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Antigen structure influences helper T-cell epitope dominance in the human immune response to HIV envelope glycoprotein gp120

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Summary

The development of an effective vaccine against HIV/AIDS has been hampered, in part, by a poor understanding of the rules governing helper T-cell epitope immunodominance. Studies in mice have shown that antigen structure modulates epitope immunodominance by affecting the processing and subsequent presentation of helper T-cell epitopes. Previous epitope mapping studies showed that the immunodominant helper T-cell epitopes in mice immunized with gp120 were found flanking flexible loops of the protein. In this paper, we show that helper T-cell epitopes against gp120 in humans infected with HIV are also found flanking flexible loops. Immunodominant epitopes were found to be located primarily in the outer domain, an average of 13 residues C-terminal to flexible loops. In the less immunogenic inner domain, epitopes were found an average of 5 residues N-terminal to conserved regions of the protein, once again placing the epitopes C-terminal to flexible loops. These results show that antigen structure plays a significant role in the shaping of the helper T-cell response against HIV gp120 in humans. This relationship between antigen structure and helper T-cell epitope immunodominance may prove to be useful in the development of rationally designed vaccines against pathogens such as HIV.

Keywords

HIV envelope protein gp120; antigen processing; protein structure; helper T lymphocyte; MHC class II

Introduction

Over the past 25 years, great strides have been made in the area of HIV/AIDS research. Much is now known about the biology and pathogenesis of the virus. In spite of this, HIV/AIDS continues to be a major health problem worldwide. In 2006, there were approximately 4.3 million new HIV cases, 400,000 more cases than in 2004, bringing the global total of people living with HIV to 39.5 million [1]. This number reflects the increase of HIV cases in every region of the world, suggesting that the epidemic is far from declining. In order to reverse this trend, an effective vaccine against HIV must be developed.

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Studies in humans and non-human primates have shown that the CD8+ cytotoxic T lymphocyte (CTL) response is essential to the control of viremia [2-6]. Neutralizing antibody has also been shown to aid in the maintenance of low viral loads, presumably through the prevention of viral transmission from cell to cell [7-9]. CD4+ helper T-cells are required for the induction and maintenance of both of these effector mechanisms [10-18]. Therefore, the ideal vaccine against HIV must be able to activate these 3 arms of the immune response in order to confer protection against infection and disease progression.

Helper T-cell activation begins when an immature CD4+ T-cell encounters an antigen presenting cell (APC) displaying a peptide bound to an MHC class II molecule (pMHCII). This contact between the CD4+ T-cell and the pMHCII complex initiates a signaling cascade that leads to the maturation of the helper T-cell. Although every sequence within a protein antigen can potentially be loaded into the MHCII molecule, it has been observed that only a few peptides are found in pMHCII complexes during an immune response against a pathogen. These peptides have been called immunodominant epitopes. The selection criteria for these immunodominant peptides are not completely understood at present.

Our previous work has shown that antigen structure modulates antigen processing and presentation of helper T-cell epitopes in mice. Immunodominant T helper cell epitopes were found to overlap flexible loops between elements of secondary structure and between protein domains[19]. This was rationalized by the fact that flexible loops more readily conform to protease active sites and therefore are preferentially cleaved by proteases during antigen processing. Hubbard, *et al.* have amply documented the correlation of local structural disorder and protease nick sites, and they have modeled the deformations that would be required for native protein segments to achieve optimal binding to protease active sites[20]. Their work shows that disordered segments of at least 12 residues are necessary for efficient cleavage. In our work, we have used 3D structural data (mostly X-ray crystal structures) to correlate immunodominant epitopes with adjacent flexible segments[19, 21-23]. We have demonstrated experimentally that reducing the proteolytic sensitivity of a flexible segment reduces the immunogenicity of an adjacent helper T-cell epitope and increases the immunogenicity of other epitopes[23, 24]. Our studies have shown that the pattern of helper T-cell epitopes and adjacent flexible loops holds for the structurally stable outer domain of HIV gp120 in Balb/c and CBA mice[25]. Hurwitz and coworkers independently found a similar pattern in C57BL/6 mice[26-28].

In this paper, we present the helper T-cell epitope mapping data on 7 patients infected with HIV. We show that the immunodominance pattern in this human population is similar to the immunodominance patterns previously observed in mice, and that this pattern is correlated to the structure of the protein antigen. Results from this work will provide a basis for understanding the impact of viral escape mutations and for designing vaccines that target HIV vulnerabilities.

Results

Immunodominance is influenced by antigen structure in humans

In order to determine the immunodominant epitopes in this study, peptides were ranked according to the total number of IFN- γ spots elicited from the PBMC samples, as each spot presumably corresponds to a single IFN- γ producing cell. Depletion studies showed that these IFN- γ producing cells included both CD4+ and CD8+ T-cells. Analysis of the nature of the T-cells producing IFN- γ in response to several stimulatory peptides in the gp120 89.6 library showed that the peptides that elicited CD8+ responses also elicited CD4+ responses[29]. Therefore, the IFN- γ data is indicative of the CD4+ T-cell response.

In the human population studied, a majority of the immunodominant peptides, in terms of the total number of IFN- γ producing cells, are found in/or overlapping the outer domain (Figure 1A). These same peptides were recognized by the greatest number of patients in the cohort (Figure 1B). Therefore, these peptides can be described as immunodominant in terms of both intensity and frequency of responses in this human population. In contrast, none of the peptides in the library produced a positive response in any of the four control PBMCs (data not shown).

Further inspection of the location of the immunodominant epitopes shows that the peptides are found in 3 main regions of the protein, namely the regions including peptides 28-32, 38-39 and the C-terminus of the protein. This pattern is strongly correlated to that previously observed from the combined Balb/c and CBA mouse data ($r^2 = 0.57$, $p < 0.001$, Figure 1C) [25]. This result reinforces the idea that dominant epitopes share a structural context that favors their presentation to helper T-cells by APC's.

Epitopes are found adjacent to flexible regions

Previous studies in mice have shown that there is a correlation of the number and location of CD4⁺ T-cell epitopes with the number and location of flexible regions in gp120, albeit with a slight offset between the location of the flexible site and that of the epitope site [25]. This pattern holds true for the human population studied here (Figure 2A).

In order to determine the amount of offset necessary to achieve maximum correlation between the structural information and the epitope data sets, Pearson correlation coefficients were calculated using a 439-amino-acid-residue window of B-factor data that was allowed to slide along the epitope data (Figure 2B). The correlation between B-factor and epitope score is weak when the entire gp120 molecule is taken into account ($r^2 = 0.10$ for all offsets). However, when the analysis is restricted to the outer domain, there is a stronger direct correlation between flexible sites and epitope sites ($r^2 = 0.43$ for offset of -12, $p = 0.002$). Thus, on the average, human helper T-cell epitopes in this population are found 12 residues C-terminal of flexible loops. These results are similar to those found in the CBA and Balb/c mouse populations previously studied ($r^2 = 0.41$ for offset of -8), and they suggest that the presentation machinery in humans and mice are similar.

For the inner domain, there seems to be no significant correlation between the locations of flexible sites and epitopes. This could be due to the lack of structural information for the V1/V2 loop. Therefore, an alternative method was employed in order to infer any possible correlation between epitope formation and antigen structure for this domain.

Epitopes are found adjacent to variable sequences

Sequence entropy is the measure of the degree of sequence variation for a particular position in a sequence alignment. It was previously observed that this measurement is directly related to the structure of the protein [30]. Regions with higher sequence entropy (i.e., more sequence variability) are found in more flexible regions, while more conserved regions are found in the well-structured core of the protein. Therefore, sequence entropy can be used as an alternative to structural flexibility obtained from crystallographic data to independently determine the correlation of helper T-cell epitope formation and antigen structure.

As anticipated, a correlation between the location of peaks of sequence entropy and the peaks in T-cell epitopes was observed (Figure 2C). In addition, a slight offset between the peaks of the two data sets was observed. In order to determine the maximum offset necessary to achieve maximum correlation, Pearson correlation coefficients were calculated by allowing a window of averaged sequence entropy to slide along the epitope data (Figure 2D). Using this analysis, no significant correlation was found for epitope sites and sequence

entropy when the entire protein was taken into account. When the analysis was restricted to the outer domain of the protein, a significant correlation was found between regions of high sequence entropy within the antigen and epitope sites ($r^2 = 0.28$ for an offset of -11, $p = 0.016$). This finding independently confirms and reinforces the results found for this domain using the structural information from the crystallographic data.

When the analysis was restricted to the inner domain, a significant correlation was found between epitope sites and the more conserved regions of the domain ($r^2 = 0.25$ for an offset of 5, $p = 0.036$). This result implies that epitopes in the inner domain form an average of 5 residues N-terminal of more structured regions of the protein. This result was not previously observed in either of the mouse populations studied[25].

Although these results are surprising, they are not completely unexpected. It has been shown in the past that the inner domain of gp120 is more flexible than the outer domain[31, 32]. A recent study in mice by Delamarre, et al. showed that less stable antigens are poor antigens for helper T-cells, most likely due to their increased susceptibility to proteolysis in the lysosome[33]. Our results suggest that this is also true for the less stable domain of HIV gp120. These results indicate that a subtle balance between flexibility and stability is involved in the determination of epitope immunodominance patterns for helper T-cells.

Discussion

A significant obstacle to the rational design of a vaccine against pathogens such as HIV is the lack of understanding of the rules governing helper T-cell epitope immunodominance. Several factors that influence the spectrum of T-cells raised against a given antigen have been identified, although their relative contributions to the final helper T-cell repertoire remain in question. These factors include the repertoire of T-cells that can recognize the peptide-MHC complex, the intrinsic affinity of the peptides for MHC II, and the pathways of antigen breakdown that make peptide segments available for binding to MHC II proteins.

Previous studies in mice have shown that antigen structure plays a significant role in sculpting the helper T-cell response. Helper T-cell epitopes against gp120 have been mapped to exposed[27], but relatively conserved[34] regions of the protein. Epitopes in the outer domain were found to be an average of 8 residues C-terminal to flexible regions of the protein[25]. It is believed that these regions become immunodominant helper T-cell epitopes because they can be easily processed and loaded into the MHC molecule.

In this paper, we have shown that antigen structure influences the helper T-cell response of humans infected with HIV. To our knowledge, this is the first study to demonstrate such a relationship. The influence of antigen structure on the shaping of the helper T-cell immunodominance pattern is most probably related to antigen processing. In order to explain the data presented in this paper, we propose the following generalized model for the processing and presentation of helper T-cell epitopes: Proteases in the early lysosome cleave highly flexible regions of the protein. Simultaneously, MHC II molecules scan these flexible regions and, with the aid of HLA-DM molecules, bind to available sequences to which they have high affinity, creating stable MHC-peptide complexes that protect the loaded peptide sequences from proteolysis. As the lysosome matures, previously structured portions of the antigen begin to unfold and become available for binding by proteases. Proteolytic digestion of these newly unfolded sequences results in the liberation of the previously formed MHC-peptide complexes and eventual exportation of these molecules to the cell surface.

In the well-structured outer domain, proteases and MHC molecules have limited access to the flexible loops due to constraints imposed on them by the spacing between well-structured segments of the protein. This results in the proteolysis of the most central portions

of the flexible loops. The MHC II molecules then bind the remaining flexible segments an average of 13 residues toward the C-terminal of the proteolyzed sections of the protein. Unfolding of the protein and subsequent proteolysis of the sequences C-terminal to the MHC-bound peptide then liberates the peptide-MHC complex and leads to presentation of the complex to helper T-cells (Figure 3A). The limited availability of proteolytic and MHC binding sites due to structure explains why helper T-cell epitopes in the outer domain tend to cluster together, regardless of MHC type.

In the more flexible inner domain, on the other hand, a large portion of the protein is unstructured and open for both proteolytic digestion and MHC binding. Again, proteases digest the central portions of the loop while MHC molecules bind to remaining available sequences to which they have high affinity. Due to the size of the flexible region in this domain, the MHC proteins have a larger stretch of sequence to select from, resulting in a widely distributed helper T-cell epitope profile that probably reflects sequence affinities for MHC proteins. Helper T-cell epitopes for this domain form an average of 5 residues N-terminal to more structured regions of the inner domain, which places the epitopes toward the C-terminal of the most flexible regions of the protein, as observed previously for the outer domain (Fig. 3B).

Why there should be a bias toward presentation of peptides C-terminal to flexible sites is not yet clear. Further mapping experiments in MHC-typed individuals will be necessary to distinguish whether this bias is based in the antigen presentation machinery or in gp120.

These results highlight the importance of the balance between structure and flexibility in the generation of helper T-cell epitopes. More importantly, this information affords us some insight into a structure-based mechanism by which we can manipulate the helper T-cell response, either by focusing it on particular portions of an antigen by introducing structure to highly flexible regions or by increasing the breadth of the helper T-cell response by increasing the flexibility of more structured and conserved regions. This may prove to be highly useful in the rational design of effective vaccines against pathogens such as HIV.

Materials and Methods

Human t-cell epitope mapping

Human T-cell epitope data were obtained in a previous study by Kleen, et al. on blood samples from 7 HIV positive patients from the Special Immunity Unit at the University Hospitals in Cleveland, Ohio. The total number (clonal mass) of IFN- γ -producing and Granzyme-B-producing cells for this population, along with the peptide-specific maps for 3 patients were previously reported [29]. In this study, we report the peptide-specific IFN- γ responses for all seven patients for the first time. All patients were on highly active anti-retroviral therapy (HAART) at the time of the study. Negative controls for these experiments consisted of peripheral blood from 4 uninfected individuals.

PBMC's from each individual were stimulated with a single peptide from a library composed of 47 overlapping 20-mer peptides that spanned the entire sequence of HIV-1 gp120 strain 89.6[25]. IFN- γ production in response to each peptide was determined via ELISPOT[29]. The number of spot forming units (sfu) per peptide, per patient was recorded using an ELISPOT reader. All samples with greater than 5 sfu per 1×10^5 cells were considered positive.

Determination of flexibility

Flexibility of regions of the protein were determined by utilizing the backbone amide-nitrogen B-factor data from the crystal structure of gp120 bound to CD4 and the X5

antibody (PDB ID 2B4C)[35]. B-factors are a measure of uncertainty in the location of a particular atom within the X-ray crystal structure [36]. It has been previously shown that areas with high backbone-atom B-factors correspond to areas of greater conformational disorder (i.e., greater flexibility). Residues present in the wild type protein, but missing from the structure were assigned an arbitrary high B-factor value (139.18 Å) that is equivalent to the highest amide nitrogen B-factor value in the crystal structure.

Determination of sequence entropy

The Shannon sequence entropy of gp120 was determined using the 972 aligned HIV-1/cpz envelope sequences found in the 2005 HIV sequence compendium[37]. The sequences in this compendium were selected to represent the entire database of over 150,000 HIV-1/cpz sequences deposited in the data bank as of 2004. The sequence of gp120 89.6 was manually aligned using the BioEdit Sequence Alignment Editor (ver 7.0.5.3) and used as the first sequence in the calculation for the Shannon sequence entropy using Entropy-ONE. The resulting sequence entropy data was then smoothed using a 17-residue sliding window. Window averages were assigned to the middle residue of each window. No values were assigned for the first and last 8 residues of the 89.6 gp120 sequence.

Statistical Correlations of epitopes to B-factors and sequence entropy

The number of individuals with lymphocytes producing IFN- γ in response to the 47 twenty-residue segments of the gp120 peptide library was correlated with the average B-factor value for the peptide sequence. The Pearson correlation coefficient (r) was used to quantify the correlation between B-factor and epitope score for sets of twenty-residue segments representing the full length protein, inner domain, or outer domain using offsets spanning the range of -20 to +20 in five residue steps. The same analysis was performed using the sequence entropy in place of B-factor data. Pearson correlation coefficients and two-tailed p-value calculations to determine the significance of the correlations were calculated using GraphPad Prism 3.0.

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Abbreviations used

HIV human immunodeficiency virus

AIDS	acquired immune deficiency syndrome
CTL	cytotoxic t-lymphocyte
APC	antigen presenting cell
MHCII	major histocompatibility complex molecule, type II
pMHC	peptide-MHCII complex
IFN-γ	interferon gamma
PBMC	peripheral blood mononuclear cells
HLA-DM	human leukocyte antigen DM
HAART	highly active anti-retroviral therapy
SFU	spot forming units

Figure 1. Majority of the immunodominant epitopes in humans are found in the outer domain Helper T-cell epitope mapping via IFN- γ ELISPOT shows that majority of immunodominant epitopes to gp120 lie in the outer domain, in or adjacent to regions of high sequence variability. The conventional domain structure of gp120 is illustrated at the top of the figure. The domain boundaries are aligned with the bar graph so that the diagram indicates the location of each peptide in the gp120 domain structure. Immunodominance patterns are the same when either (A) the number of IFN- γ producing cells or (B) the number of individuals with a positive response to a particular peptide is taken into account. (B) Stacked bars show the number and identity of the individuals that respond to each peptide. Responses to the inner domain, are almost unique in each human studied and are not significantly correlated with previous mouse data, indicating that the response in this domain may be influenced by MHC II specificity. (C) T-cell proliferation assays revealed a similar helper T-cell epitope immunodominance pattern in mice.

Figure 2. Helper T-cell epitopes are found adjacent to flexible and variable segments of gp120
 (A) The epitope mapping data (empty bars) is superimposed on the graph of the average B-value for the corresponding peptide (solid line). (B) The Pearson correlation coefficient (r) between the two data sets was calculated for offsets that spanned the range of -20 to $+20$ in five-residue steps. A maximum correlation was observed when the outer domain data (solid circles) was offset by -12 residues from the B-factor data, indicating that epitopes in the outer domain are found an average of 12 residues toward the C-terminal of peaks of flexibility. (C) The epitope data (empty bars) was also compared to the average sequence entropy for each peptide (solid line). (D) Calculation of the Pearson correlation coefficients (r) revealed a maximum correlation (r^2) at an offset of $+5$ for the inner domain (solid squares) and an offset of -11 for the outer domain. These results indicate that epitopes in the inner domain are found an average of 5 residues toward the N-terminal of conserved regions of the protein while those in the outer domain are found an average of 11 residues toward the C-terminal of peaks of sequence variability. No significant correlations were found for the whole protein (empty circles). Asterisks indicate correlation values with a p-value < 0.05 .

Figure 3. Antigen structure affects processing and presentation of helper T-cell epitopes against gp120

Proposed models for the processing and presentation of helper T-cell epitopes in the outer domain (A) and inner domain (B). Details for each model are found in text. Structured regions of the outer domain are represented with black rectangles while structured regions of the inner domain are represented by black ovals. Flexible regions are represented by thick black lines and proteolyzed sections are represented by thick dashed lines. Proteolytic enzymes (●), MHC II molecules (●●) and HLA-DM (⦿) are also shown.