Clustering of Th Cell Epitopes on Exposed Regions of HIV Envelope Despite Defects in Antibody Activity

Scott A. Brown,* John Stambas,*, Xiaoyan Zhan,† Karen S. Slobod,‡ Chris Coleclough,*¶ Amy Zirkel,* Sherri Surman,*, Stephen W. White,‡ Peter C. Doherty,*¶ and Julia L. Hurwitz2*¶

A long-standing question in the field of immunology concerns the factors that contribute to Th cell epitope immunodominance. For a number of viral membrane proteins, Th cell epitopes are localized to exposed protein surfaces, often overlapping with Ab binding sites. It has therefore been proposed that Abs on B cell surfaces selectively bind and protect exposed protein fragments during Ag processing, and that this interaction helps to shape the Th cell repertoire. While attractive in concept, this hypothesis has not been thoroughly tested. To test this hypothesis, we have compared Th cell peptide immunodominance in normal C57BL/6 mice with that in C57BL/6μMT/μMT mice (lacking normal B cell activity). Animals were first vaccinated with DNA constructs expressing one of three different HIV envelope proteins, after which the CD4+ T cell response profiles were characterized toward overlapping peptides using an IFN-γ ELISPOT assay. We found a striking similarity between the peptide response profiles in the two mouse strains. Profiles also matched those of previous experiments in which different envelope vaccination regimens were used. Our results clearly demonstrate that normal Ab activity is not required for the establishment or maintenance of Th peptide immunodominance in the HIV envelope response. To explain the clustering of Th cell epitopes, we propose that localization of peptide on exposed envelope surfaces facilitates proteolytic activity and preferential peptide shuttling through the Ag processing pathway.

The Journal of Immunology, 2003, 171: 4140–4148.

T helper epitope processing is generally initiated by the interaction of an exogenous Ag with the membrane of an APC (dendritic cell, macrophage, or B cell), followed by internalization of Ag. Ag uptake can be mediated by Ab binding in B cells, by macropinocytosis in dendritic cells, and by phagocytosis in macrophages (1, 2). Ab may also enhance Ag uptake in non-B cells by FcR binding on APC membranes. Once Ags are drawn into APCs, vesicle fusions expose proteins to lysosomal enzymes and to MHC class II molecules. Proteolytic enzymes (e.g., cathepsins and asparagine endopeptidase) mediate the cleavage of both exogenous Ag and MHC-associated invariant chains (3, 4). Ag fragments that successfully compete with class II-associated invariant chain peptides bind to the class II peptide binding groove, after which they may be further truncated as they are shuttled to the cell membrane (5, 6).

To explain why a subset of peptides within each protein Ag is preferentially targeted by T cells, comprehensive studies of MHC-peptide interactions have been performed (7–13). These studies have been helpful in showing that certain amino acid residues within a peptide can have a substantial influence on epitope selection, because they bind tightly to pockets within the MHC peptide binding groove. Algorithms have been formulated and have proven useful in some cases to predict which peptides within a protein sequence will be strong immunogens for T cells. However, in many cases the predictions based on algorithms have proven incorrect, suggesting either that the algorithms are imperfect or that factors other than peptide sequence can influence Ag processing.

As a demonstration of one alternative influence on peptide processing, we have shown a striking correlation between HIV envelope Th cell peptides and the three-dimensional positioning of peptides within the folded envelope protein. Specifically, we found that Th epitopes are clustered within hot spots on one exposed face of HIV gp120 (14, 15). Many of these T cell epitopes overlap with known Ab binding domains (16, 17). Studies of influenza and parainfluenza viral membrane proteins have similarly shown that immunodominant Th cell epitopes can be localized to exposed protein surfaces (18–21). These relationships have been explained by two nonmutually exclusive theories. 1) Exposed regions of protein, relatively free from complex secondary structure, are primary substrates of proteolytic enzyme activity. Once released, fragments are preferentially shuttled through the Ag processing pathway for association with MHC class II molecules (14, 15). 2) Abs bind the outer surfaces of proteins and protect associated fragments from degradation. Select peptides are thus preserved for downstream Ag processing (18, 19, 22, 23).

To discriminate between these two possibilities, we have tested T cell responses in wild-type C57BL/6 and C57BL/6μMT/μMT mice. C57BL/6μMT/μMT animals have an inactivating mutation in the membrane exon of the μ heavy chain and cannot form a pre-B cell receptor. Unlike other mouse strains with the μMT/μMT mutation, the C57BL/6μMT/μMT mice have a complete block of the development of mature B lymphocytes and Ab activity (24, 25). Here we compare T cell responses toward HIV envelope protein

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*Departments of Immunology, †Infectious Diseases, and ¶Structural Biology, St. Jude Children’s Research Hospital, Memphis, TN 38105; Departments of ‡Pediatrics and ¶Pathology, University of Tennessee, Memphis, TN 38163; and §Department of Microbiology and Immunology, University of Melbourne, Melbourne, Victoria, Australia

Received for publication April 7, 2003. Accepted for publication August 11, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Grant P01-AI45142), National Cancer Institute Cancer Center Support Core Grant P30-CA21765, The James B. Pendleton Charitable Trust, and the American Lebanese Syrian-Associated Charities. P.C.D. was also supported by an F. M. Burnet Fellowship from the Australian National Health and Medical Research Council.

2 Address correspondence and reprint requests to Dr. Julia L. Hurwitz, Department of Immunology, 332 North Lauderdale, Memphis, TN 38105. E-mail address: julia.hurwitz@stjude.org

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Ags in C57BL/6 and C57BL/6(MT/ImT) animals. We show that the absence of normal Ab activity has essentially no effect on Th cell epitope immunodominance. Ab appears not to be a major influence in the molding of Th cell epitope hierarchy in the HIV envelope system. Therefore, to explain epitope clustering, we support the first of the two theories mentioned above, that exposed protein fragments are uniquely susceptible to proteolytic activity and are thus preferentially shuttled through the Ag-processing pathway.

Materials and Methods

Mice
Female C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions in a BL1 or BL2 containment area at St. Jude Children’s Research Hospital animal facilities, as specified by the Association for Assessment and Accreditation for Laboratory Animal Care guidelines. The C57BL6(MT/ImT) breeding colony, maintained by Charles River (Wilmington, MA), supplied male mice. All mice were <6 mo of age at the initiation of the immunization protocols.

DNA vectors and purification
HIV gp140 envelope genes (comprising gp120 and external gp41 sequences) were from primary isolates, 1007, 1035, and UG92005 (UG 14, 26), GenBank accession no. AF321563, AF532615, and AF338704. The constructs used for DNA vaccination were made by independently incorporating each envelope gene into a vector containing the CMV enhancer/promoter, CMV intron A, tissue plasminogen activator leader, and bovine growth hormone poly(A) sequence (27). Plasmids were purified using an endotoxin-free Giga Prep kit (Qiagen, Valencia, CA) and were resuspended in sterile PBS at 1 mg/ml for injection.

Peptide organization and synthesis
The predicted amino acid sequences from 1007 and UG92005 gp140 envelope genes (comprising 14 aa) to –14 aa) to –14 aa) to –14 aa) to

Immunoassay and assay conditions for analyses of CD4 ELISPOTs
The mice were injected three times at 1-mo intervals with 100 µg of rDNA (1 mg/ml), given at a dose of 50 µg in each gastrocnemius muscle. Approximately 1 mo after the last injection, mice were sacrificed, spleens were removed, and CD4+ T cells were enriched for assay. Briefly, the cells were treated with anti–CMV intron A, tissue plasminogen activator leader, and bovine growth hormone poly(A) sequence (27). Plasmids were purified using an endotoxin-free Giga Prep kit (Qiagen, Valencia, CA) and were resuspended in sterile PBS at 1 mg/ml for injection.

Ab analyses
Mice were immunized with three doses of 1007 envelope given at 3-wk intervals by either of two methods: 1) mice received DNA (100 µg) i.m., followed by recombinant vaccinia virus (107 PFU) i.p., and finally by CHO-derived recombinant protein in CFA i.p.; or 2) mice received three consecutive doses of rDNA (100 µg/dose) i.m. Three weeks after the last immunization, sera were collected from five mice per group and pooled. Samples were tested in triplicate by ELISA. To perform the ELISA, 96-well plates (BD Biosciences, Franklin Lakes, NJ) were coated overnight at 4°C with 2 µg/ml of purified CHO-derived 1007 envelope protein in PBS. The plates were washed three times with 0.05% Tween 20 in PBS, blocked with 1% BSA/PBS at room temperature for 1 h, and washed an additional three times. Samples were diluted in 1% BSA/0.05% Tween 20/PBS to a final volume of 50 µl and were incubated in wells for 2 h at room temperature. After three washes, alkaline phosphatase-conjugated anti-mouse IgG1 (50 µg/ml; Southern Biotechnology Associates, Birmingham, AL) diluted 1/1000 in 1% BSA/0.05% Tween 20/PBS was added for 1 h at room temperature. Following three washes, the assay was developed with 75 µl/well of p-nitrophenyl phosphate (Sigma-Aldrich) substrate (33 mg/ml in diethanolamine buffer) and was read at OD405.

Structural analysis
Peptide determinants were mapped within the envelope crystal structure (32, 33). Figures were prepared with MOLSCRIPT (34) and were rendered with RASTER3D (35).
hybridomas were first assayed for reactivity toward envelope expressed by recombinant vaccinia virus-infected cells and then tested for responses to overlapping peptides spanning the entire envelope sequence. Our use of hybridoma technology simplified these epitope-mapping studies, because of the clear distinction between positive and negative responses (see Fig. 1 for sample results) and the ease of assay reproducibility. Results from such analyses demonstrated that the Th peptide targets clustered within four hot spots on the envelope protein (three hot spots were in gp120, and one was in gp41). Mapping of the gp120 peptides on the
known crystal structure of gp120 revealed that all were localized on one exposed face of the molecule (14, 33). To ensure that the results described above were not unique to 1) hybridoma technology, 2) the D-V-P priming regimen, or 3) the original screen with vaccinia virus recombinants, we chose to repeat experiments using a prime-boost-boost regimen with DNA immunizations alone (without vaccinia virus or purified protein boosters) and analyze the responses with IFN-γ ELISPOT assays (rather than hybridoma testing). We first immunized C57BL/6 mice with a DNA construct encoding one of two different envelope proteins, either a 1007 (clade B) or a UG92005 (clade D) envelope (see Fig. 2 for protein sequences). Immunogens were independently tested in mice using three consecutive injections with 100 μg of DNA, administered at 1-mo intervals. Approximately 1 mo after the final immunization, mice were sacrificed for testing. Responding Th cell populations were next tested by an ELISPOT assay against overlapping 9–15 mer peptides spanning each of the protein sequences.

Mice were first tested individually against pools of overlapping peptides (see Fig. 2 for pool designation) to identify hot spots for Th epitopes and to define mouse-to-mouse variability. Representative results are shown in Fig. 3 for mice immunized with the 1007 envelope. As demonstrated in Fig. 3A, not all mice responded identically. The highest activity in these mice was against pools, D, G, and H in the gp120 molecule and against pool L in gp41.

**FIGURE 3.** IFN-γ ELISPOT defines Th cell determinants on the 1007 envelope in DNA-vaccinated mice. ELISPOTS were performed in wells containing 1 x 10⁶ CD4⁺ enriched splenocytes and 5 x 10⁵ APCs from C57BL/6 mice immunized with the 1007 envelope by DNA immunization. A, T cells were incubated with overlapping peptides (~7.5 μM) in pools or with no peptide (Neg.) for 24 h. B, Peptides from positive pools were individually tested (~10 μM) with splenocyte pools from six mice. Plates were developed, and spots per well were counted. SEMs are shown for replicate wells.
We next tested the responses to each of the individual peptides within positive pools. In this case, cells from multiple immunized mice were pooled for study. As shown in Fig. 3B, one peptide or two adjacent peptides within each pool elicited a response. The peptides identified, described in the context of variable (V) and constant (C) regions of gp120, were PKVSFEPIHYCAP (V2-C2 region of gp120), IIGDIRQAHCVSRE and RQAHCNISKEWEGT (V3 region of gp120), SNNTVGNPIILPCRI and GNPIILPCRIKQIIN (V4-C4 region of gp120), and TPPWNASWSNKSL (gp41). Representative results from mice immunized with the UG92005 envelope are shown in Fig. 4. Major responses were toward pools G, I, L, and M (Fig. 4A). Individual peptide tests (Fig. 4B) revealed responses to IVGNIRQAHCNVSKA and RQAHCNVSQAKWNNT (V3 region of gp120), GKAMAYPIAGLIQC and APPIAGLIQC SSNIT (V4-C4 region of gp120), TNVWNASWSNKSL (gp41), and IEESQNQQNEQEL and NQKKNEQELLELDK (gp41).

The positions in the envelope sequence of all Th target peptides from both 1007- and UG92005-primed animals are shown in Fig. 5. ELISPOT analyses, as represented by yellow bars, were identified toward C2, V3-C3, V4-C4, and gp41. In Fig. 5 it is also shown a comparison of the ELISPOT analyses with our results from previous hybridoma studies (red). The results from the two studies were very similar. Apparently the results from hybridoma studies were not substantially biased by the technological approach. Also, although the vaccination protocol (D-V-P) used for the hybridomas was different from that used for the ELISPOT (DNA priming alone), the spectra of peptide specificities defined by the two approaches were overlapping.

FIGURE 4. IFN-γELISPOT defines Th cell determinants on the UG envelope in DNA-vaccinated mice. ELISPOTs were performed in wells containing 1 × 10⁶ CD4⁺ enriched splenocytes and 5 × 10⁵ APCs from C57BL/6 mice immunized with UG92005 envelope by DNA immunization. A, T cells were simulated with overlapping peptides (~7.5 μM) in pools or in no peptide (Neg.) for 24 h. B, Peptides from positive pools were individually tested (~10 μM) with splenocyte pools from five mice. SEMs are shown for replicate wells.
In Fig. 6, the Th cell peptide targets defined above are mapped onto the three-dimensional structure of the HIV gp120 crystal. Two orientations of the gp120 envelope molecule (blue) bound to CD4 (gray) are shown in A, B and C, D, respectively. Epitopes defined by our ELISPOT analyses are highlighted in yellow (A and C), and epitopes described by our hybridoma study are shown in red (B and D). Mapped peptides include those in regions C2, C3, V4, and C4 (peptides in exposed V2 and V3 loops could not be mapped because these loops were deleted from the protein crystal). As demonstrated in all four panels, Th cell target peptides were clustered within nonhelical strands on one face of the gp120 glycoprotein.

**Priming of Th cells in the presence or the absence of an Ab response**

The clustering of Th epitopes on outer protein surfaces has been described in other viral systems and has been hypothesized to be a consequence of Ab-mediated Ag processing (18, 38). As shown in Fig. 7A and as previously described (36), mice immunized with the D-V-P protocol generated strong binding (and neutralizing) Abs toward the HIV envelope. As shown in Fig. 7B, mice immunized with DNA alone generated weak, but significant, Ab responses. We therefore questioned whether these Ab responses, either strong or weak, were responsible for the patterning of envelope-specific Th cell responses. To address this question, experiments were designed to evaluate the Th cell response pattern in the absence of normal B cell activity.

C57BL/6/μMT/μMT mice were chosen for this study because, unlike other mouse strains with the same mutation, these animals have a complete block of mature B cells and Ab development. C57BL/6/μMT/μMT test mice and C57BL/6 controls were immunized with the 1007 or UG92005 envelope using the DNA immunization regimen described above. Due to the small sizes of C57BL/6/μMT/μMT spleens, individual mice of this strain could not be tested. Instead, ~1 mo after the final immunization, 25 animals were sacrificed, and all spleens were pooled for testing. CD4+ T cells were enriched, and cells were screened for reactivity against overlapping peptides. In Fig. 8 are shown results for the C57BL/6/μMT/μMT and C57BL/6 mice tested following 1007 (Fig. 8A) and UG92005 (Fig. 8B) immunizations. Results were strikingly similar between the two mouse strains. Responses in the DNA-primed C57BL/6/μMT/μMT mice were shared with those in the DNA-primed C57BL/6 mice and the D-V-P-primed C57BL/6 mice described previously (14). Clearly, the lack of normal Ab activity in the C57BL/6/μMT/μMT animals did not influence Th cell epitope immunodominance.

**FIGURE 5.** Th cell epitopes are located in hot spots in gp120 and gp41 proteins. The relative positions of Th cell epitopes (defined in Figs. 3B and 4B) are displayed as yellow bars in the context of the envelope variable regions and the peptide pools from which they were dissected. The relative positions of target peptides defined by hybridoma analyses are shown as red bars.

**FIGURE 6.** Anatomical location of hot spots to exposed, nonhelical loops and strands of the envelope protein. Th cell hotspots are highlighted on the crystal structure of the gp120 molecule (blue), complexed to CD4 (gray). Peptides defined by ELISPOT analyses are outlined in yellow, while peptides defined by hybridoma analyses are outlined in red (14). Front (A and B) and side (C and D) views of the crystal are shown.
Because of the limited numbers of T cells, a complete analysis of individual peptides could not be performed with cells from the C57BL/6/H9262/MT/H9262/MT mice. Instead, peptides defined as targets for Th activity in the C57BL/6 animals (Figs. 3 and 4) were individually tested on C57BL/6/H9262/MT/H9262/MT spleens, and all were shown to elicit positive responses (data not shown).

Highly restricted pattern of Th cell activity occurs in the absence of a normal Ab response

Our previous work with a third envelope protein, designated 1035, revealed Th cell epitope skewing even more pronounced than that for 1007 or UG92005 envelopes. Th cells primed with the 1035

**FIGURE 7.** Ab responses following D-V-P and immunization with three consecutive DNA injections (D-D-D). Mice were primed with three consecutive immunizations at 3-wk intervals. D-V-P mice received 100 µg of DNA (expressing the 1007 protein) i.m., followed by 107 PFU recombinant vaccinia virus (expressing the 1007 protein) and finally 5 µg of purified 1007 protein in CFA i.p. D-D-D mice received 100 µg of DNA (expressing the 1007 protein) every 3 wk, while naive mice received no injection. Serum samples taken ~3 wk after the last injection were tested by ELISA for 1007 envelope-specific Abs. Sera from five mice per group were combined for assay in triplicate. Serum dilutions are shown on the x-axis.

**FIGURE 8.** Matched patterns of Th cell epitope immunodominance in the presence or the absence of Ab. T cells from C57BL/6 and C57BL/6H9262/MT/H9262/MT mice were compared for responsiveness to peptide pools. C57BL/6 data are the averages of results from six mice tested individually. C57BL/6H9262/MT/H9262/MT data are from pooled T cells tested in replicate. Peptide concentrations were 7.5 µM for each individual peptide within a pool, and plates were incubated for 48 h before developing. Each well contained 1 × 106 CD4+ enriched splenocytes. Responses toward envelopes 1007 (A) and UG92005 (B) are shown for C57BL/6 (light gray bars) and C57BL/6H9262/MT/H9262/MT (dark gray bars) animals.

**FIGURE 9.** Patterns of T cell activity toward the 1035 envelope among C57BL/6H9262/MT/H9262/MT mice. C57BL/6H9262/MT/H9262/MT mice were immunized with the 1035 envelope by DNA immunization, and T cells were screened on envelope peptides. A, Results are from splenocytes combined from 25 C57BL/6H9262/MT/H9262/MT mice immunized with 1035. Peptide concentrations were 7.5 µM for each individual peptide within a pool, and plates were incubated for 48 h before developing. Each well contained 1 × 106 CD4+ enriched splenocytes. B, No peptide (Neg.) or individual peptides were tested on three pooled spleens from C57BL/6H9262/MT/H9262/MT mice to confirm their immunogenicity.
envelope were almost exclusively targeted toward the sequence PKVSFEPIPHYCAP in the gp120 molecule (26). One additional, strong response was observed toward gp14 sequence TNPVPN ASWSNKSL. To determine whether Ab influenced this unusual skewing, we tested C57BL/6/KSLE, predicted to be Th cell targets by our C57BL/6 studies (26), and C57BL/6/KSLE peptides, respectively (1035 peptide pools were produced in the same register as described for 1007 and U929005 sequences; Fig. 2). Essentially the results were the same for C57BL/6 and C57BL/6aMT/mT mice despite the lack of normal B cell activity in the latter strain (25). As described above, there was a limitation in cells from C57BL/6aMT/mT animals, so that a large number of peptides could not be individually tested. However, peptides PKVSFEPIPHYCAP and TNPVPNASWSNKSL, predicted to be Th cells targets by our C57BL/6 studies (26), were tested with C57BL/6aMT/mT T cells, and each elicited a response (Fig. 9B).

Discussion
The goal of this study was to define the role, if any, that Ab plays in the establishment of Th cell epitope immunodominance in an HIV envelope priming system. Despite the fact that epitope immunodominance characterizes essentially all T cell responses, the factors responsible for the creation and maintenance of peptide hierarchy are not fully understood. It was originally proposed that the peptide sequence was responsible for peptide immunodominance, because certain amino acid residues were preferentially bound to pockets of the MHC peptide binding groove (7–13). However, our studies and those of others have highlighted additional influences on Th cell epitope selection. We recently found that peptide immunodominance correlated with the location of peptides within the three-dimensional structure of the HIV envelope target Ag. Specifically, we showed that immunodominant peptides on the envelope gp120 molecule were exclusively localized to a single exposed face of the molecule (14). Other groups working with T cells from HIV-infected humans or envelope-primer mice have also identified responsiveness toward peptides in these regions (15, 39–43).

Are Abs influencing the processing of envelope peptides, thus explaining the phenomenon of epitope clustering? Abs have clearly been shown to affect Ag processing pertinent to Th activity, both quantitatively and qualitatively (3, 44). One recent demonstration of Ab contributions to Ag processing and Th cell responsiveness was with tetanus toxin-specific Abs. Anti-tetanus toxin Abs were shown to stabilize or footprint fragments of tetanus toxin during the proteolysis associated with Ag processing. In a dose-dependent manner, Abs could either enhance or suppress Th cell responses in vitro toward peptides within protected domains (3).

The precise mechanisms by which Abs function in the protection or shutting of fragments through Ag processing compartments is unclear. The results described above were confounded when mutant Ags, lacking major asparagine endopeptidase processing sites, were designed. Specifically, a mutation was made in Asn1184 of a tetanus toxin C fragment (TTCF), mediating an alteration of the footprinting pattern for TTCF and TTCF-specific Ab. It was expected that this change in footprinting would alter Ag presentation and associated Th cell responses. Surprisingly, there was no detectable effect of this mutation on T cell activity. Conversely, a different mutation (Asn1219), with no known effect on footprinting, had a deleterious effect on Ag processing and T cell function.

Complicating the relationship further is the fact that a B cell, when acting as an APC, need not share Ag specificity with a cognate T cell. For example, a B cell expressing hemagglutinin-specific Abs can entrap whole influenza virus and effectively present matrix peptides to responding Th cells (45, 46). Additionally, B cells with entirely irrelevant Ag specificities can act effectively as APCs (3). Thus, while Ag-specific Abs enhance protein uptake and have the potential to alter epitope presentation in vitro, their influences are not absolute and might be easily overwhelmed by other factors.

Our data directly address the potential for Ab to alter Th epitope immunodominance in vivo. Results suggest, at least for our HIV envelope system, that epitope immunodominance can be established in the absence of normal Ab activity. Thus, influences other than B cell activity must be considered as explanations for the patterning of epitope hot spots in the HIV envelope protein. The immunodominance (or lack thereof) of any given peptide-specific T cell response is a reflection of complex variables, including peptide sequence, peptide context, mechanisms of Ag processing, and T cell repertoire. Based on the results described in this and other reports, we suggest that peptide context is of particular importance for establishment of immunodominance in the HIV envelope system. We consider that the clustering of Th cell peptide targets may reflect the accessibility of these peptides to proteolytic cleavage. Clearly, secondary protein structure impacts resistance to proteolysis (as with hen egg-white lysozyme (4)). The HIV gp120 envelope is unique in that it is riddled with disulfide bonds and is heavily glycosylated, with carbohydrate accounting for >50% of the total m.w. Other proteins with limited three-dimensional structure may be easily unfolded, such that both internal and external peptides are equally available for Ag processing. For the envelope protein, however, peptides buried in the core may be relatively resistant to proteolysis, while peptides on exposed surfaces may be facile substrates. Exposed regions are probably among the last to become structured, and consequently are preferentially accessible to fragmentation. Peptides may be "peeled away" from the envelope core, leading to rapid trafficking through endosomal compartments for eventual association with MHC class II molecules.

Proteolytic activity may be further influenced by a high frequency of asparagine residues, the potential sites of glycosylation and targets of lysosomal asparagine endopeptidase (3), on exposed envelope protein surfaces. As an illustration of this concept, Sjoland et al. (42) have shown that the removal of glycosylation sites in the C-terminal region of the gp120 molecule is sufficient to render an adjacent peptide nonimmunogenic.

Our studies of epitope immunodominance contribute not only to the definition of processing mechanisms, but also to the design of vaccines. Our results suggest that peptide location within a protein influences the processing and presentation of T cell epitopes, regardless of Ab influence. Vaccine approaches that involve either the production or the injection of native proteins may thus be preferable to those using shuffled or linked peptides (47–49). In the latter instance, it is possible that mechanisms of processing and presentation may be altered when peptides are removed from their normal context (50). Vaccines that maintain native envelope structures and preserve natural processing mechanisms might best mimic the live viruses they are designed to eradicate.

Acknowledgments
We thank Bart Jones, Brita Brown, and Pam Freiden for excellent technical assistance. We thank the World Health Organization and Dr. James Bradac (AIDS Research and Reference Reagent Repository, Rockville, MD) for virus U929005, from which a DNA sequence was derived. The DNA expression cassette, with which vaccines were made, was kindly provided by Drs. James Mullins and Harriet Robinson.
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