T-LYMPHOCYTE EPITOPE IDENTIFICATION AND THEIR USE IN VACCINE DEVELOPMENT FOR HIV-1

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1. ABSTRACT

Cellular immune responses mediated by CD8+ cytotoxic T-lymphocytes (CTL) and CD4+ helper T-lymphocytes (HTL) are needed to effectively control and clear many viral pathogens, including HIV-1. Thus, vaccines for HIV-1 capable of inducing CTL and HTL responses are now the focus of multiple academic and industry-based research and development programs. The use of defined, minimal CTL and HTL epitopes in vaccines has several potential advantages. Firstly, it is possible to use epitopes that are conserved thus targeting the majority of viral variants within a given clade or across clades. Secondly, epitopes from multiple viral structural or accessory gene products can be included in vaccines, which supports the induction cellular immune responses with significant breadth. Finally, dominance relationships between epitopes can be altered to increase immune recognition of subdominant epitopes. HTL and CTL epitopes from HIV-1 have recently been identified and characterized in numbers that are large enough to support their use in experimental vaccines. Initial studies with prototype DNA vaccines encoding epitopes indicate the need to include intracellular targeting sequences, to direct the encoded gene products to different cellular compartments, and amino acid spacer sequences between epitopes to optimize the processing, and subsequent presentation, of individual epitopes. Vaccines composed of CTL or HTL epitopes are now being developed for clinical testing.

2. INTRODUCTION

2.1. Correlation between T-lymphocyte responses and HIV-1 infection status or disease prognosis

Infection with HIV-1 results in a disease state characterized by progressive immune dysfunction, ultimately resulting in acquired immunodeficiency syndrome (AIDS). Although disease progression occurs in the presence of HIV-1 specific immune responses, there is increasing evidence that cellular immune responses, immune responses mediated by CD4+ helper T-lymphocytes (HTL) and CD8+ cytotoxic T-lymphocytes (CTL) can control HIV-1 replication. The role of HIV-1-specific CTL and HTL in controlling HIV-1 replication, and subsequent progression to AIDS, was demonstrated in different patient populations (see subsequent sections). The data from these different population groups support the theory that broadly reactive cellular immune responses, consisting of both HTL and CTL, are needed to control

infection and protect against disease progression. In a small percentage of individuals, T-lymphocyte responses may actually clear infection. Thus, the current focus on vaccine formats and immunization strategies designed to induce cellular immune responses is clearly warranted (1).

2.1.1. T-lymphocyte responses in acutely infected individuals

Studies of acute HIV-1 infection documented an early expansion of CD8+ T-lymphocytes, which included readily measurable CTL activity specific for HIV-1 structural and regulatory gene products. Several weeks after infection, an increase in the numbers of circulating HIV-1-specific CTL is readily detected and this rise is temporally associated with the initial *in vivo* decline of viremia (2,3).

2.1.2. T-lymphocyte responses in chronically-infected individuals

Data also support the hypothesis that CTL are involved in controlling HIV-1 replication and delaying disease progression in chronically-infected individuals (4-7). Infected individuals that do not progress to AIDS over extended periods of time are referred to as long-term non-progressors (LTNP). These individuals are characterized by the presence of low viral loads and potent, broadly reactive HIV-1 specific CTL responses (8-14). The breadth of CTL responses may be critical to control of the virus in these persons since CTL responses focused on limited numbers of viral gene products are more often associated with viral persistence and rapid disease progression. HTL also appear to play a critical role in establishing effective immunity to HIV-1 (15, 16). The presence of strong HIV-1 Gag p24-specific HTL is often inversely correlated with viral load in chronically infected individuals and HTL proliferative responses to HIV-1 p24 were observed more frequently in LTNP (17,18). Similarly, higher numbers of CD4+ T-lymphocytes capable of producing cytokines in response to HIV-1 antigens, cytokines characteristic of the Type-1 immune responses such as interleukin-2 (IL-2) and gamma-interferon (gamma-IFN) were identified in HIV-1 infected patients with non-progressive disease (19).

2.1.3. T-lymphocyte responses in exposed but uninfected individuals

A limited number of HIV-1 exposed, but uninfected, individuals have been identified. These individuals have documented exposure to HIV-1 but remain uninfected, based on assays designed to detect antibodies to HIV-1 proteins or by direct measurement of HIV-1 viral DNA and RNA (20-27). Exposure to HIV-1 is documented by the presence of HIV-1 specific HTL and CTL responses. This finding supports the theory that coordinated T-lymphocyte responses to HIV-1 can, in rare instances, protect against the establishment of HIV-1 infection.

2.1.4. Structured treatment interruptions (STI) as a method of increasing HIV-1 specific T-lymphocyte responses

The immune system can be protected from HIV-1 pathogenesis if Highly Active Anti-Retroviral Therapy (HAART) is started during the acute infection stage (28-30). Initiation of HAART prior to noticeable loss of immune capabilities, particularly the loss of CD4+ HTL, supports the development and maintenance of both CTL and HTL capabilities and responses to HIV-1 antigens are typically more robust. STI can be defined as a cyclic and controlled cessation of HAART in infected patients. The earliest studies were initiated by the infected patients themselves, and thus were not actually structured or controlled. As antiviral drugs levels dropped *in vivo*, T-lymphocyte responses increased and this observation demonstrated the potential of the immune system to respond despite the presence of HIV-1 (31, 32). In a controlled clinical experiment, eight patients who initiated HAART while acutely infected were removed from drugs and the rebound of their virus and HIV-1 specific immune responses were characterized. While rebound of viral replication was observed after HAART was stopped, both HTL and CTL responses increased dramatically. Following one or more cycles of STI, the immune responses in 5 of the 8 patients appeared to be of sufficient magnitude to control the virus in the absence of HAART (33). In similar studies completed in chronically infected persons, cellular immune responses specific for HIV-1 were also increased by STI although benefit to the patient was not clearly demonstrated (34, 35). The data from these studies support the hypothesis that STI can be used to augment T-lymphocyte responses and that these responses can contribute to the control of viremia in some individuals.

3. T-LYMPHOCYTE EPITOPES

A clear understanding of how T-lymphocytes recognize antigen has emerged over the past decade. It is now well established that small fragments of protein antigens are generated, defined as peptide epitopes, which bind to Major Histocompatability Complex (MHC) molecules expressed on the cell surface. These epitope MHC complexes represent the ligands recognized by T-lymphocytes through the function of T-cell receptors (TCR) (36, 37). The CD8+ CTL recognize epitopes bound to MHC Class I molecules whereas the CD4+ HTL recognize epitope peptides bound to MHC Class II antigens. In humans the MHC Class I cell-surface expressed molecules are referred to as HLA-A, -B or –C and the MHC Class II as HLA-DR, -DP and -DQ.

3.1. CTL epitope identification

Sequencing of naturally processed peptides bound to MHC Class I molecules provided a means to identify the amino acid residues required for allele-specific binding of putative CTL epitopes (38-40). Additional data obtained using X-ray crystallographic analysis of MHC-peptide complexes, allowed for the structural characterization of 'binding pockets' within the peptide binding cleft of MHC molecules. The definition of epitope anchor motifs is the key of most, if not all, epitope prediction methods. More refined epitope anchor motif definitions were then developed using data obtained from *in vitro* peptide-MHC binding assays. It is now well known that the main anchor residues of peptides that bind to HLA molecules typically occur at position two and the

Table 1. HLA superfamily motifs and alleles

Supertype	Motif[*]	Prototype Allele	Additional Supertype Alleles
A2	X (AIVLMT) X_{6-7} (AIVLMT)	HLA-A*0201	HLA-A*0202, A*0203, A*0204, A*0205, A*0206, A*0207, A*6802, A*6901
A3	X (AVILMST) X_{6-7} (RK)	HLA-A*0301, A*1101	HLA-A*3101, A*3301, A*6801
B7	X (P) X_{6-7}(ALIMVFWY)	HLA-B*0702	HLA-B*3501-03, B*51, B*5301,B*5401, B*0703-05, B*1508, B*5501-02, B*5601-02, B*6701, B*7801

Amino acid sequence motifs of peptides that bind to HLA supertypes A2, A3 and B7. Individual amino acids defining the motifs used for epitope identification are illustrated with an X; X_{6-7} is shown to indicate that the motif anchor positions, shown in the parentheses ( ) are separated by 6-7 amino acids. Any single residue contained within the parentheses ( ) is acceptable at the motif anchor position. The prototype allele is the allele to which a peptide must bind to be considered a supertype peptide. For each HLA supertype, the additional alleles that are members of that supertype family are listed. The single letter amino acid code is used (A = alanine, R = arginine, I = isoleucine, L = leucine, K = lysine, M = methionine, F = phenylalanine, P = proline, S = serine, T = threonine, W = tryptophan, Y = tyrosine and V = valine).

carboxyl terminus of peptides 8-11 amino acids in length (41-48).

Amino acids at other positions can contribute to peptide-MHC binding affinity; these sites are commonly referred to as secondary anchors (49). The analysis of data on both primary and secondary anchors led to the definition of statistically based algorithms, generally referred to as polynomial algorithms, for estimating the likelihood that peptides can bind to HLA Class I molecules. These algorithms are based on the premise that the overall affinity of peptide-HLA interactions can be approximated as a linear polynomial function. They are, therefore, designed to account for the impact of amino acids at the primary anchor positions as well as secondary anchors. Appropriate binding thresholds can be chosen as a function of the degree of stringency of prediction desired and then, potential epitopes identified (50,51). This approach is commonly referred to as a matrix-based method.

In addition to the Epimmune matrix based method, other computer-based approaches for predicting epitopes have been developed. These include variations of the motif scanning and matrix approaches (52-58), neural networks (50) and threading algorithms (59, 60). The Epimmune method and software is proprietary and as such, access is limited to groups collaborating with Epimmune scientists. However, access to other epitope prediction methods is provided through the Los Alamos Molecular Immunology Database and other 'not for profit' services (56,57).

Initially, CTL epitope identification methods were developed using common HLA alleles, such as HLA-A2.1. Motifs defined using different HLA molecules were found to be similar and this lead to the definition of HLA supertype families (61). The biological effect of this supertype relationship was first demonstrated for HIV-1 epitopes in a study where the HLA-A3 and -A11 repertoires were demonstrated to be overlapping, not only with each other but also with HLA-A31, -A33 and -A6801 (62, 63). This binding specificity was defined as the HLA-A3 supertype. A significant overlap in peptide binding repertoires was also demonstrated amongst several serologically distant HLA-B alleles (64,65), and multiple HLA-A2 alleles (66, 67), resulting in the definition of the HLA-B7 and HLA-A2 supertype families (Table 1).

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. Recognition of epitopes by CTL in supertype manner has been demonstrated to occur naturally in infectious diseases and cancer (62,68-73). Each of the known HLA Class I supertypes include a relatively common set of HLA alleles and due to this, the use of these supertypes relationships when identifying epitopes allows for the selection of those most likely to be targets for the immune system in a genetically diverse population. The demonstration of supertype binding for epitope peptides was a critical observation for the identification and subsequent use of CTL epitopes in vaccines. As would be expected, the predicted population coverage of a vaccine based on supertype-restricted epitopes is significantly greater than one based on allele-specific epitopes.

3.2. HTL epitope identification

A similar approach is utilized to identify potential HTL epitopes, focusing on the identification of peptides that bind to MHC Class II molecules. There exists a significantly higher level of variation in the motif definition and peptide length, since peptide binding to MHC Class II molecules is generally more promiscuous. However, at Epimmune we have identified highly predictive peptide binding motifs for the major supertypes, HLA-DR3 and HLA-DR1,4,7, which include most of the common HLA-DR types (74). These motifs have been used to identify HLA-DR-restricted epitopes from several viruses.

3.3. Direct measurement of epitope peptide binding to HLA-A, HLA-B and HLA-DR molecules

Epitope predictions based on primary amino acid sequence data are useful but certain limitations exist. The major limitation is that a significant number of motif-positive peptides identified using predictive algorithms will fail to bind with high affinity to MHC molecules. Thus, it is logical to increase the accuracy of the identification process using epitope prediction in conjunction with laboratory assays to directly measure the affinity of the binding between peptides and MHC molecules (38-40). The inclusion of peptide-MHC binding data to the epitope selection process is critical for the identification HLA supertype-binding epitopes for both MHC Class I and Class II epitopes and those most likely to be immunogenic.

Peptide binding assays are based on the use of purified HLA-A, -B or -DR molecules and radio-labeled peptides with known binding affinity. At Epimmune, we have assembled a library of >100 different HLA specificities, in the purified protein form. To determine the binding affinity of an unknown peptide, increasing concentrations are allowed to compete with the known peptide for binding to the HLA molecule. The assay endpoint is based on the ratio of bound and unbound radio-labeled peptide and actual binding affinities can be calculated. We routinely use binding affinity threshold values, based on IC_{50} values, of < 500 nM for CTL epitopes and <1 micro-M for HTL epitopes, for initial selection of epitopes, since peptides binding with these affinity levels are most likely to be immunogenic (75).

4. IDENTIFICATION AND CHARACTERIZATION OF HIV-1 CTL AND HTL EPITOPES

The use of vaccines to induce protective or therapeutic cellular immune responses to viruses, such as HIV-1, is complicated by the presence of immunologically variable regions within the virus. To be truly effective, the vaccine must induce responses that recognize immunologically conserved regions, as this will allow for the vaccine to be used against many viral variants. The use of highly conserved epitopes allows for the induction of immune responses to conserved viral sequences. There are several other potential advantages associated with the use of defined epitopes in vaccines:

(1) There exists significant potential for increasing the margin of safety since the immunization strategy mimics the natural antigen processing and presentation that would be associated with infection by HIV-1, without the use of any intact viral gene products that may alter immune function.

(2) The large degree of flexibility, with respect to epitope selection, allows for both structural and regulatory gene products to be targeted by the vaccine.

(3) The potential for increased vaccine immuno-genicity through the delivery of pure epitopes in high concentration should increase the magnitude of immune responses.

(4) The potential of selecting subdominant epitopes, which would normally be minimally recognized by the immune system, provides an opportunity to increase the functional breadth of the responses.

In addition to the use of data generated from the computer-based epitope predictions and peptide binding measurements, there are other criteria that can be applied to refine the epitope identification and selection process. For our current generation of experimental HIV-1 vaccines, we focused on:

(1) Conservation of epitope sequences between multiple HIV-1 clade B isolates and other clades.

(2) Restriction of epitopes to HLA alleles represented by the most common HLA-supertypes, rather than individual HLA alleles, focusing on the HLA-A2, -A3/11 and -B7 supertypes.

(3) Selection of epitopes from multiple structural and/or nonstructural viral gene products.

(4) Epitope immunogenicity, defined using appropriate types of T-lymphocytes from HIV-1 infected individuals.

Our program for HIV-1 epitope identification is structured to include these properties and as such, the identified epitopes are believed to be well suited for use in vaccines designed for use in the general population.

To identify putative epitopes, 64 intact HIV-1 amino acid sequences from the Los Alamos database were searched for the presence of HLA-A2, -A3 and -B7 Class I binding motifs and HLA-DR 1,4,7 supertype and HLA-DR3 Class II epitope binding motifs. Motif positive sequences were selected for further study with a preference for those conserved amongst clade B viral isolates and commonly found in non-B clades. We defined epitope conservation based on the presence of the entire epitope sequence, which is a very stringent criterion given the known promiscuity of both anchor residues and TCR contact residues. This conservation requirement was a very effective way to reduce the number of epitopes to be evaluated further. For example, approximately 20,000 HLA-A2 supermotif sequences were identified but fewer than 300 were highly conserved. This limited number of potential epitopes was synthesized as peptides and evaluated in HLA binding assays.

A subset of high affinity binding peptides was tested *in vitro* using lymphocytes from HLA-defined, HIV-1 infected persons to document recognition by CD8+ CTL. *In vitro* induction of primary CTL responses was also used to document the presence of the appropriate TCR repertoire in normal individuals (76,77). Assays to measure proliferation and cytokine production by CD4+ HTL were used to similarly document immune system recognition of HTL epitopes (78). Recognition of the epitope peptides by CTL or HTL from HIV-1 infected persons also demonstrates that the epitopes identified using our search process are in fact, generated *in vivo* during the course of infection. This program allowed us to identify a candidate set of epitopes for potential use in HIV-1 vaccine clinical development.

It should be noted that the selected set of epitopes is not representative of those most likely to be immunogenic as the result of HIV-1 infection. The main differences are that many epitopes recognized following HIV-1 infection are immunodominant and derived from variable regions of the viral proteins. These epitopes are also typically restricted in a HLA allele-specific manner, not a supertype manner. Immune responses to dominant CTL epitopes are not necessarily correlated with better prognosis (79) so their inclusion in vaccines is not always warranted. Obviously, epitopes from variable regions will not induce responses to a large percentage of the HIV-1
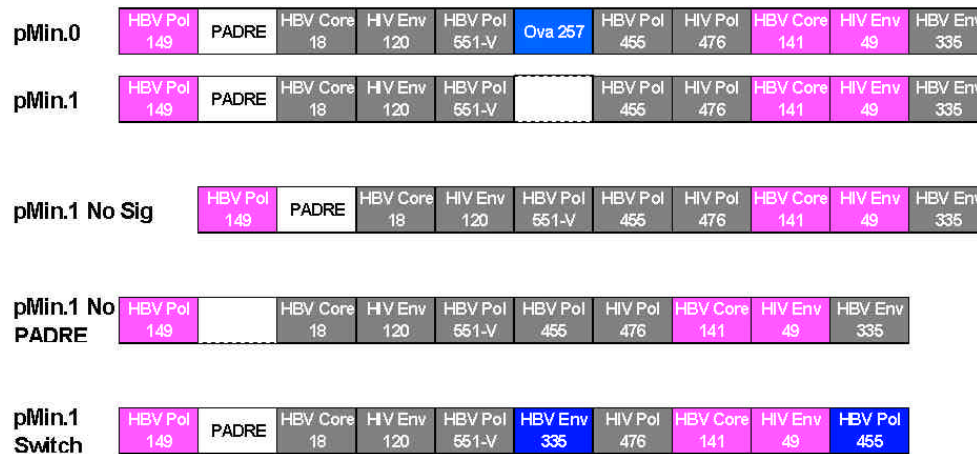
Figure 1. pMin series of experimental epitope-based DNA vaccines (termed EpiGenes). Experimental vaccine constructs encoding well known HLA-A2 and HLA-A3 restricted CTL epitopes from HBV and HIV-1.

variants circulating and epitopes with HLA allele-specific restriction will severely limit population coverage of vaccines composed of them.

5. DESIGN AND DEVELOPMENT OF VACCINES COMPOSED OF MULTIPLE EPITOPES

There are several vaccine delivery methods amenable for use with epitopes. Synthetic peptides representing CTL or HTL epitopes derived from HIV-1 have been tested in clinical trials delivered in ISA-51, a clinical grade Incomplete Freund's Adjuvant (80,81) or as lipidated peptides (82). The numbers of epitopes that can be included in a vaccine based on these formats is generally limited to only a few and vaccines based on peptides can be difficult to manufacture and maintain as stable formulations. We believe the incorporation of multiple CTL epitopes restricted to numerous HLA types into a vaccine is key to developing this approach for generalized use in human vaccines and therapeutics. Thus, we elected to investigate other vaccine delivery formats.

An alternative approach to multi-epitope vaccine design is to construct minigenes and to produce these as DNA plasmid-based vaccines or as large polypeptides or recombinant proteins. Multi-epitope vaccines have evolved in a relatively predictable step-wise manner. For example, a construct encoding two Lymphocytic Choriomeningitis virus CTL epitopes restricted by two different mouse MHC haplotypes was developed and, as predicted, immunization with this vaccine induced protective immune responses in the relevant strains of mice (83,84). In a similar study, CTL, HTL and antibody epitopes from Herpes Simplex virus were combined and used to induce protective immune responses (85). More recently, ten contiguous CTL epitopes were delivered using a recombinant Vaccinia virus vector and this vaccine was capable of inducing protective immune responses against Sendai virus, Cytomegalovirus and an experimental tumor (86,87). A mixture of CTL, HTL and antibody epitopes from Vesicular Stomatitis virus, Respiratory Syncytial virus, Mengovirus, Sendai virus Lymphocytic

Choriomeningitis virus and *Mycobacterium tuberculosis* have also been used to induce protective immune responses specific for all of the study pathogens in the appropriate strains of mice. Of interest was the observation that protection against the Lymphocytic Choriomeningitis virus appeared to be mediated by CTL whereas antibodies to the Mengovirus were protective. The induction of CTL specific for multiple epitopes in the context of several different murine MHC Class I molecules has also been achieved, which is the first step towards the use of this type of vaccine in random-bred species (86-88). These studies demonstrated the potential utility of the multi-epitope vaccine approach for the simultaneous induction of immune responses specific for different antigenic targets and restricted to different MHC allelic products (89,90).

To provide a realistic level of protection in humans, even more complex vaccines are likely to be needed. To address this issue, vaccines containing up to 20 HLA-restricted CTL epitopes from HIV-1 or *Plasmodium falciparum* were produced and delivered as DNA vaccines and using a viral vector. These vaccines contained 'marker' epitopes restricted to rhesus macaque and mouse MHC to allow the immunogenicity of the constructs to be evaluated in animal models (91,92). Studies focusing on the marker epitopes have demonstrated the immunogenicity of these vaccines and they are now being evaluated in human clinical studies. Limitations associated with this approach are that the immunogenicity of the majority of the HLA restricted CTL epitopes could not be demonstrated nor was the contribution of HTL epitopes tested.

To address these limitations, we produced experimental DNA vaccines containing multiple CTL epitopes and tested them for immunogenicity using mice transgenic for HLA-A2 and –A11 (93). The epitope component of vaccines were synthesized using a process based on primer extension with overlapping oligonucleotide PCR primers, averaging 70 nucleotides in length with 15 nucleotide overlaps. These vaccines encode HLA-A2 and HLA-A3/11 supertype restricted HIV-1 and HBV CTL epitopes characterized by moderate to high
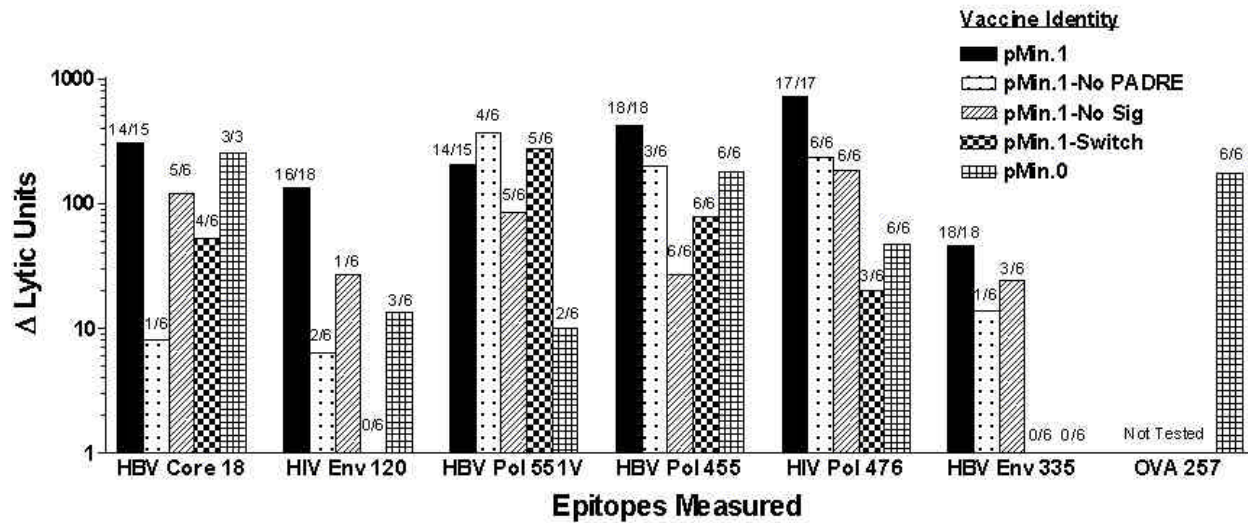
Figure 2. Immunogenicity of modified pMin.1 constructs that address variables critical for *in vivo* immunogenicity. Modified versions of the pMin.1 DNA construct were tested for CTL immunogenicity in HLA-A2 transgenic mice to determine the effect of deleting the ER signal sequence (the No Sig construct), deleting the HTL epitope (No PADRE), switching a weakly immunogenic epitope to a different position (Switch) and the inclusion of a dominant mouse epitope, Ova 257 (pMin.0). Shown is the CTL activity of splenocytes obtained from DNA vaccine immunized mice after a single *in vitro* stimulation with the different epitopes encoded in the DNA construct. The data are expressed as the geometric mean of lytic units per 10^6 effector cells. The number above each bar represents the frequency of CTL-positive cultures, 10^6 splenocytes/culture (numerator) and the total number of cultures tested (denominator).

binding affinity and known immunogenicity. The parent experimental vaccine is referred to as pMin (Figure 1) (93). Variations of this vaccine were also produced and tested; including a vaccine with a murine marker CTL epitope from ovalbumin (pMin.0), an endoplasmic reticulum (ER) targeting/signal sequence (pMin.1 –vs- pMin.1 No Sig), the universal HTL Pan HLA-DR Epitope (PADRE) (94) (pMin.1 –vs- pMin.1 No PADRE) and a modified epitope order (pMin.1 Switch).

The immunogenicity of these different experimental DNA vaccines was assessed using several assays to measure CTL responses, including a standard ^{51}Cr-release assay, an ELISpot to measure gamma-IFN production by freshly purified CD8+ T-lymphocytes and an *in situ* ELISA which includes an *in vitro* epitope-specific restimulation step.

The results of this study revealed several important properties of multi-epitope CTL vaccines delivered using the DNA plasmid format. Firstly, the inclusion of a mouse CTL epitope from ovalbumin as a marker was found to be detrimental (Figure 2). Responses to the HLA-A2.1 epitopes were reduced for 5 of 6 tested, 4 of these by a $\log_{10}$, (compare responses induced using pMin.1 to pMin.0). Thus, the use of a marker epitope to document the immunogenicity of a complex multi-epitope vaccine in HLA transgenic mice may not provide accurate data. Secondly, the ER signal sequence and PADRE generally increased the immunogenicity of epitopes in the vaccine (compare pMIn.1 to pMin.1-No Sig and pMin.1-No PADRE). Although the effect on some epitopes was

only two-fold, the immunogenicity of others was increased by factors of 3->10.

In a separate experiment, the effect of epitope order on their immunogenicity was completed, with surprising results. The position of only two HLA-A2 restricted epitopes was switched and the immunogenicity of one of the epitopes was lost. Also of interest was the observation that the immunogenicity of an epitope that was not moved was also lost. These data led us to the hypothesis that the types of amino acids flanking individual epitopes may affect proteosome processing and presentation efficiency.

6. OPTIMIZATION OF CTL EPITOPE VACCINES

The immunogenicity data obtained using the pMin family of prototype vaccines clearly demonstrate the types of variation that can be associated with this type of product. We have identified two sources of variation that can significantly alter epitope immunogenicity:

(1) The presence of dominant epitopes that may be introduced as marker epitopes to that may be introduced in error, the result of the junction between two well-defined epitopes, referred to as 'junctional epitopes.'
(2) Efficiency of epitope processing and subsequent presentation.

6.1. Avoiding the creation of junctional epitopes

Of major concern is the presence of epitopes that may dominate the immune response. While this effect was demonstrated through the incorporation of a dominant
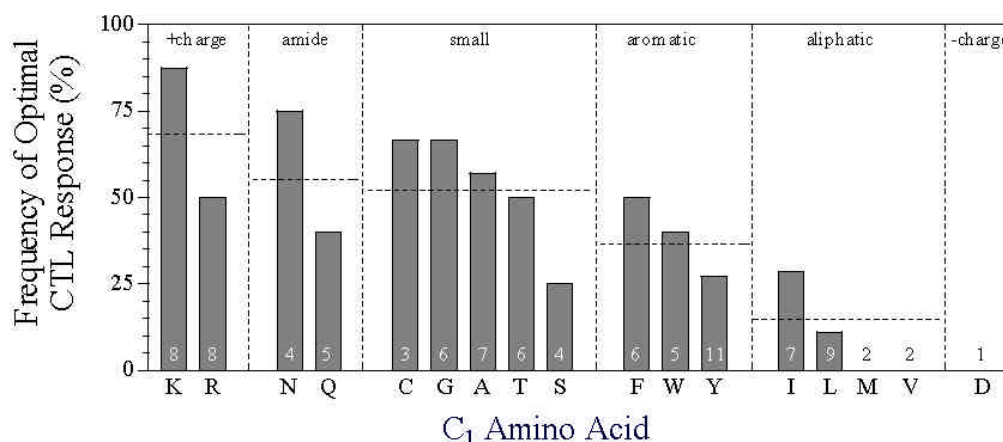
Figure 3. Influence of the C1 flanking amino acid on epitope immunogenicity. A database incorporating CTL responses from a variety of minigenes representing 94 epitope/C_1 amino acid combinations was analyzed to determine the frequency (%) of instances in which a particular combination was associated with an optimal CTL response. CTL responses were considered optimal if they were statistically comparable to responses induced using peptide forms of the epitope emulsified in IFA. The number of times a given epitope/C1 amino acid combination was observed is also provided as the numerical values superimposed on the data bars.

murine epitope (OVA), there is the possibility of inadvertently creating neo-epitopes that consist of components of two adjacent epitopes, referred to here as junctional epitopes. The presence of junctional epitopes may have no effect, or alternatively, they could significantly alter vaccine immunogenicity should they dominate immune responses or induce responses against a self-protein.

The existence and effects of junctional epitopes has been documented in vaccine settings for HTL epitopes (95,96). The effect of a junctional epitope was observed in a vaccine where two different HLA Class II restricted epitopes were juxtaposed in a synthetic peptide. This junctional epitope was very immunogenic in clinical trials; the effect was so marked that responses to the authentic vaccine epitopes were 'silenced.' Responses against junctional HTL epitopes were also observed in humans following immunization with a synthetic lipopeptide, which was composed of an HLA-A2-restricted HBV-derived immunodominant CTL epitope and a universal Tetanus Toxoid-derived HTL epitope (97). In this second study, effects on immunogenicity were not obvious. The creation of junctional epitopes remains a potential concern and we, therefore, design vaccines using an epitope order to limit the numbers of junctional epitopes that are formed.

6.2. Use of amino acid spacers to optimize epitope processing

At least three factors are likely to play a role in variation associated with the induction of CTL responses induced using epitope-based vaccines:

(1) The efficiency with which an epitope is generated through intracellular processing and then presented bound to MHC Class I antigens.
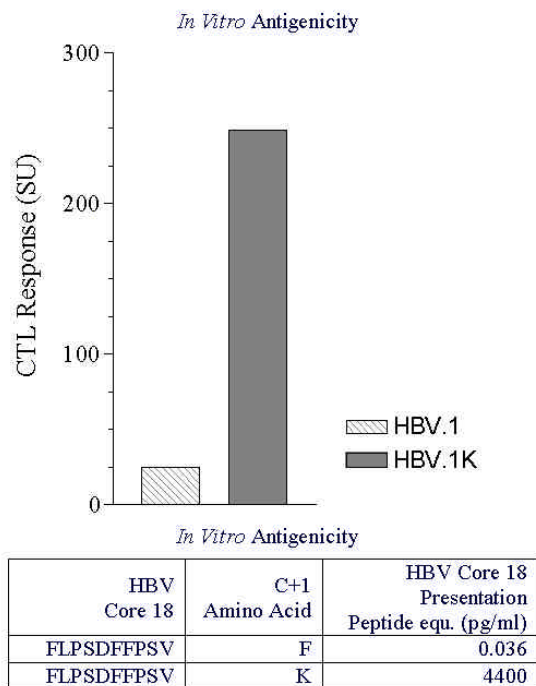
(2) The binding affinity of the epitope to MHC Class I molecules.

(3) The existence of a suitable TCR repertoire in the potential vaccinee.

It is possible to design vaccines using epitopes with high MHC binding affinity and defined immunogenicity, thus, the efficiency of epitope processing may be the dominant variable.

The influence that amino acids flanking CTL epitopes have on the efficiency of processing and presentation is the subject of debate (98,99). Evidence supporting the effects of flanking amino acids was provided in experiments where CTL immunogenicity was significantly influenced by the addition of single C-terminal flanking amino acids (98, 100). These data support the hypothesis that proteosome processing can be affected. However, profound effects associated with amino acids flanking the N-terminus of mouse influenza virus epitopes have also been observed, suggesting that the efficiency of epitope trimming in the ER may be involved (101). The variation of epitope immunogenicity we observed, when different epitope orders were compared, supports the concept of flanking amino acid effects and points, in particular, to a C-terminus effect.

To address this question, we analyzed immunogenicity data obtained from HLA-A2, -A11 and –B7 transgenic mice immunized with a number unrelated experimental multi-epitope DNA vaccine constructs. A total of 94 different epitope/flanking residue combinations were analyzed. We did not identify a significant association between an individual amino acid or chemical class of amino acids flanking the N-termini of epitopes and immunogenicity. However, significant effects of the C-terminus flanking amino acids, the C1 residue, were identified. Positively charged amino acids, such as K or R , were most frequently associated with optimal CTL responses (Figure 3) and

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In Vitro Antigenicity

HBV Core 18	C+1 Amino Acid	HBV Core 18 Presentation Peptide equ. (pg/ml)
FLPSDFFPSV	F	0.036
FLPSDFFPSV	K	4400

Figure 4. Increased epitope immunogenicity is correlated to the level of epitope presentation. CTL responses to the HBV Core 18 epitope encoded in HBV.1 and HBV.1K DNA vaccines were measured using HLA-A2 transgenic mice and the *in situ* ELISA to measure gamma-INF production by purified CD8+ T-cells after restimulation *in vitro* with the HBV Core 18 peptide (left). The amount of the HBV Core 18 epitope presented on the surface of target cells was determined using HLA-A2 expressing cells transfected with the HIBV.1 or HBV.1K minigenes and CTL lines derived from HLA-A2 transgenic mice immunized with HBV Core 18 peptide emulsified in IFA. Peptide-pulsed target cells were used to establish a standard curve and data are presented as peptide equivalents. The magnitude of CTL responses was directly correlated with the amount of epitope presented.

amino acids, such as N and Q, or small amino acids, such as C, G, A, T, and S were also associated with moderate epitope immunogenicity. Conversely, epitopes flanked by aromatic and aliphatic amino acids induced optimal responses at much lower rates. The influence of C1 amino acid on epitope immunogenicity was found to be statistically significant but no significant influence on epitope immunogenicity was noted when similar analyses were performed for amino acids more distal to the epitope.

To directly evaluate the effect of different amino acids in the C1 flanking position, we produced two multi-epitope constructs, referred to as HBV.1 and HBV.1K. These HBV experimental constructs were based on the pMin.1 and encode the epitopes without intervening spacers. For HBV.1, the HIV-1 epitope directly following the highly immunogenic HBV Core 18 epitope was replaced with the HBV Pol 562 epitope. This altered the C1 amino acid from a K to an F. The second construct, HBV.1K, was produced

by the insertion of an additional epitope, HBV Pol 629, between the HBV Core 18 and HBV Pol 562 epitopes; a change that replaced the C1 amino acid with a K residue. When the immunogenicity of the HBV Core 18 epitope presented in these different contexts was evaluated in HLA-A2 transgenic mice, we found that this epitope was virtually non-immunogenic in HBV.1 but strongly immunogenic in HBV.1K (Figure 4).

If the variation of immunogenicity for the HBV Core 18 epitope is the result of differential proteosome processing efficiency, as is predicted, then differences in the levels of epitope presentation should be detectable. To test this hypothesis, we utilized an *in vitro* antigenicity assay (93). In this assay, Jurkat cells, stably transfected to express HLA-A2, were co-transfected with an episomal vector expressing either HBV.1 or HBV.1K. These cell lines were then used as target cells in assays to test CTL lines produced against HBV Core 18 by immunization of HLA-A2 transgenic mice with this epitope, in peptide form and emulsified in IFA. The Jurkat target cells pulsed with increasing amounts of the HBV Core 18 peptide were used for comparison, in the form of a standard curve. We found that the HBV Core 18 epitope was presented at $>10^5$ higher levels when a K was in the C1 position, rather than an F. These data demonstrate the striking effect that amino acids at the C1 position can exert on efficiency of epitope presentation in multi-epitope DNA vaccines (102).

These studies allowed for the development of vaccine design rules to minimize junctional epitopes and to optimize epitope-processing efficiency. As with the identification of potential epitopes using computerized motif searches, vaccine design can be facilitated using a computer. At Epimmune, the incorporation of preferred flanking amino acids to optimize proteosome-processing and a motif searching function have been incorporated into a basic computer program. The program accepts, as input, the particular set of epitopes, a ranking of desirable C1 amino acids and the motifs to be scanned, to identify junctional epitopes. The program can simulate the construction of a multi-epitope vaccine, examining epitope pairs, as it proceeds, to optimize proteosome-processing and avoid the occurrence of junctional epitopes. The variables available to the computer include epitope order, the ability to insert certain amino acids between epitopes to augment proteosome processing and, if needed, additional amino acids as spacers to disrupt junctional epitopes. Using this program, we can evaluate 6×10^5 epitope configurations/second. This computational speed is essential as a complete analysis of a 10-epitope construct requires the examination of $>3.6 \times 10^6$ combinations (10 factorial). This is a task that can be completed in six seconds using the computer but the required analysis time increases very rapidly as the number of epitopes is increased. For example, a fourteen-epitope construct requires two days of analysis time. A complete analysis is not required as many of the potential products the computer designs are highly related, often differing only by the position of one epitope. Thus, members of different "vaccine families" can be selected for construction and testing. An optimized HIV-1 vaccine based on 21 CTL

epitopes restricted to HLA-A2, -A3/11 or –B7 is currently under development.

7. CONSTRUCTION OF MULTI-HTL EPITOPE VACCINES

The design process and evaluation of HTL epitope-based vaccines must include different features to address the properties of HTL epitopes, including the highly promiscuous manner in which they bind to MHC Class II molecules and the antigen processing pathways most commonly utilized. To address both of these issues, we are experimenting with universal spacers, such as one consisting of GPGPG. The logic behind the design of this spacer is that neither G or P are routinely used as primary anchors, at positions 1 or 6 in the core region of an HTL peptide epitope, by any know murine or human MHC Class II molecule. The gap of five amino acids introduced by this spacer separates adjacent epitopes so the amino acids of two epitopes cannot physically serve as anchors in the 1 and 6 positions (103). This type of spacer is also predicted to introduce a beta-turn, which should enhance processing between epitopes (104).

Other experimental vaccine components being evaluated include signals to target the HTL epitope-containing protein to compartments of the cell known to optimally generate HTL epitopes. The most common target site is the lysosomal compartment. The inclusion of the targeting segment from lysosomal associated membrane proteins (LAMP) or the lysosomal integral membrane protein (LIMP) to the immunogen portion of DNA vaccines, or experimental vaccine products delivered using viral vectors, augment HTL responses (105-108). While most studies completed to date involved the use of intact gene products as the vaccine immunogen, the benefit of lysosomal targeting HTL epitopes in experimental vaccines has also been reported (109,110).

8. SUMMARY AND CONCLUSIONS

The widespread belief that vaccine induced protection against HIV-1 will require the induction of potent cellular immune responses has supported the development of numerous vaccine technologies. Amongst these is the use of highly characterized CTL and HTL epitopes. The major potential advantage associated with the use of epitopes in HIV-1 vaccines is the ability to direct immune responses to highly conserved regions of the viral genome. However, potency of the product and safety are also significant considerations. Vaccine formulations composed of a small number of epitopes can be produced using synthetic peptides but delivery technologies, such as DNA vaccines, viral vectors or recombinant proteins, appear to be better suited for use with larger numbers of epitopes. The process of designing and building vaccines composed of epitopes is complicated by the need to optimize proteosome-processing and subsequent presentation of epitopes. However, there are numerous technological tools and design rules that can be employed to correct deficits, such as the incorporation of intracellular targeting or secretion signals coupled with the use of amino acid spacers to maximize proteosome processing efficiency. Vaccines based on CTL and HTL epitopes using DNA plasmid and recombinant protein delivery formats are in preclinical development and will likely be tested clinically later in 2002.

9. ACKNOWLEDGMENTS

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