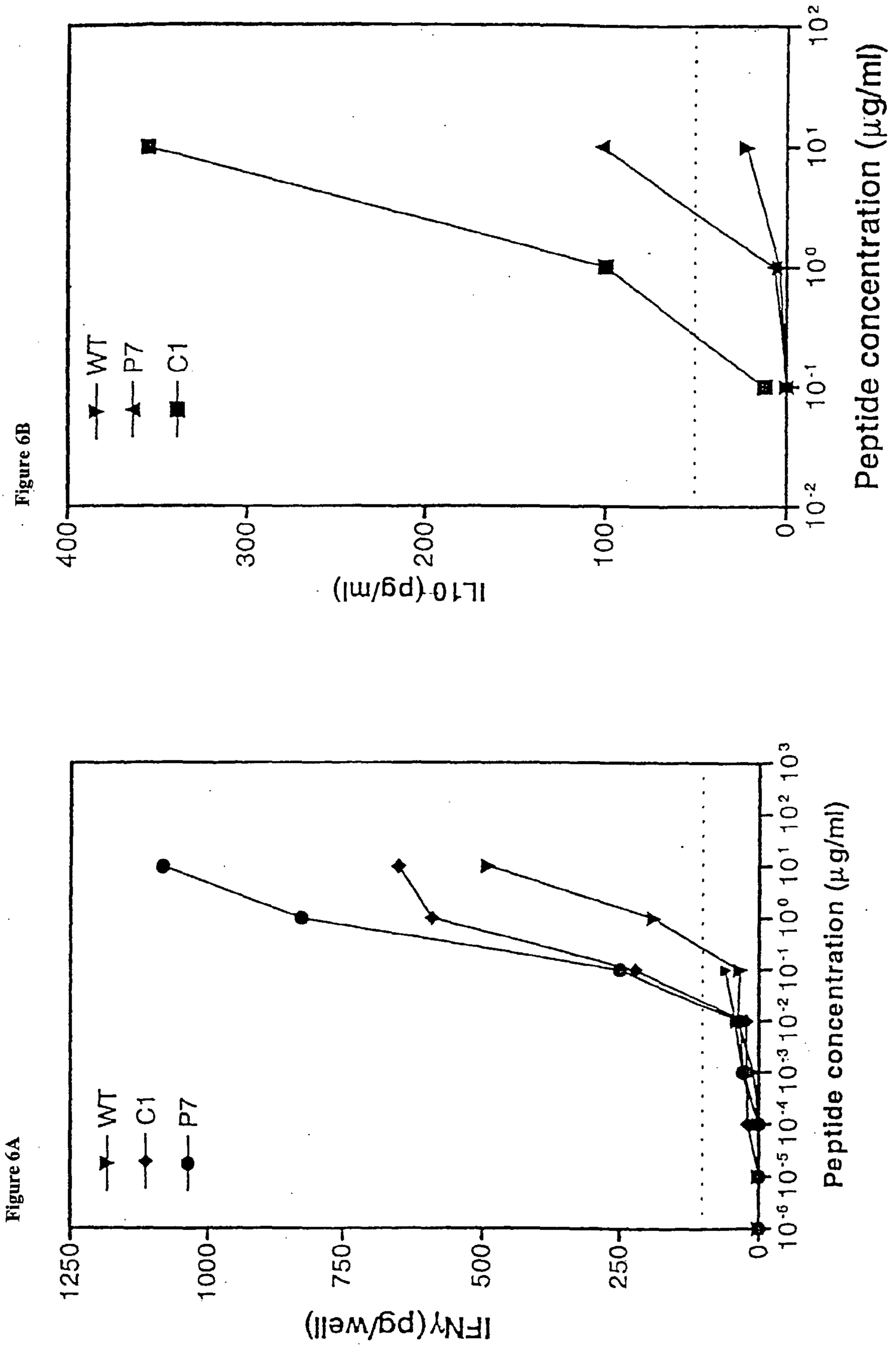


Figure 6A

Figure 6B

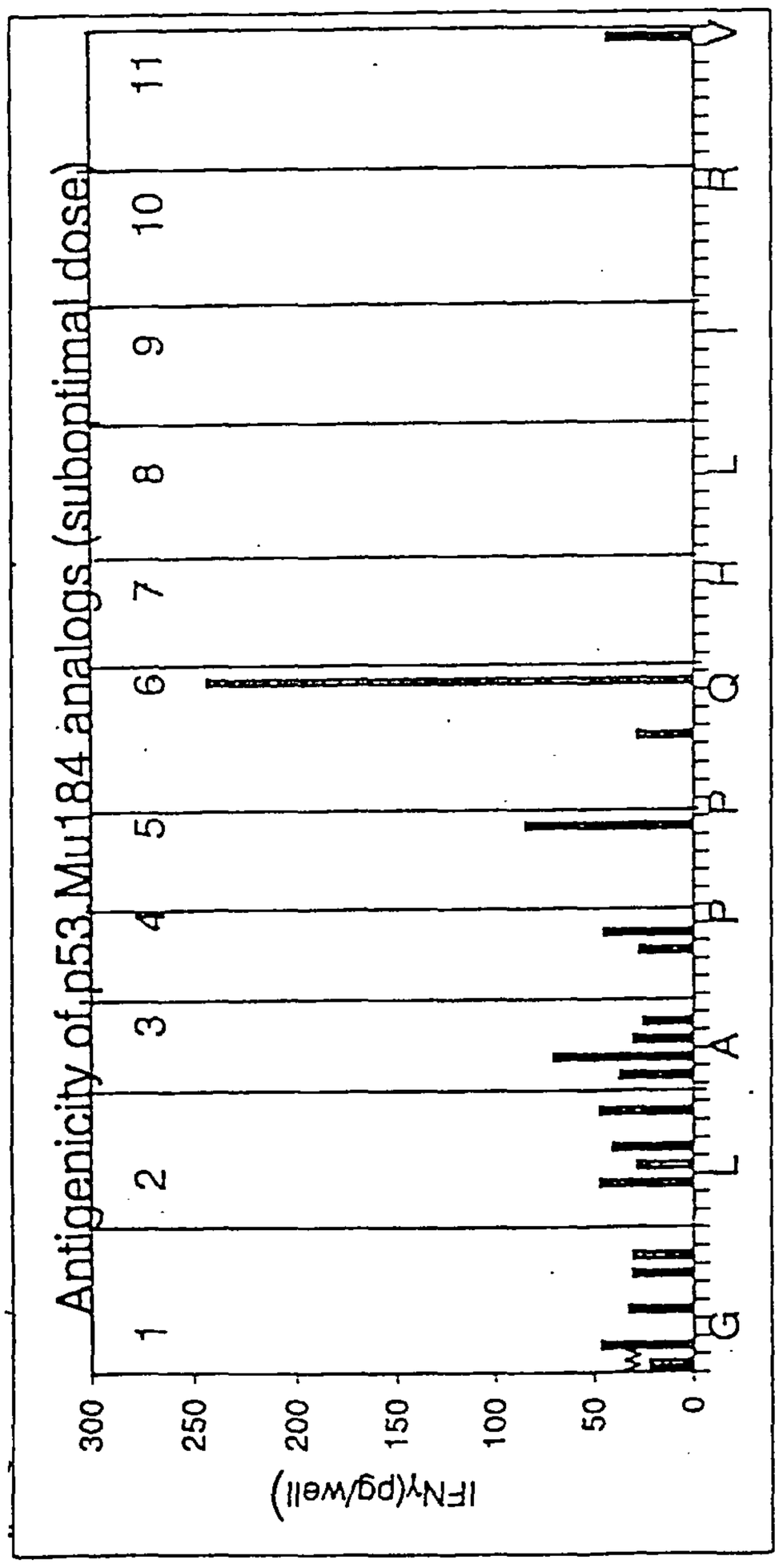

Antigenicity of p53 Mu184 analogs (suboptimal dose)
IFNγ(pg/well)
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
G
L
A
P
P
Q
H
L
R

Figure 7

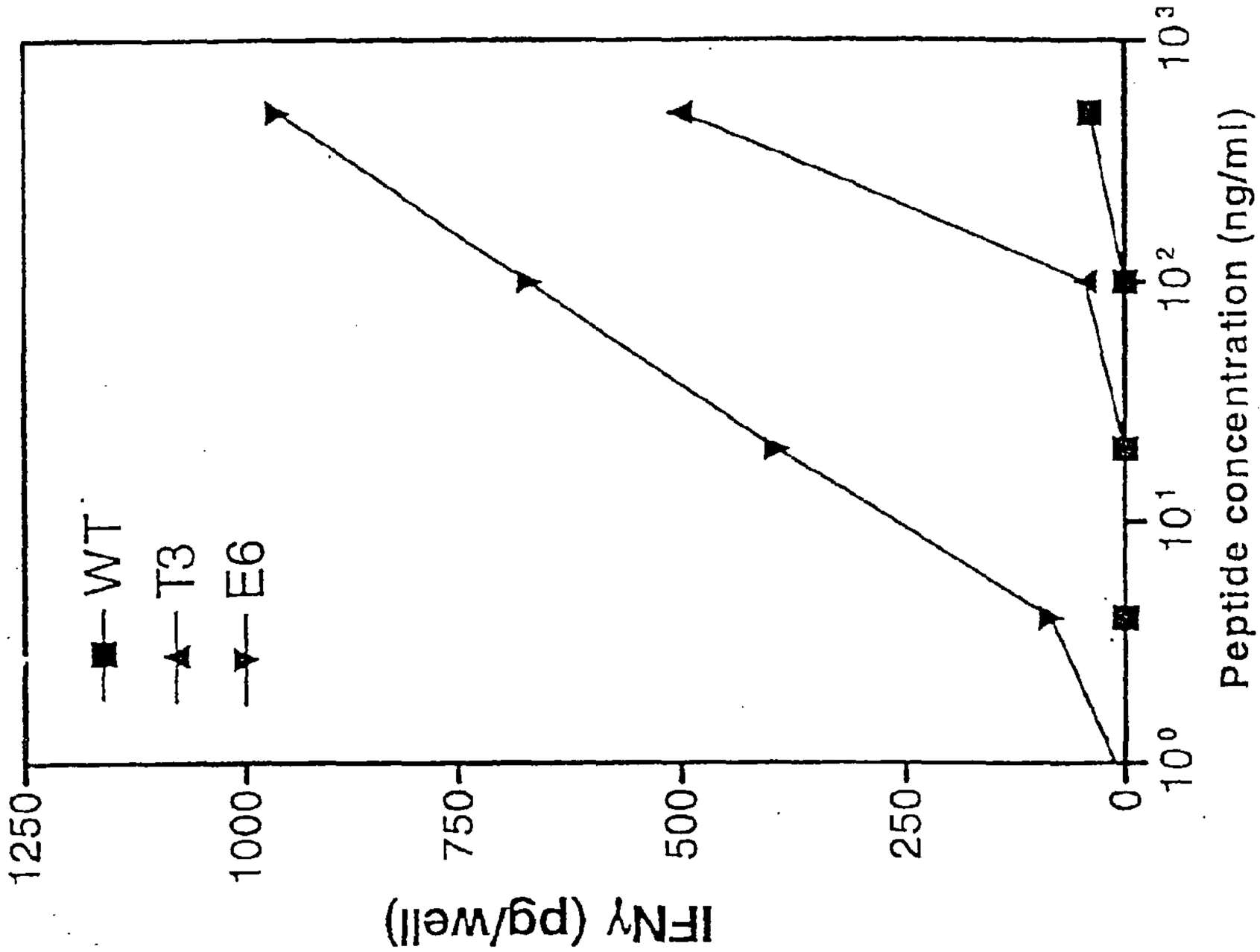
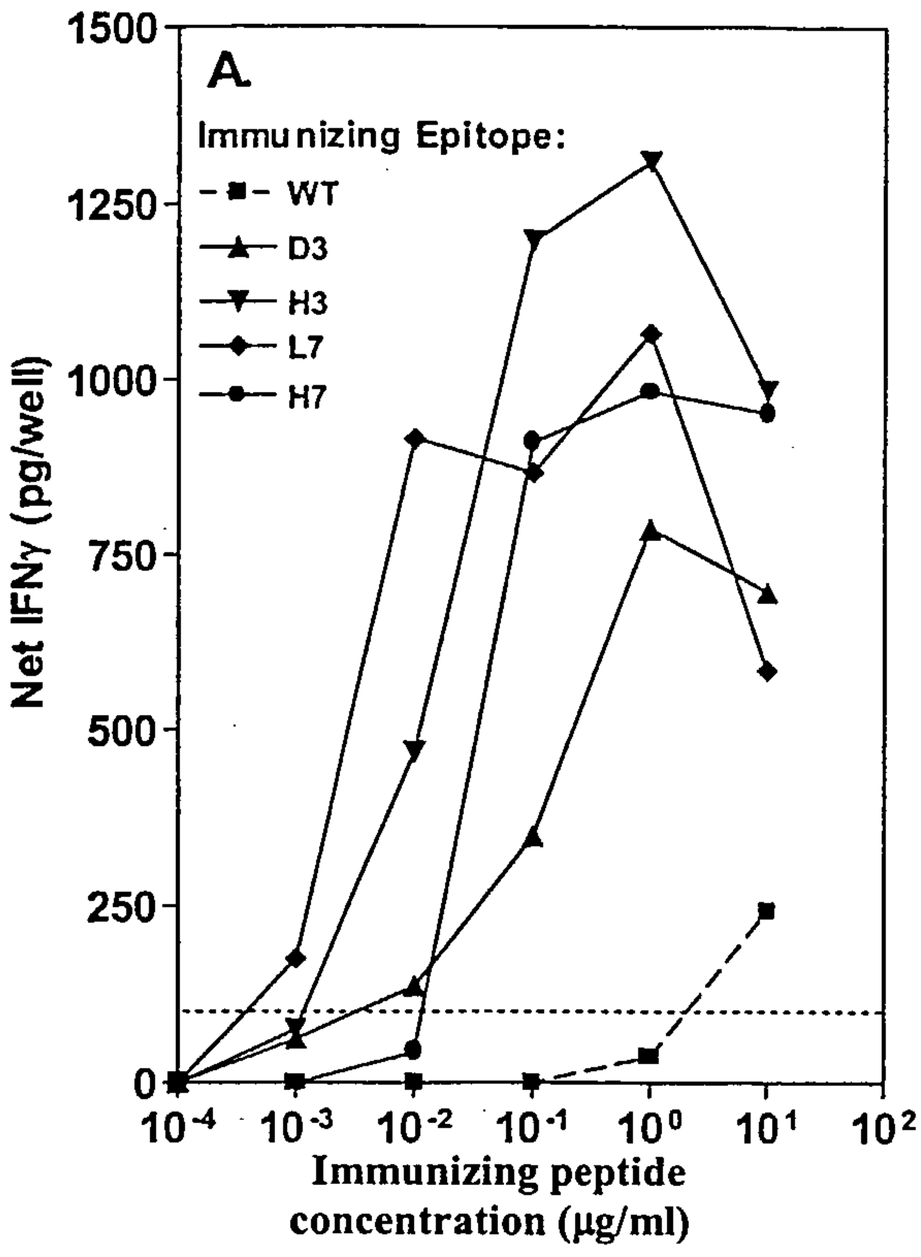
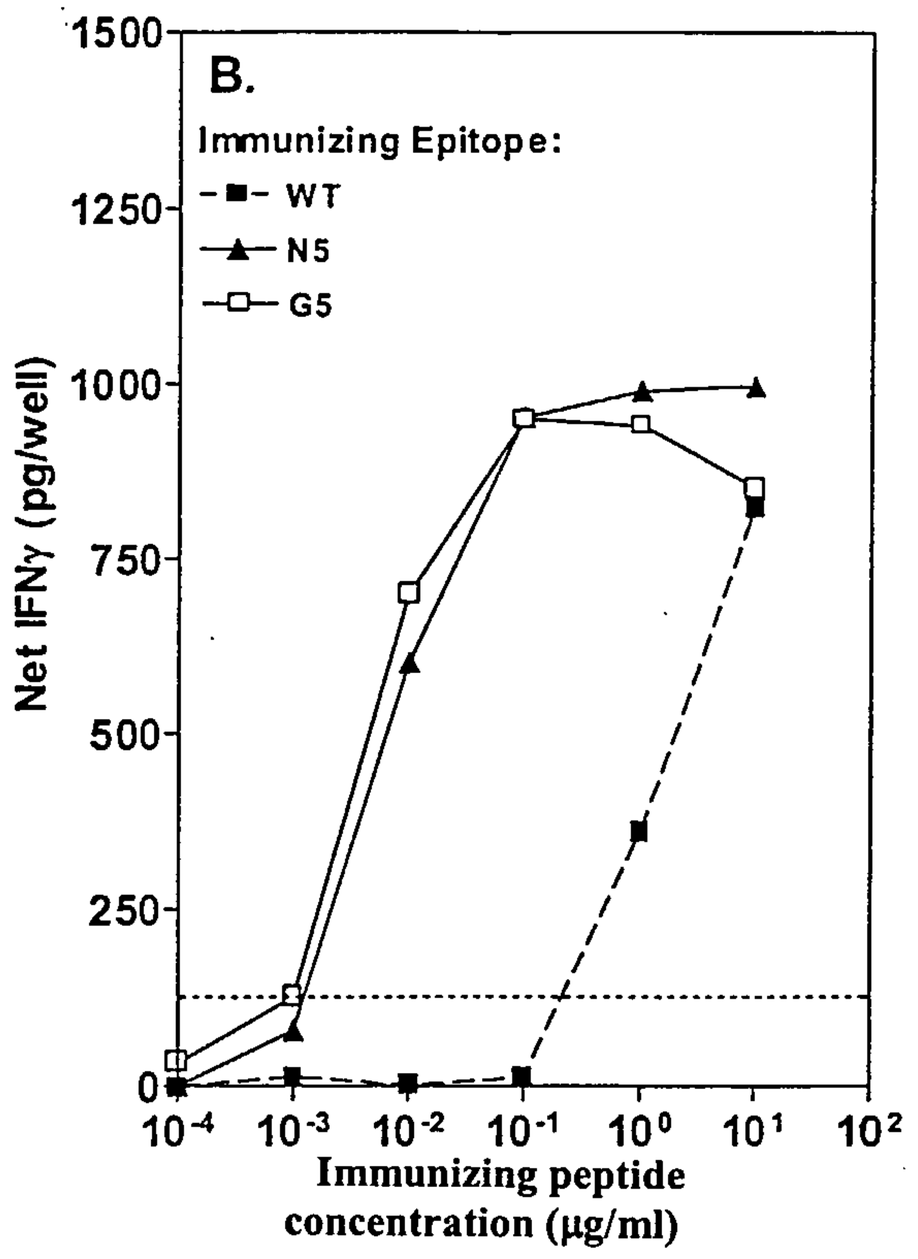
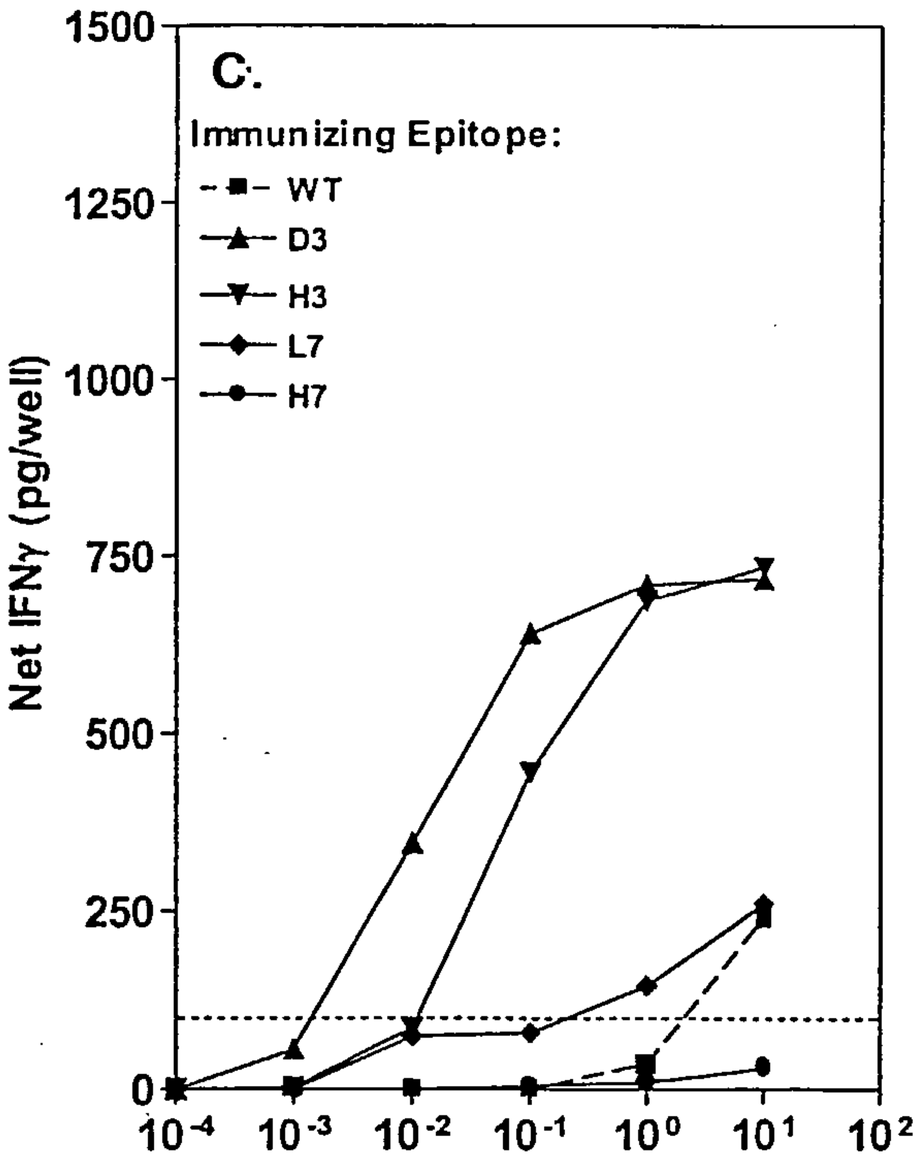

Dose titration of p53.Mu184 analogs
WT
T3
E6
IFNγ (pg/well)
1250
1000
750
500
250
0
10^0
10^1
10^2
10^3
Peptide concentration (ng/ml)

Figure 8

Figure 9


A.
Immunizing Epitope:
WT
D3
H3
L7
H7
Net IFNγ (pg/well)
1500
1250
1000
750
500
250
0
10-4 10-3 10-2 10-1 100 101 102
Immunizing peptide concentration (μg/ml)


B.
Immunizing Epitope:
WT
N5
G5
Net IFNγ (pg/well)
1500
1250
1000
750
500
250
0
10-4 10-3 10-2 10-1 100 101 102
Immunizing peptide concentration (μg/ml)


C.
Immunizing Epitope:
WT
D3
H3
L7
H7
Net IFNγ (pg/well)
1500
1250
1000
750
500
250
0
10-4 10-3 10-2 10-1 100 101 102
Wildtype peptide concentration (μg/ml)

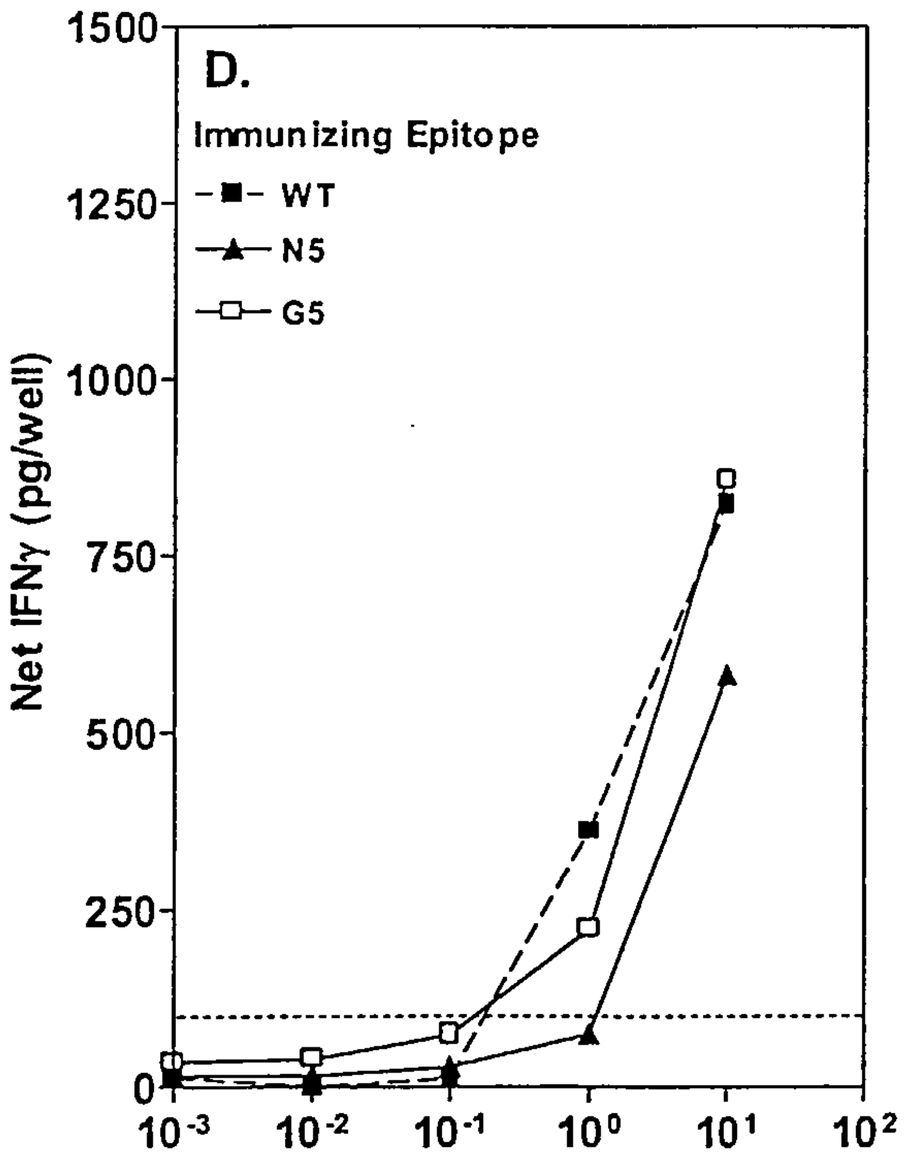

D.
Immunizing Epitope
WT
N5
G5
Net IFNγ (pg/well)
1500
1250
1000
750
500
250
0
10-3 10-2 10-1 100 101 102
Wildtype peptide concentration (μg/ml)

Figure 10

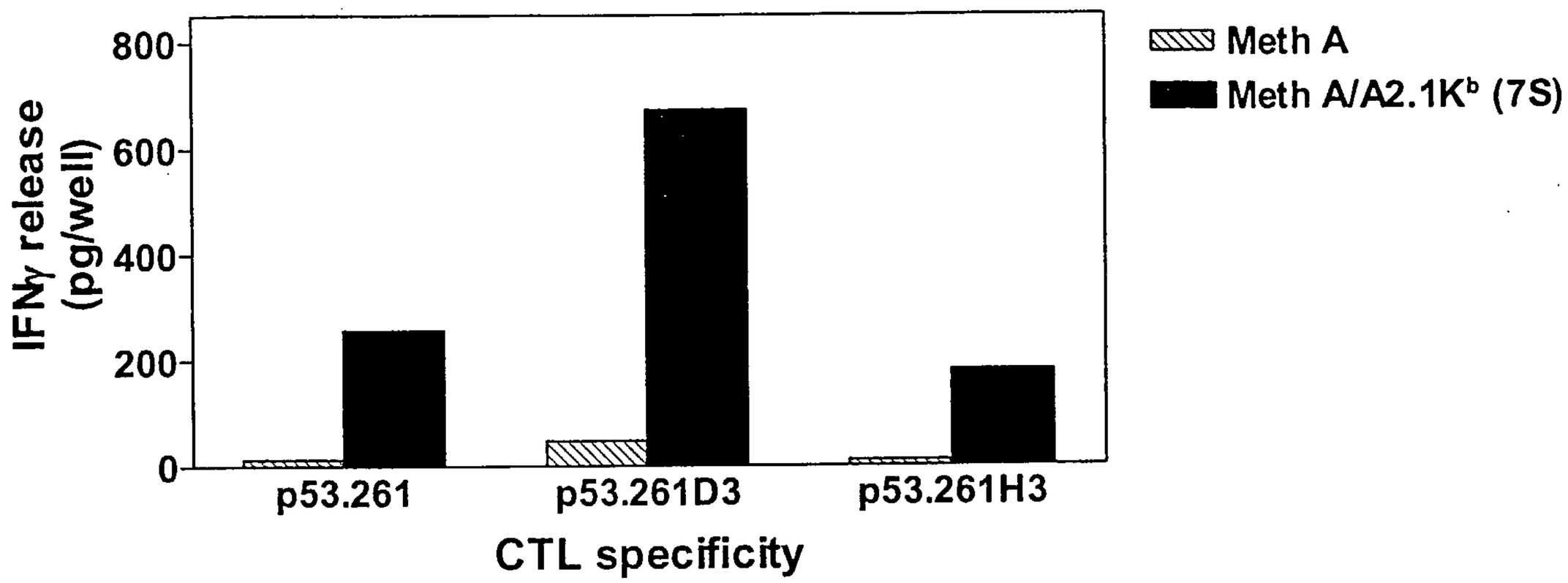

800
600
400
200
0
IFNγ release (pg/well)
p53.261
p53.261D3
p53.261H3
CTL specificity
Meth A
Meth A/A2.1K^b (7S)

Figure 11

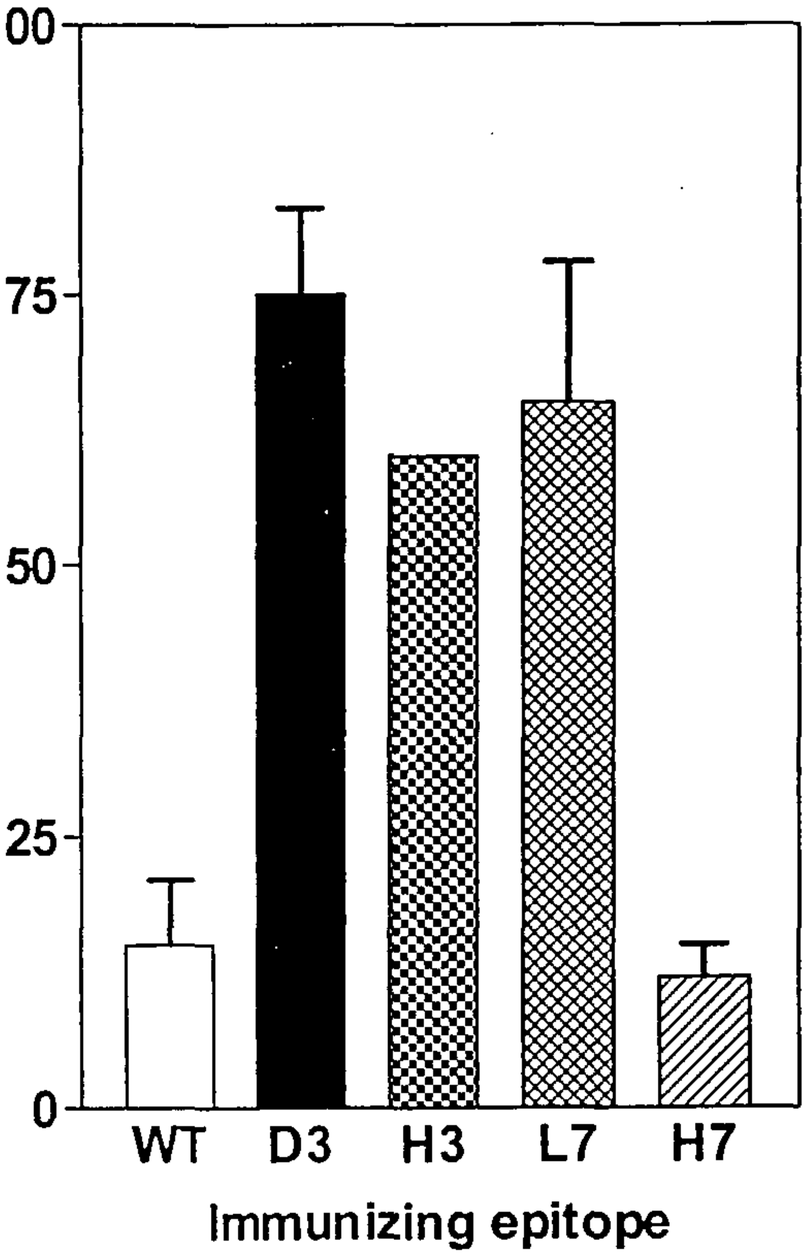
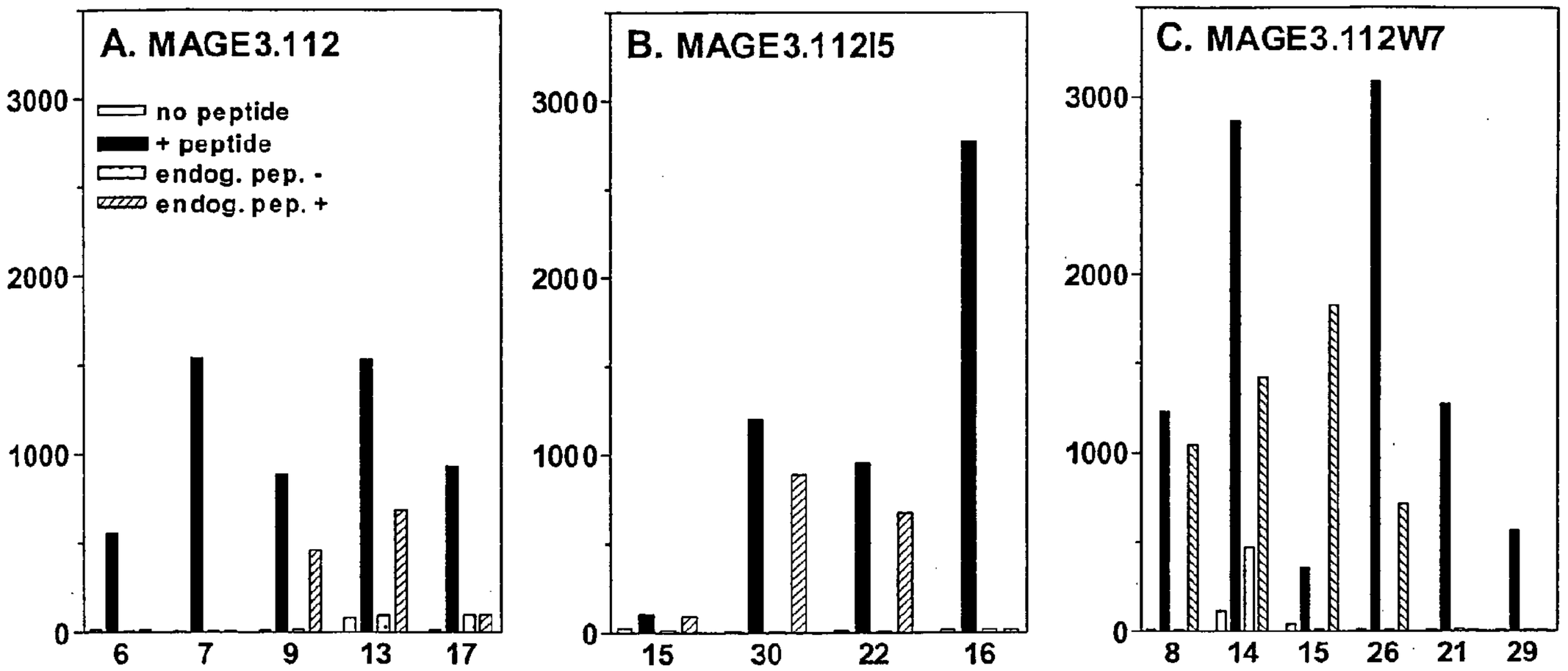

100
75
50
25
0
Net spots/10^6 CD8^+ cells
WT
D3
H3
L7
H7
Immunizing epitope

Figure 12


A. MAGE3.112
no peptide
+ peptide
endog. pep. -
endog. pep. +
3000
2000
1000
0
6
7
9
13
17
B. MAGE3.112I5
3000
2000
1000
0
15
30
22
16
C. MAGE3.112W7
3000
2000
1000
0
8
14
15
26
21
29
Well number