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This grant addresses 2 major problems in HIV synthetic peptide vaccine development: 1) the ability of synthetic peptides to mimic conformational determinants of HIV envelope proteins, and 2) the design of optimally immunogenic multivalent peptide immunogens capable of being recognized by MHC Class I and II types of outbred populations. In Technical Aim (T.A.) #1, a peptide mimitope of the 48d human mab has been found that binds to fusin+ T cells, and in binding to human cells, ceases exposure of the 48d binding site. Immunogenic gp120 C4-V3 peptides have been mutated based on structural predictions that improve the immunogenicity of C4-V3 peptides. New potentially neutralizing determinant peptides from HIV gp120 and gp41 have been made and are being tested in a novel water based adjuvant strategy utilizing intranasal immunization for optimal systemic antibody response generation. In T.A.#2, the 4 C4-V3 peptides from HIV MN, RF, EV91 and CANOA as well as C4 mutant peptides have been studied using NMR, their solution conformers determined, and structure-function relationships made with studies in T.A.#1. In T.A.#3, an assay has been developed to predict HIV peptide binding to specific HLA molecules.

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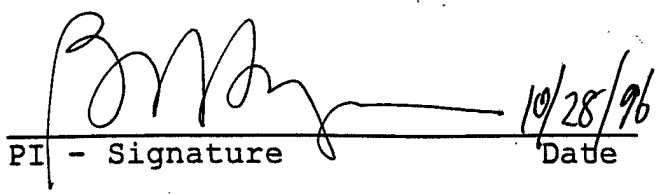

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Overview of Activities of the Duke DAMD17-94-J-4467 Grant

Principle Investigator: Barton F. Haynes, MD

1995-1996 has been a time of enormous change in the field of HIV biology with several major discoveries greatly affecting the field. The discovery that the envelope of HIV primary isolates is different, with regard to neutralizing epitopes expressed, from laboratory-adapted HIV strains has reoriented our work to focus on the envelope of HIV primary isolates. The discoveries of HIV co-receptors on T cells and monocytes to be chemokine G-protein-coupled receptors, has opened a new area of research for those involved in HIV pathogenesis and vaccine development. The discovery of the importance of non-MHC restricted chemokine production to control of HIV, and that the co-receptor for monocytotropic HIV is the beta chemokine 5 receptor, has targeted HIV vaccine developers on the portion of HIV gp120 that binds to HIV co-receptors on host cells. Finally, the importance of inducing protective immunity at mucosal surfaces remains a high priority, and much progress has been made in understanding the correlates of protective immunity to HIV in humans.

Because of these remarkable advances in HIV biology, the Duke Army Vaccine Grant team has moved to build on these breakthroughs in HIV biology and to apply these new insights to facilitate development of a practical vaccine for HIV. Also, the Duke Army Grant team has restructured personnel to accommodate Thomas Palker's leaving Duke this year to join Merck.

Regarding reorganization of the Duke Army Vaccine Grant team to accommodate Tom Palker's departure, as stated in the original grant, Dr. Barton Haynes has taken over the leadership of the programs and the grant. To take Tom Palker's place as PI on Project 1, Dr. Haynes has recruited to the Duke faculty Dr. Herman Staats, who has just finished his post-doctoral fellowship with Dr. Palker, has already worked on Project 1 for three years, and made outstanding contributions to working our immunization schedules for induction and measurement of mucosal immune responses induced by C4-V3 immunogens.

The programmatic changes that have taken place include reorganization of Project 1 to study HIV env conformation determinants that are important in HIV interaction with the HIV co-receptors important for HIV primary isolate and monocytotropic HIV entry into CD4+ T cells and monocytes/dendritic cells. A major effort is being made in Projects 2 and 3 to correlate HIV env immunogen function with immunogen structures.

Achievements of the Duke Army Grant Team over the past year include: 1) discovery that the SP10Can0(A) V3 loop motif has both structural and immunogenic similarities to HIVMN (Projects 1 and 2), 2) demonstration that the 48d-defined conserved epitope that is expressed on gp120 after CD4 ligation, may be involved in HIV env interaction with uninfected host cells, i.e. bind to the HIV co-receptor (Project 1), 3) determination of solution structures of the HIV peptide multivalent V3 immunogen presently in human vaccine trials (HIV EV91, RF, MN and Can0) (Project 2), 4) demonstration of the MHC binding capabilities of HIV env CTL epitopes within the V3 loops of peptides of HIVCan0, EV91, RF and MN as well as V3 loop motifs from Clave B and Clave E, 5) design of a number of immunogenic peptides from HIV env gp120 and gp41 that induced antibodies to native proteins, for study in the Army Grant for their ability to induce neutralizing antibodies against primary isolates when formulated in water and/or saline-based adjuvant preparations, and 6) discovery of a V3 neutralizing epitope in the SIV envelope (Project 1).

While there is still uncertainty over the nature of the most promising practical HIV vaccine immunogen construct, the new discoveries mentioned above have provided an enormous boost to the HIV vaccine development field, and guarantee that this will be a scientifically key year in the life of HIV vaccine development. The investigators in the Duke Army Grant Team remain both excited about, and committed to, development of a preventive HIV vaccine.

DAMD17-94-4467 PROGRESS REPORT - YEAR 2: STRUCTURAL AND FUNCTIONAL STUDIES OF EXPERIMENTAL HIV SYNTHETIC PEPTIDE IMMNOGENS

Technical Aim 1: To use existing and new human and mouse monoclinal antibodies as templates for definition of native gpl20 conformations and determinants of synthetic peptides that bind to these antibodies.

Principle Investigator: Herman Staats, Ph.D.
Co-Investigator: Barton F. Haynes, M.D

INTRODUCTION

The hypotheses to be tested in Technical Aim 1 are:

Regarding B cell anti-HIV immune responses, synthetic peptides can be designed that mimic not only linear determinants of neutralizing epitopes of gpl20 but also mimic more conserved broadly neutralizing HIV conformational determinants.

Regarding T cell anti-HIV immune responses, synthetic peptides can be designed that optimize antigen processing and/or antigen-presentation by MHC molecules on antigen-presenting cells.

Regarding MHC-restricted binding of HIV peptides, the HIV synthetic peptide mixtures can be designed to stimulate T cell responses in individuals of ethically diverse ancestry in multiple geographic locations.

BODY

1. The gpl20 V3 Peptide Of HIV Isolate CanO Mimics A Highly Conserved Confirmational Determinant of Native gp120 That Is Exposed Following gp120-CD4 Binding.

CD4 ligation of HIV envelope gpl20 results in conformational changes in gpl20 that lead to exposure of the fusogenic domain of gp41 and fusion of HIV with the host cell membrane. An important conserved confirmational determinant at or near the CD4 binding site exposed on gpl20 subsequent to CD4 binding is defined by two human mabs, 17b and 48d. These mabs do not block the binding of anti-CD4 binding site mabs, but themselves are blocked by CD4 binding site antibodies. Similarly, mabs 17b and 48d do not block CD4 binding to gpl20, rather 17b and 48d mab binding to gp120-is upregulated following CD4 binding. To determine if synthetic peptide mimetopes could be designed that reflect conserved confirmational determinants on the surface of gpl20, we have screened gpl20 hybrid synthetic peptides comprised of C4 and V3 sequences from ten divergent HIV isolates for their ability to bind to mabs 17b and 48d in solid phase ELISA assays. We found that mab 48d selectively bound only to the HIV CANO C4-V3 peptide (mean+/-SEM OD, 1.180+/-.050; mean E/C=12.8+/-1.1, n=8), whereas the 17b mab bound none of the peptides tested. Treatment of the C4-V3 peptide with 8M urea abrogated mab 48d peptide binding (mean E/C pre-urea treatment=13.6+/-1.6, mean E/C post-urea treatment=3.4+/-, n=4, $p<.002$). Analysis of mab 48d to bind equimolar aounts of C4, C4-V3, or V3 HIV Can0 peptides demonstrated that 48d bound better to the HIV Can0(A) C4-V3 peptide (2 fold increase in binding, n=8, $p<.02$), and did not bind to the C4 peptide alone. We have recently shown that the SP10 peptide binds to the surface of HSB-2 T cells and that HSB-2 T cells are positive for expression of the T cell tropic HIV co-receptor, LESTR/fusin, using monoclonal antibody 12G5 from James Hoxie. Moreover, when the SP10 Can0 V3 peptide binds to fusin-positive HSB-2 T cells the 48d monoclonal antibody is no longer able to bind to the SP10 Can0(A) V3 peptide, implying that the 48d binding site on the Can0 peptide is involved in interacting with host cell proteins. Current work involves determination if the anti-fusin monoclonal antibody, 12G5 can inhibit the interactions of the SP10 Can0 peptide with both mitogen-induced activated peripheral blood T cells

as well as with the HSB-2 T cell line. Thus, these data suggest that the primary sequence of the HIV Can0 V3 loop exists in a conformer that mimics a conformational determinant of native gp120 exposed on the surface of gp120 subsequent to CD4 binding. Structural studies of mab 48d-Can0(A) V3 peptide complexes (in Project 2) should provide important information regarding the nature of gp120 conformational changes that are required for env-mediated cell fusion (see ref. in appendix).

2. Modification of Anti-Envelope Antibody Responses and Peptide Solution Conformations by Amino-Acid Sequence Variation in Human Immunodeficiency Virus gp120 kDa Envelope C4-V3 Immunogenic Peptides.

Immunogenic peptides have been developed that elicit B and T cell responses to various strains of human immunodeficiency virus (HIV) (1-4). These peptides consist of two segments derived from non-contiguous regions of the gpl20 *env* protein. One segment contains 16 residues from the fourth constant (C4) domain of gpl20, and includes a potent T-helper epitope, termed T1 (5).Linked to the carboxyl terminus of this gpl20 C4 region-derived peptide is a 23-residue segment from the third variable (V3) domain of gpl20, that includes a B cell neutralizing antibody epitope for cell line-adapted HIV strains (1, 6, 7), a T-helper epitope (1), and two cytotoxic T cell (CTL) epitopes (8,9). These C4-V3 peptides induce antibodies that bind to native gpl20 and neutralize the particular cell line-adapted strain of HIV from which the V3 segment was derived, with some cross-strain neutralization activity often found (1,4).

A prototype polyvalent HIV experimental immunogen comprised of the conserved C4 region of gpl20 and the V3 regions of HIV isolates MN EV91 and RF has been constructed and currently is in clinical trials in humans (4). Thus, understanding secondary and higher order structures of the components of this polyvalent immunogen, as well as defining strategies to optimize gpl20 immunogen antigenicity, is of critical importance to HIV vaccine design and development efforts. In addition, recent data suggest that the HIV V3 region may be involved in gpl20 interactions with the recently described HIV co-receptors, LESTR/fusion and chemokine receptor type 5 (CCR-5).

To understand the physical basis of immunogenicity of the four C4-V3 peptides in this multivalent prototype immunogen and to understand structural principles necessary for HIV V3based immunogen design, we have used nuclear magnetic resonance (NMR) to characterize conformations of the multivalent immunogen C4-V3 peptides in solution (10,11 Vu et al., in preparation). It was found that the V3 segments of each of the four C4-V3 peptides displayed evidence of preferred solution conformations, with some features shared and other features differing among the four peptides. The C4 segment, which is of identical sequence in all the peptides, showed in each case a tendency to adopt nascent helical conformations (10, 11, Vu et al., in preparation).

The C4 domain of gpl20 is part of the CD4 binding site, and antibodies recognizing the C4 sequence block CD4-V3-gpl20 binding (12). However the C4 sequence as a peptide does not elicit antibodies that bind native gpl20. This led us to speculate, based on the hypothesis that solution conformations of peptides may determine immunogenicity, that the nascent helical conformations exhibited by the C4 segment might reflect a conformation not native to HIV gpl20. Amino-acid sequence homology between C4 and a human IgA CH1 domain has been noted. By comparison to the structure of mouse IgA (16) the C4-homologous region of IgA has a β strand secondary structure (10). Thus, while the C4 gpl20 peptide in solution adopts nascent helical conformations, the native structure of the gpl20 C4 region may be quite different (ie have a β strand secondary structure).

Thus, we have constructed C4-V3RF(A) peptides with amino acid substitutions designed to minimized C4 a-helical peptide conformation and promote β strand C4 secondary structures with conformers in order to induce anti-native gp120 antibodies with the modified C4 peptide (Table 1). In addition, we tested to see if any of these mutated C4-V3 pepides would enhance gp120 peptide

immunogenicity, and therefore augment anti-HIVRF gp120 V3 loop antibody responses. Finally, the solution conformers of each peptide studied immunologically were solved using NMR to correlate peptide with peptide immunogencity.

We found that substitution of G for E at position 9 of the C4 peptide, decreased the C4 peptide tendency for formation of nascent helicals (see report of Project 2). Nonetheless, no β strand stable coformers were noted in C4 E9V V3RF(A), and antibodies to this and all other C4 V3RF(A) peptides tested did not bind to the C4 region of native gpl20IIIB. However, substituting V for E at position 9 of C4 resulted in the C4 E9V V3RF(A) peptide inducing 2 logs higher anti-gp120 V3 loop antibody levels after 2 immunizations compared with the original C4-V3RF(A) peptide (Table 2). Table 3 shows that the decreased antibody responses induced by the K12E C4-V3RF peptide to the HIVRF gp120 V3 region was not due to decreased immunogenicity at the T cell level of the C4K12E-V3RF(A) peptide. Thus, these data form the basis for future rational experimental HIV immunogen design to maximize anti-HIV envelope responses.

3. Design of Immunogens to Induce Neutralizing Antibodies of HIV Primary Isolates Grown in Peripheral Blood Mononuclear Cells.

In another grant (NIAID- funded National Cooperative Vaccine Development Group Grant [AI-3535]), we synthesized a large number of the putative HIV env neutralizing epitope peptides from HIV gp120 or gp41 and immunized guinea pigs intramuscularly in incomplete Freund's adjuvant (oil-based adjuvant) (Table 4). Peptides reflective of HIVIIIB, MN, BAL have been tested in neutraliation assays against the above 3 isolates and against a panel of HIV primary isolates with V3 loop and other neutralization determinant sequences that match those of the immunizing peptides. Table 4 summarizes our results to date in the characterization of these sera. As can be seen, we have raised a number of sera that recognize epitopes of HIV gp120 that are expressed on the surface of gp120 of lab-adapted HIV strains. To date, the only sera that neutralizing HIVMN or IIIB are anti-V3 antisera. However, none of these antisera including the anti-V3 antisera have neutralized HIV primary isolates. Of interest is the observation that we have made C4-V3 peptides reflective of HIV 89.6 HIV primary isolates/SHIV chimeric virus and demonstrated that anti-C4-V3 89.6 V3 peptide antibodies neutralizes the 89.6 non-pathogenic SHIV strain but not the 89.6P pathogenic strain. Therefore, in this Army Vaccine Development grant, we will take advantage of the recent breakthroughs made by Staats and Palker in demonstrating that intranasal immunization in aqueous adjuvant formulations with cholera toxin induces the same level of systemic antibody as does immunization intramuscularly in the presence of oil-based adjuvants. To address the critical question as to why we are not getting antibodies that neutralize HIV primary isolates with these env fragments, we will test the notion that the conformation of the peptides in oil is not the appropriate conformation for inducing antibodies that block infection of HIV primary isolates and that immunization in saline adjuvant formulatons intranasally will be the system in which to test the notion of importance of water-based peptide conformations. Importantly, in Project 2 all of the preferred peptide conformers solved by the Spicer NMR structural biology team have been in aqueous solutions. These studies will be particularly important with regard to anaylsis of antibody responses to the Can0 peptide epitope, since rhesus monkey antisera raised against the C4-V3 Can0(A) peptide have not neutralized HIV primary isolates with the appropriate HIV primary isolates. However, we have yet to match those primary isolate sequences with this specific V3 loop sequence of the HIV Can0 isolate.

Therefore, it is of particular interest given the discovery that the Can0 V3 peptide is a mimitope for the 48d monoclonal antibody which defines a conserved epitope expressed on gp120 after ligation with CD4 and putatively is involved in the interaction of gp120 with HIV co-receptors on host cells. We will determine if immunization with the Can0 peptide in saline or water-based adjuvant formulations intranasally yields more broadly reactive neutralizing antibody responses compared to immunization with the peptide in oil-based adjuvants.

Thus, the environment in which an antigen is presented to the immune system may greatly affect the subsequent immune response. For example, subcutaneous immunization with HIV peptides formulated in complete Freund's adjuvant (CFA) depends upon formulation of the peptide in a hydrophobic oil environment. Although high-titered anti-peptide antibody responses may be induced with this protocol, the presentation of antigen in a hydrophobic environment (as compared to an aqueous, hydrophilic environment) may alter the conformation of the antigen and therefore alter the specificity of the immune response induced. We have recently determined that intranasal immunization with the hybrid C4-V3 HIV peptide TlSP10MN(A) and the mucosal adjuvant cholera toxin induced high titered serum anti-peptide IgG responses (Table 5). For intranasal immunization, the peptide was diluted to the appropriate concentration in phosphate-buffered saline and administered to the nasal mucosa of C57BL/6 mice. Intranasal immunization was as effective as subcutaneous immunization for the induction of serum anti-TlSP10MN(A) IgG titers (Table 6). Therefore, we have utilized two immunization protocols that induce comparable serum IgG titers after HIV peptide immunization. One protocol depends upon presentation of the antigen in a hydrophobic environment (CFA) while the other utilizes a hydrophilic environment for antigen presentation (intranasal immunization).

To determine if the vaccine delivery vehicle affects the induction of antibodies capable of neutralizing HIV-1 primary isolates, guinea pigs will be intranasally immunized with our HIV-1 peptides and cholera toxin as a mucosal adjuvant. Guinea pigs will be intranasally immunized with 200 Rg HIV-1 peptide and 1 ug cholera toxin on day 0, 14, 28, and 42. Serum samples will be collected on day 28 and 49. Anti-HIV peptide serum induced by intranasal immunization will be compared to anti-HIV peptide serum induced by subcutaneous immunization with peptide formulated in CFA for its ability to: 1) bind to HIV antigens in Western blot, 2) bind to the surface of HIV-infected cells, and 3) neutralized lab-adapted and primary HIV-isolates.

4. <u>The V3 Domain of SIVmac251 gp120 Contains a Linear Neutralizing Epitope.</u>

Antisera to 21 synthetic peptides containing hydrophilic sequences of simian immunodeficiency virus strain mac251 (SIVmac251) gp120 and gp32 were tested for the ability to neutralize SIVmac251. Goat antisera raised to peptides SP-1 and SP-1V containing the carboxy-terminal portion of the V3 domain of SIVmac251 gpl20 between amino acids (a.a.) 327-339 inhibited syncytium formation (90% inhibition at a 1/1024 dilution) and cell killing of CEMx174 cells by SIVmac251 (50% inhibition of cell killing at a dilution of 1/5832), SIVDeltaB670 (1/5687 and SIVsmH4 (1/740). Neutralizing antibodies to SIVmac251, SIVDeltaB670 and SIVsmH4 could be adsorbed by peptides containing a neutralizing V3 sequence of SIVmac2S1 gpl20 (GLVFHSQPIND, amino acids 329-339) but not by peptides lacking this sequence. This V3 neutralizing region corresponds to a homologous V3 neutralizing site within HIV-2 gpl20 reported by Bjorling et al. 1991, 1994). Antibodies in 20 of 31 sera obtained from rhesus macaques infected with SIVmac251 reacted with a peptide containing the entire V3 sequence of SIVmac251 gpl20; whereas no sera contained antibodies reacting with the V3 neutralizing site between amino acids 329-33-9. Low levels of antibody-mediated recognition and subsequent lack of selective pressure against this linear V3 neutralizing site might in part explain why sequences within this region do not vary during the course of SIV (see ref. in appendix).

CONCLUSIONS

The implications of this research are that these intense efforts are ongoing to produce peptide immunogens in native conformations that will induce neutralizing antibodies against HIV primary isolates in both the US and other parts of the world. We have made progress on this project with discovery that the SP10Can0(A) V3 loop motif has both structural and immunogenic similarities to HIVMN, demonstration that the 48d-defined conserved epitope that is expressed on gp120 after CD4 ligation, may bind to the HIV co-receptor, determination of solution structures of our HIV peptide multivalent V3 immunogen presently in human vaccine trials demonstration of the MHC binding capabilities of HIV env CTL epitopes within the V3 loops of peptides, design of a number

of immunogenic peptides from HIV env gp120 and gp41 that induced antibodies to native proteins, and discovery of a V3 neutralizing epitope in the SIV envelope. The investigators in the Duke Army Grant Team remain both excited about, and committed to, development of a preventive HIV vaccine.

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TABLE 1

Peptide	Sequence	
	1 16	17 39
C4-V3 RF	KQIINMWQEVGKAMYA	TRPNNNTRKSITKGPGRVIYATG
E9G	KQIINMWQGVGKAMYA	TRPNNNTRKSITKGPGRVIYATG
E9V	KQIINMWQVVGKAMYA	TRPNNNTRKSITKGPGRVIYATG
K12E	KQIINMWQEVGEAMYA	TRPNNNTRKSITKGPGRVIYATG
	C4	V3

TABLE 2

Comparison of the Ability of C4-V3 Peptides To Induce HIV gp120 Anti-C4 and Anti-V3 Antibodies in Balb/c Mice

Peptide Immunogen	Bleed After Boost	N	Peptide on Plate in ELISA For Anti-Peptide Antibody					
			C4	V3RF(A)	C4-V3RF(A)	C4E9G-V3RF(A)	C4E9V-V3RF(A)	C4K12E-V3RF(A)
			Geometric Mean Titer					
C4-V3RF(A)	2	6	2	1,584	2,239	1,195	1,584	1,412
C4-V3RF(A)	3	6	1	22,387	10,000	14,125	6,309	11,220
C4E9G-V3RF(A)	2	6	2	6,310	7,079	5,623	3,162	3,548
C4E9G-V3RF(A)	3	6	1	56,234	17,782	11,220	8,912	17,782
C4 E9V-V3RF(A)	2	5	14	151,356	131,825	87,096	87,096	114,815
C4E9V-V3RF(A)	3	5	15	131,825	151,356	173,780	131,825	151,356
C4K12E- V3RF(A)	2	6	1	8	8	1	3	3
C4K12E-V3RF(A)	3	6	5	120	239	371	169	169

Data represent the reciprocal of endpoint dilutions at which the E/C was ≥ 3.0 in anti-peptide ELISA as described in the Methods.

TABLE 3

Comparison of the Ability of C4-V3 Peptides To Induce Anti-HIV gp120 Peptide ^{3}H-Thymidine Incorporation in Splenocytes from Naive and Immunized Mice

Peptide Immunogen	Peptide Used As Stimulator in ^{3}H-Thymidine Incorporation Assay						
	N	C4	V3RF(A)	C4-V3RF(A)	C4E9G-V3RF(A)	C4E9V-V3RF(A)	C4K12E-V3RF(A)
	Mean ± SEM Δ CPM per 10^{6} Splenocytes in Culture						
None (Naive Balb/c)	6	613 ± 322	408 ± 140	149 ± 84	114 ± 85	74 ± 47	187 ± 165
C4-V3RF(A)	6	2,289 ± 1,332	995 ± 353	8,390 ± 1,424[a]	8,067 ± 1,728	6,242 ± 1,787	6,198 ± 1,343
C4E9G-V3RF(A)	6	408 ± 95	708 ± 325	2,103 ± 1,170	3,559 ± 2,310[b]	998 ± 340	1,101 ± 399
C4E9V-V3RF(A)	5	84 ± 52	1,463 ± 473	933 ± 4,528	11,743 ± 3,830	24,824 ± 5,581[c]	10,269 ± 3,592
C4K12E-V3RF(A)	6	3,430 ± 2,796	4,417 ± 2,217	8,670 ± 3,865	13,237 ± 8,563	7,513 ± 2,951	12,644 ± 4,138[d]

Data represent peak ^{3}H-thymidine responses at 7 days as described in reference ___.

Δ CPM = CPM experimental - experimental control.

[a] $p < .001$ vs naive mice; p = NS vs C4-V3RF(A) or C4K12E-V3RF(A) stimulated C4K12E-V3RF(A) immunized splenocytes.

[b] p = NS vs naive mice.

[c] $p < .001$ vs naive mice.

[d] $p < .02$ vs naive mice.

Table 4. HIV-1 Envelope Peptides Used in the Touchpoint Study

Name of peptid	Targeted region	GP #	ELISA titer	Surface binding		Western blot			Neutralization	
				CEM/IIIB	CEM/MN	r-gp41, or r-gp120	CEM/IIIB	CEM/MN	MN	MN
GTH1-SP10MN(A)	V3	2	51200	Positive	n/d	Both positive	Both positive	n/d	>3200	200
GTH1-SP60	gp41	3, 4	25600, 51200	Both negative	n/d	n/d	Both negative	n/d	-	-
GTH1-SP61	gp41	5, 6	51200, 102400	Both negative	n/d	n/d	Both negative	n/d	-	-
GTH1-SP62	gp41	17, 18	102400, 102400	Both negative	Both negative	Both negative	Both negative	n/d	-	-
GTH1-SP70	V2	7, 8	<50, <50	Both negative	n/d	n/d	Both negative	n/d	-	-
GTH1-SP75	gp41	9, 10	<50, <51	Both negative	n/d	n/d	Both negative	n/d	-	-
GTH1-SP80 NL4-3	V1	11, 12	3200, 12800	Both negative	n/d	n/d	Both negative	n/d	-	-
GTH1-SP80 BAL	V1	19, 20	102400, 25600	Both negative	Both negative	Both negative	Both negative	n/d	-	-
SP400-BAL	gp41	13, 14	204800, 204800	Both positive	n/d	Both positive	Both negative	n/d	-	-
SP410-BAL	C5	15, 16	409600, 204800	Both positive	n/d	Both positive	Both positive	n/d	-	-
SP420-BAL	C2	Insoluable	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
T1-SP420-BAL	C2	Insoluable	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
T1-SP10(A)BAL	V3	21, 22	25600, 12800	Both negative	Both positive	Negative, positive	Negative, positive	Both positive	1800	-
T1-SP430-BAL	C2	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	
T1-SP440-BAL	C1	23, 24	25600, 12800	Both negative	Both negative	Positive, negative	Positive, negative	Both negative	32, -	-
T1-SP450-BAL	C1	25, 26	25600, >102400	Both negative	Both negative	Both positive	Both positive	n/d	-	-
SP-200	gp41	P1-3	51200, 102400, 102400	n/d	n/d	n/d	n/d	n/d	n/d	-
SP-201	gp41	P4-6	102400, 102400, 102400	n/d	n/d	n/d	n/d	n/d	n/d	-
SP-202	gp41	P7-9	102400, 51200, 12800	n/d	n/d	n/d	n/d	n/d	n/d	-
SP-203	gp41	P10, P12	102400, 51200	n/d	n/d	n/d	n/d	n/d	n/d	-
SP-204	gp41	P13, P14	1600, 25600	n/d	n/d	n/d	n/d	n/d	n/d	-

* HIV envelope peptide sequence in bold

TABLE 5

Group	Day 7	Day 14	Day 21	Day 28	Day 35
1ug CT used as adjuvant with					
50 ug T1SP10MN(A)	<64	8,192	65,536	65,536	524,288
50 ug T1SP10MN(A)	<64	1,024	32,768	32,768	262,144
50 ug T1SP10MN(A)	<64	<64	256	512	2,048
No adjuvant used with	<				
50 ug T1SP10MN(A)	<64	2,048	4,096	8,192	32,768

Serum anti-T1-SP10MN(A) IgG titers (reciprocal) after intranasal immunization with indicated amount T1-SP10MN(A) with or without 1 ug cholera toxin (CT) as a mucosal adjuvant. C57BL/6 mice were immunized on day 0,7,14, and 28.

TABLE 6

Mouse Strain	Serum Anti-T1SP10MN(A) IgG End-point Titers (Reciprocal)
BALB/c	262,144
C57BL/6	131,072

Peak serum anti-T1-SP10MN(A) IgG titers after subcutaneous immunization with 50 ug T1-SP10MN(A) in CFA. Titers expressed as reciprocal of highest dilution giving a positive response.

DAMD17-94-4467 PROGRESS REPORT - YEAR 2: STRUCTURAL AND FUNCTIONAL STUDIES OF EXPERIMENTAL HIV SYNTHETIC PEPTIDE IMMNOGENS

Technical Aim 2: Structural Studies Using NMR

Principle Investigator: Leonard D. Spicer, Ph.D.
Co-Investigators: Robert de Lorimier, Ph.D. and Hai Vu, Ph.D.

INTRODUCTION

Technical Aim 2 involves the use of highfield NMR spectroscopy to study the immunogenic peptides targeted in this grant. The strategy is to determine the structural requirements for the design of immunogens capable of inducing broadly cross-reactive anti-HIV neutralizing antibodies and anti-HIV T cell responses against HIV isolates grown either in T-cell lines or PBMC. These studies include both peptides already being characterized for immunogenic properties and cross reactivity and new peptide constructs based on iterative evaluation of structure/function and design. We have completed our first cycle as described in the proposal by characterizing the initial four peptides from the T1SP10 RF(A), CAN0(A), MN(A), and EV91(A) sequences, and have correlated those results with immunogenic data. Based on those findings, three new peptides were designed to enhance reactivity. These have been synthesized and characterized immunologically and by NMR. Peptides based on gp41 epitope sequences were also redesigned to enhance specific structural characteristics and were tested immunologically. We are on schedule for this project.

BODY

A. The four T1SP10 peptides CAN0(A), EV91(A), MN(A), and RF(A) have been completely assigned with respect to their proton resonances using a combination of 2-dimensional NMR experiments including COSY, TOCSY and NOESY. Their conformational elements have also been characterized from NOE connectivity maps and coupling constants. The RF(A) and CAN0(A) studies have been published (de Lorimier et al., 1994; Vu et al., 1996), and a manuscript describing EV91(A), MN(A) and a comparison of all four peptides, will soon be submitted for publication (Vu et al., in preparation). Results are summarized below.

Short- and medium-range NOEs were observed in all four peptides which indicated that the peptide conformations are not completely random; i.e. certain conformations are preferred. The T1 segment, which is identical in sequence in all four peptides, shows evidence of a tendency to form helical conformations, especially in the region from residue Val10 to Tyr15. On the other hand the SP10 segments, which differ in sequence, show different conformations in each peptide. The GPGX sequence at the tip of the V3 loop forms a Type II b-turn in peptides CAN0(A) and MN(A), but a Type I b-turn in RF(A) and EV91(A). The RPXX sequence toward the C-terminal end of the SP10 sequences forms a Type I b-turn in RF(A) and MN(A), a Type II b-turn in EV91(A), and is in an extended conformation in CAN0(A). Residues just preceding the GPGX turn in RF(A) and CAN0(A) are extended, but in EV91(A) form another turn. And residues immediately following the GPGX turn are helical in EV91(A) and MN(A), but extended in RF(A) and CAN0(A).

To further evaluate the organization of the peptides, particulary with respect to potentially important side chain orientations, the above-listed conformational elements suggested by NMR data were used to create models of the peptides. The initial structures were optimized to avoid steric hindrance of the side chains and their surface potential energies were minimized before molecular dynamics calculations were performed. The refined models were compared with the published x-ray crystal structure of the antibody-bound MN V3 loop (Rini et al., 1993). An exposed patch of apolar side chains immediately before the b turn of the bound MN peptide was reported in the crystal study and shown to interact extensively with residues in the antigen-binding

pocket. This hydrophobic patch was observed in our modeling of CAN0(A), but not in EV91(A) or RF(A). The RF(A) model showed a disruption in the middle of this apolar surface by the protrusion of a charged side chain from a lysine residue. The corresponding hydrophobic region in the EV91(A) model was seen to be twisted and appeared more compact than its extended counterparts in CAN0(A) and MN(A). The structural similarity of CAN0(A) and MN(A), and their differences with EV91(A) and RF(A), parallels their immunogenicities in that CAN0(A) and MN(A) can induce more broadly cross-reactive antibodies (Haynes et al, 1995). We therefore suggest that the flat apolar surface observed in the x-ray structure of MN(A) and in our CAN0(A) model may act as a key conformational motif in inducing antibodies that are cross-reactive. Peptides EV91(A) and RF(A) lack this motif, which may explain why they induce only type-specific antibodies.

B. Significant progress has been made in designing and characterizing peptide variants to enhance immunogenicity toward HIV. One such study, based on designed variants of the T1SP10RF(A) peptide, is complete (de Lorimier et al., in preparation). As noted above, the T1 sequence exhibits helical conformations, but it has sequence homology to a region of IgA which is b strand (de Lorimier et al., 1994). We previously hypothesized that this potential disparity in conformation might explain the absence of B-cell immunogenicity in the T1 portion of T1SP10 peptides, even though the T1 portion of native gp120 is antigenic. Thus we proposed to alter the sequence of T1 by single amino-acid substitutions that might reduce its helical tendency in solution and thus render it immunogenic for antibodies which recognize the T1 epitope of gp120. Three variants of T1SP10RF(A) were designed and synthesized: Glu^{9}->Gly (E9G), Glu^{9}->Val (E9V), and Lys^{12}->Glu (K12E). In order to prevent peptide dimerization all three variants lacked the single cysteine which was present in the original RF(A) peptide at the junction between T1 and SP10. Hence a control peptide (T1SP10RF(A)C-) was produced which had the native T1 sequence but no Cys residue. All four peptides (control and three variants) were studied for immunogenicity and conformation.

In terms of B-cell immunogenicity, none of the T1 sequence variants elicited detectable antibodies which bind to the T1 epitope of gp120. One possible explanation for this finding is that the sequence alterations removed determinants important for recognition of native T1. Another is that more stringent constraints are required for T1 to be immunogenic for a B-cell response. In terms of T-cell immunogenicity, measured by antibody titer and splenocyte proliferation, the T1 variants induced very different responses. Variant E9V was a much more potent inducer of T-helper responses than the original T1 sequence, while K12E was significantly less immunogenic. Since T1 is an important MHC class II epitope it may be expected that alteration of its sequence would affect function. In fact these results corroborate those of another study which examined T-cell immunogenicity in sequence variants of T1 (Boehncke et al., 1993).

To determine whether the designed conformations were exhibited by these peptides we studied them by NMR using the same methods previously employed for analyzing T1SP10 peptides. First resonance assignments were determined, then analysis of NOEs showed the position and relative population of nonrandom conformations in the peptide sequence. Peptide K12E yielded spectra with poor signal-to-noise, and the high viscosity of its solution suggested that it aggregates. To obtain usable spectra of K12E trifluoroethanol was added to 20%, and the temperature was raised to 45 C. Analysis of T1SP10RF(A)C- showed that its conformation was nearly identical to T1SP10RF(A), including a helical propensity in T1. Variant E9V retained this helical character, while variant E9G lacked any NOEs indicative of a helical tendency. Variant K12E showed helical conformations in T1, but this may have been due to the presence of trifluoroethanol in the solution, which is known to induce helical conformations. Thus in at least one case, E9G, the designed solution conformation was attained.

C. The so-called Katinger epitope of gp41, which includes the linear sequence ELDKWAS, is a target for design of immunogenic peptides. The ELDKWAS sequence is predicted to be helical

in gp41, so in collaboration with Dr. T. Palker, we devised sequence variants which might favor helical conformations of this peptide in solution. One such alteration substitutes the serine with amino-isobutyric acid (Aib), a non-natural analog of alanine that promotes helix formation. An ELDKWAS-based peptide bearing this substitution was synthesized and found to be recognized better by HIV patient serum than non-derivitized peptide. Studies will be conducted to compare solution conformations of Aib-substituted and non-substituted ELDKWAS peptides.

CONCLUSIONS

The objectives of Technical Aim 2 through Year 2 have been achieved. The original four T1SP10 peptides have been characterized by NMR with respect to solution conformations. Molecular modeling of these peptides based on NMR results suggests a correlation between structural features and immunogenic properties. To test one hypothesis derived from these studies, a set of peptides based on T1 sequence variants of T1SP10RF(A) was synthesized and characterized for solution conformations and immunogenicity. The results showed that conformational features can be designed with some success. In terms of design of function, it appears that in order to confer B-cell immunogenicity to T1 requires more that single amino-acid substitutions, but that T-cell immunogenicity can be profoundly affected by such substitutions. Design and characterization of conformations in peptide immunogens based on gp41 have begun.

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DAMD18-94-4467 PROGRESS REPORT - YEAR 2: STRUCTURAL AND FUNCTIONAL STUDIES OF EXPERIMENTAL HIV SYNTHETIC PEPTIDE IMMUNOGENS

Technical Aim 3: To measure the specificity and binding affinity of peptide-HLA interactions, the stability of these complexes and their immunogenicity.

Investigator: Donna D. Kostyu, Ph.D.

INTRODUCTION

More than 400 HLA alleles have now been identified, and for vaccination protocols involving synthetic immunogens, these highly polymorphic molecules may play a significant role. The failure of a peptide to bind to an HLA molecule in an appropriate manner may lead to poor Tc and B cell responses. Peptides may induce tolerance rather than immunity and may present an epitope that is not a naturally processed form. Even though peptide binding motifs and anchor residues have been identified for multiple HLA alleles, there are still numerous underlying complexities at the HLA-peptide level that obscure the simple prediction of suitable peptides for vaccines.

The aim of this project was therefore to establish a simple, specific, predictive peptide-HLA binding assay and to correlate results with functional assays measuring T cell recognition of peptide-HLA complexes. This includes defining the restricting alleles for the multivalent HIV peptide vaccines currently in clinical trials and any modified HIV peptides synthesized as part of this project, the route of presentation, and manner of T cell recognition (CD4 or CD8). Such a systematic analysis should facilitate the development of experimental peptide immunogens and help assess the safety, efficacy and immunogenicity of peptide vaccines prior to clinical trials.

BODY

A. PEPTIDE-HLA INTERACTIONS DEFINED BY BINDING

Binding Assay. Class I-peptide interactions have been defined in a peptide and b_2-microglobulin (b2m) reconstitution assay, modifying the procedure originally reported by Zeh et al (1). HLA molecules on C1R cells transfected with a single class I allele or reference T and B cell lines expressing multiple alleles are denatured with a pH 3.1 buffer, then reconstituted in the presence of 50-100 uM peptide and 250 nM b2m. Loss and gain of conformation is monitored with a PE-labelled w6/32 monoclonal antibody for transfected cells and allele-specific, biotin-labelled monoclonal antibodies and an avidin-FITC secondary reagent for other cells.

The 39mer peptides T1SP10MN(A), T1SP10EV91(A), T1SP10RF(A), T1SP10CANOA(A), the substituted T1SP10RF(A) peptides E9G, E9V and K12E, and the T1, SP50 and AP10 subregion peptides have been tested for their ability to reconstitute HLA-A1, A2, A3, A9 (24) and B5 (51), B7, B8, B12 (44), and B35 molecules. These alleles represent 67% and 50% of the serologically defined HLA-A and HLA-B alleles respectively in Caucasians .

Precise, accurate, and reproducible binding was difficult to achieve under the experimental conditions reported by Zeh and coworkers and we have spent considerable time and effort in achieving these goals. A representative binding assay is shown in Figure 1, where the increase in mean fluorescent channel or intensity (MFI) in pH treated cells incubated with b2m and peptide compared to pH treated cells incubated with b2m alone is used as an indication of peptide binding. This ratio (MFI of cells + peptide + b2m/ MFI of cells + b2m) allows comparison of results between assays. A theoretical ratio of 1.0 indicates no binding; ratios $\geq$ 1.19 (mean + 3 SDs) are considered positive. Controls include untreated

cells exposed to an isotype control antibody (negative control), untreated cells exposed to the conformation-dependent antibody (positive control), pH treated cells alone to demonstrate effectiveness of pH stripping (the MFI of pH treated cells is routinely <10% of the MFI of untreated cells), and pH treated cells incubated with b2m alone to control for occasionally weak b2m-induced conformational changes. Binding assays are performed at 37C in the presence of 0.02 mM monensin to block class I transport through the golgi and prevent new expression of class I. Mage3 and flu matrix peptides which bind to HLA-A1 and A2, respectively, have been synthesized and included for specificity controls.

Based on these procedures, we have been able to identify numerous potential CTL epitopes in the subregion peptides T1, SP50 and AP10 and in the longer 39mer peptides as described below. Binding results are shown in Table 1, where the ratios reflect the means of from 1 to 5 individual assays, and are summarized in Table 2.

T1 peptide. The T1 region contains a potent Th epitope in rats and mice. We find numerous potential CTL epitopes in this peptide as well, noting binding of T1 to HLA-A1, A2, A24, B8, B12 and B51. We can identify two potential HLA-A1 binding sequences based on reported HLA-A1 motifs, i.e. the nonamer WQEVGKAMY and the longer 11 residue NMWQEVGKAMY as shown in Table 2. Underlined residues indicate predicted anchor residues (see ref 2-8). There are five potential sequences in T1 that may be recognized by HLA-A2. There is a potential sixth HLA-A2 recognition site (RIHIGPGRA) overlapping the SP50-AP10 region. As testing has been limited to previously synthesized subregion peptides, we are unable to test this sequence directly. The T1 peptide binds to HLA-A24, perhaps using the M at position 6 as an amino terminal anchor. However, none of the residues reported for carboxy terminal anchors (FWILV) are present (5). The peptide also binds to HLA-B8, possibly using the internal sequence QEVGKAMYA where the K at P5 serves as one anchor and the A as the carboxy terminal anchor. T1 binding to HLA-B12 (B*4402) may be accounted for by the eight residue sequence QEVGKAMY which matches the predicted motif of E at P2 and Y at the carboxy terminal end. B12 has been included in a supermotif of alleles (B37, B41, B44, B45, B47, B49, B50, B60 (40012) and B61 (4006)) all of which bind peptides containing an acidic D or E at P2 and an aromatic or hydrophobic residue at the C terminal end (4). This would suggest that T1 may be recognized by a variety of other HLA-B alleles. For HLA-B51 (*5101), the T1 peptide binds but the usual amino and carboxy terminal anchors (APG at P2 and FI at the carboxy terminal end) are absent.

Although T1 is predicted to contain two potential HLA-A3 binding sites, an 8 mer NMWQEVGK , and an 11 mer NMWQEVGKAMY , little or no binding has been observed in repetitive assays. It may be that the peptide isn't processed correctly, the 8 and 11 residue lengths are inappropriate, or that other residues in the peptide are deleterious. The occurrence of a D, E or P at position 1 and a D or E at position 3 have been reported to be deleterious for HLA-A3 binding peptides (3). Sidney and coworkers have described an HLA-A3 supermotif of alleles that includes HLA-A*0301, 1101, 3101, 3301, 3401, 6601, 6801, and 7401, based on their ability to bind similar peptides and the occurrence of the same amino acids in the B and F pockets of the HLA-A molecule (4). Whether any of these other alleles will bind the T1 peptide is not yet known.

It should be noted that the binding of T1 to several HLA alleles is remarkably strong. It may be that the T1 peptide is processed by proteases present during the binding assay and multiple binding peptides are generated. It is also possible that the T1 peptide adopts a unique conformation that greatly facilitates HLA class I binding.

SP50 peptides. Six SP50 peptides were tested, several binding to HLA-A1, A2 and B7. Binding of SP50MN to HLA-A1 was very weak; the usual anchor residues for HLA-A1 are not present. Four of the SP50 peptides (MN, RF, MAL and ELI) bound weakly and only sporadically to HLA-A2. The reason for the inconsistent binding is not known; an appropriate carboxy terminal anchor (I or T) is present but no reported amino terminal anchor (LMIVAT). All six SP50 peptides bound to HLA-B7, correlating with the presence of reported anchors of a P at P2 and an L or I at the carboxy terminal end.

Safrit et al. (9) identified HLA-B7 restricted CTL clones in two patients shortly after seroconversion; the epitope mapped to the sequence RPNNNTRKSI, which is equivalent to the SP50 region here.

We would have predicted that the SP50 peptides would bind to HLA-B51 (B*5101), as reported amino and carboxy terminal anchors are present. No binding was observed.

AP10 peptides. Five AP10 peptides were tested, some binding to HLA-A2, HLA-A24 and HLA-B7. AP10MN(A) and AP10CANOB(A) bound weakly to HLA-A2, while AP10RF(A) and AP10EV91(A) gave borderline values. All have appropriate anchors if a long peptide is considered. Chen et al. (10) report that the carboxy terminal end may not be as critical for 12 mers and that other residues close to the carboxy-terminal end can serve as auxiliary anchors. Weak binding may also be due to the charged R and K residues at P7 which are reported to be deleterious. AP10MN(A) and AP10CANOA(A) bound to HLA-A24; AP10RF(A) binding was marginal. All of the AP10 peptides have an F or I at P9 to serve as a carboxy terminal anchor. The M at P3 in AP10CANOA(A) might serve as an amino terminal anchor generating an 8 residue peptide. For MN and RF, no obvious amino terminal anchor is identifiable.

We find it odd that the AP10 region contained predicted motifs for several HLA alleles, but no binding was observed, i.e. AP10CANOA(A) and HLA-A1 (IHMGPGKAFYTTG), AP10CANOB(A) and HLA-A3 (HMGPGKAF), AP10RF(A) and AP10EV91(A) and HLA-B8 (ITKGPGRVIYATG AND IPIGPGRAFIATS) and AP10EV91(A) and HLA-B51 (IPIGPGRAFIATS).

The 39mer peptides. Since these peptides contain the T1, SP50 and AP10 subregions, they should theoretically be recognized by HLA-A1, A2, A24, B7, B8, B12, and B51 molecules. We initially thought they would be too long to be active in a peptide-binding assay. However, T1SP10MN(A) binds well to HLA-A1, A2, B12 and B51 (but not to A24 or B8). In contrast, T1SP10RF(A), T1SP10EV91(A) and T1SP10CANOA(A) are routinely negatively at 50 uM in 30-90 minute binding assays at 37C. We have been perplexed as to why one 39-mer peptide would be active and the other three inactive. A protease present in the binding assay may cleave the 39mer into shorter, active pieces. Or the RF, EV91 and CANOA peptides may exist in a formation that makes them not susceptible to proteolytic cleavage under our conditions; sequence differences in the V3 region would have to affect the efficiency of processing. Alternatively, the 39mer MN peptide may contain trace amounts of short, highly reactive peptides that are in fact responsibile for binding. Initial mass spectroscopy of the T1SP10MN(A) peptide and a new analysis of the diluted peptide we use in the binding assay showed no evidence of small peptide contaminants. Recent experiments with the T1SP10RF(A) mutants E9G, E9V and K12E indicate that E9G and E9V are also active in our binding assay. The K12E peptide fails to bind at 50-100 uM, but binds weakly at 200 uM.

Time, temperature, and concentration. Peptides bind to empty class I molecules within 15 minutes at 37C, reaching maximal levels between 30 and 45 minutes (Figure 1). This is true of the 39mer peptide T1SP10MN(A) and the shorter subregion peptides. HLA-B7 molecules reconstituted with the SP50MN peptide are stable for at least 3 hours at 37C. Binding assays performed at 22C for 2-4 hours do not always give comparable binding, perhaps reflecting different class I loading pathways or affinities. Peptides are active at low concentrations (<25 uM) (Table 3).

Protease-dependent binding. Class I molecules have been shown to occasionally bind long peptides, e.g. $\geq$12 residues (7,11,12). However, since most of the potential anchor residues for class I binding of the 16 mer T1, the 13 mer AP10, and the 39 mer T1SP10MN(A) peptides are internal, it seems possible that the binding we observe is dependent on proteases present during the binding assay. Assays are performed in Iscove's media without protease inhibitors. Proteases may be normal membrane constitutents or they may be activated as a result of pH treatment. It is also possible that small numbers of cells damaged during pH treatment release intracellular proteases. To determine how dependent the binding assay is upon proteases, pH treated cells have been pre-treated with protease inhibitors and protease inhibitors have been present during the peptide binding assay. Some inhibition of peptide-HLA interactions has been noted and this work is currently in progress.

Since peptides are reported to have a short half life in serum due to proteases (13), we were interested in the stability of these peptides in plasma. Peptides were pre-incubated with an equal amount of heparinized plasma for up to 60 minutes at 37C, then added to pH treated cells in a normal binding assay. We found little or no evidence of peptide degradation during that time, suggesting that plasma itself would not prevent the rapid binding of peptides to empty class I molecules at the cell surface in vivo.

Localization of binding to the cell surface. There are several reasons why we believe our binding assay is measuring cell surface peptide-HLA interactions and not intracellular interactions. Binding is detectable within 15 minutes at 37C, assays are performed in the presence of monensin to block movement of HLA class I through the golgi, and binding is dependent on the addition of exogenous b2m. We have also fixed cells in 1% formalin after pH treatment but prior to the addition of peptide to prevent translocation across the cell membrane. Fixation had no effect on the binding of SP50MN/RF peptides to HLA-B7 (binding ratios for fixed and nonfixed cells were 1.55 and 1.60 respectively) or of the T1 peptide to HLA-A2 (ratios for fixed and nonfixed cells were 3.7 and 3.5 respectively).

B. PEPTIDE-HLA INTERACTIONS DEFINED BY FUNCTIONAL RESPONSES

For peptide vaccines, there are several pathways in which peptides may meet HLA class I molecules. One involves extracellular processing and loading onto empty class I molecules on the cell surface. This pathway might operate in vivo and our binding assay may be a suitable model for this. The classical pathway depends upon introduction of the peptide into the cytoplasm, processing by a proteasome, translocation into the endoplasmic reticulum by TAP molecules, competition with endogenous peptides, and stable transit through the Golgi to the cell surface. Alternate pathways have been proposed that involve receptor recycling or golgi-independent cytoplasmic pathways. It is therefore possible that the 39mer peptides unreactive in our binding assay are suitably immunogenic when introduced into a cytoplasmic class I pathway, especially when administered with adjuvant.

To identify functional responses to the 39 mer and subregion peptides, we initially set up traditional cytotoxic and proliferative T cell assays. The results were uninspiring, poorly reproducible, and days away from the initiating events. We consequently have begun to study immediate responses following peptide exposure. Peripheral blood lymphocytes and monocytes have been cocultured with peptides for 8 to 48 hours, and activation assessed by the expression of CD69. Five parameter immunofluorescence compares untreated vs. peptide treated cells, using a FL-labelled CD69, a Cy-labelled CD4 or CD8, and PE- labelled CD16 monoclonal antibodies, while gating on large refractive cells. Initial work has demonstrated the feasibility of this method in identifying small numbers of peptide-reactive cells and these experiments will continue into the next year.

C. HIGH RESOLUTION TYPING OF CLASS I ALLELES

We have developed and tested 15 primer sets and >60 probes for exons 2 and 3 of HLA-A and exon 2 of HLA-B. This allows low resolution of HLA-A and HLA-B in general and high resolution typing for many alleles. Analysis of exon 3 of HLA-B is in progress.

DISCUSSION

The peptide binding assay has proven adaptable. C1R cells transfected with a single allele can be used for specific binding analyses and this requires only a fluorescent-labelled framework monoclonal antibody. Unfortunately, only a few transfected lines are available and the number of HLA class I alleles that may be desireable to study grows readily. A variation of the binding assay uses biotin-labelled monoclonal antibodies and a secondary avidin-FITC reagent to identify peptide interactions with a single HLA molecule on cells expressing multiple alleles. This has proven to be sensitive and reproducible and allows us to test a wide variety of cell lines under more physiologic conditions. It will enable us to identify allele-specific binding on cell lines of any ethnic group; analysis is limited only by

the availability of class I monoclonal antibodies . Another adaptation uses a peptide that is directly labelled with biotin, followed by avidin-FITC. We synthesized a prototype peptide (APRK(biotin)LVYLL) based on the HLA-B7 binding peptide identified by Sette and coworkers which binds to HLA-B7 with an affinity of <5 nM (6). We find this peptide binds HLA-B7, B8, B51, and other alleles sharing similar pockets. While the biotin-labelled peptide is easy to detect and the addition of a biotin at an internal residue does not interfere, it is not clear how advantageous it is in terms of defining specificity.

As others have noted, it is difficult to predict peptide-HLA interactions based only on sequence information and motifs. Although we can correlate binding patterns in most cases with sequences containing appropriate anchors, there are some peptides that bind in the absence of expected anchors and other peptides that failed to bind where expected. It would be possible to identify the actual peptide binding sequences in the T1 and AP10 regions by synthesizing series of truncated peptides to define minimal length, substituting suspected anchor residues or suspected deleterious residues, or sequencing stripped peptides from pH reconstituted cells. However, these are lengthy approaches and we have chosen instead to concentrate on functional studies at this point.

We believe that the class I-restricted sequences present in the T1, SP50 and AP10 regions should lead to functional Tc responses if presented to the immune system in an appropriate manner. The binding we observe with these peptides occurs at similar times, temperature, and concentrations as the control flu and Mage3 peptides which are known to bind with high affinity and which can induce CTLs. HLA-B7 and HLA-A2 restricted Tc epitopes have been described by others in the C4/V3 regions (7,14-16). The fact that we find fast binding of short peptides to empty class I molecules on the surface of B and T cell lines at 37C and the minimal effect of 50% plasma on the half-life of peptides suggests that peptides at least up to 16 amino acids in length may be in an immunogenic form already and may bind to APC at the cell surface. What happens to longer peptides is not clear. Ossendorf (17) found processing of long peptides into an immunogenic form depended on recognition of enzymatic cleavage sites in flanking residues. Safrit et al. (9) and Koenig et al. (18) found >25 mer peptides unable to sensitize BCLs for lysis even when the minimum CTL epitope was contained, regardless of time, temperature and concentration. And of the 40mer RF derivative peptides, E9V and E9G induce B and T cell responses but K12E is functionally inert. One hypothesis may be that K12E has lost a Th epitope. An alternative hypothesis is that the peptide is not processed correctly because of conformation. Thus, an additional goal in the coming year is to compare binding and functional responses in the context of peptide length.

CONCLUSIONS

We have established a suitable and adaptable binding assay, identified prospective CTL epitopes in the T1, SP50 and AP10 subregions, and initiated functional assays to identify CD4 and CD8 responses to the 39-mer peptides and subregion peptides. The project is progressing along predicted lines and there are no changes anticipated at this point.

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Table 1

Binding of the T1SP10 Peptides to Common HLA Alleles

Peptides:	HLA-A1	HLA-A2	HLA-A3	HLA-A24	HLA-B7	HLA-B8	HLA-B12	HLA-B35	HLA-B51
T1SP10MN(A)	2.49	1.77	1.18	1.02	0.77	0.86	2.59	nt	1.54
T1SP10RF(A)	0.98	1.09	1.13	0.92	0.71	0.87	1.04	nt	1.15
T1SP10EV91(A)	1.03	1.15	1.07	0.95	0.98	0.94	0.96	nt	1.00
T1SP10CANOA(A)	0.90	1.12	1.00	0.94	0.81	0.95	1.05	nt	1.08
T1SP10RF(A) E9G	*1.78	1.16							1.20
T1SP10RF(A) E9V	1.33	1.58							1.04
T1SP10RF(A) K12E	1.03	0.91							1.04
T1	5.43	24.54	1.15	2.05	1.12	2.61	5.10	nt	6.31
SP50MN	1.31	1.31	1.01	1.12	1.55	1.01	1.02	nt	0.89
SP50RF	0.90	1.31	0.98	1.03	1.67	1.05	1.07	nt	1.01
SP50EV91	1.07	1.04	0.98	0.98	1.66	1.04	1.03	nt	0.99
SP50CANOA	0.90	1.00	0.90	1.07	1.19	1.02	1.09	nt	0.95
SP50MAL	1.03	1.20	0.98	1.00	1.76	0.96	0.99	nt	1.08
SP50ELI	1.05	1.30	0.98	0.98	1.73	1.01	1.03	nt	1.00
AP10MN	0.90	1.24	1.00	2.14	1.03	0.95	0.95	nt	0.90
AP10RF	0.95	1.13	0.99	1.16	1.07	0.94	0.97	nt	0.95
AP10EV91	0.97	1.16	0.96	0.96	1.36	1.04	1.04	nt	0.85
AP10CANOA	1.02	1.03	0.98	2.00	1.00	0.92	1.05	nt	0.83
AP10CANOB	1.07	1.19	0.96	0.80	0.99	0.92	1.08	nt	1.00
FLU	0.99	2.40	0.79	nt	nt	0.90	0.91	nt	0.92
MAGE3	2.60	1.24	1.00	0.86	1.07	nt	0.96	nt	nt

Ratios are MFI (cells + peptide + b2m) / MFI (cells + b2m only). Binding was detected with allele-specific monoclonal antibodies for HLA-A1, A2, A3 , A24, B8, B12 and B51 on pH-treated human B cell lines. Binding to HLA-B7 was detected using the w6/32 monoclonal antibody and the C1R B cell line transfected with either B7. Each is a representative experiment of from one to seven binding assays. Ratios > 1.19 are considered positive binding.

*Another HLA-A1+ cell failed to bind E9G or E9V and may be a different HLA-A1 subtype.

Table 2

Prediction of Tc Epitopes in the C4 and V3 Regions Based on Binding Assays and Published Motifs

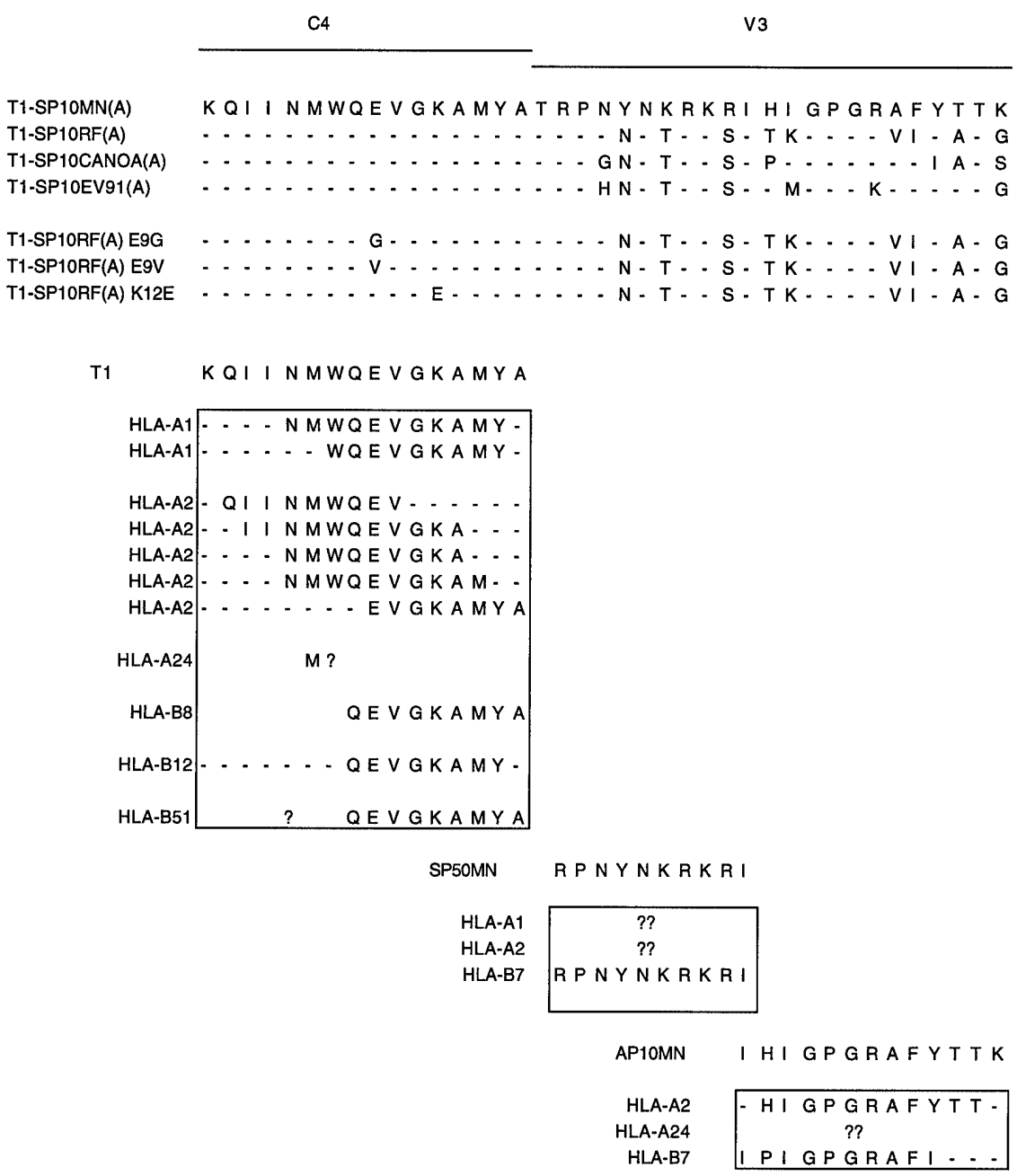


Table 3

Peptide Binding is Concentration Dependent

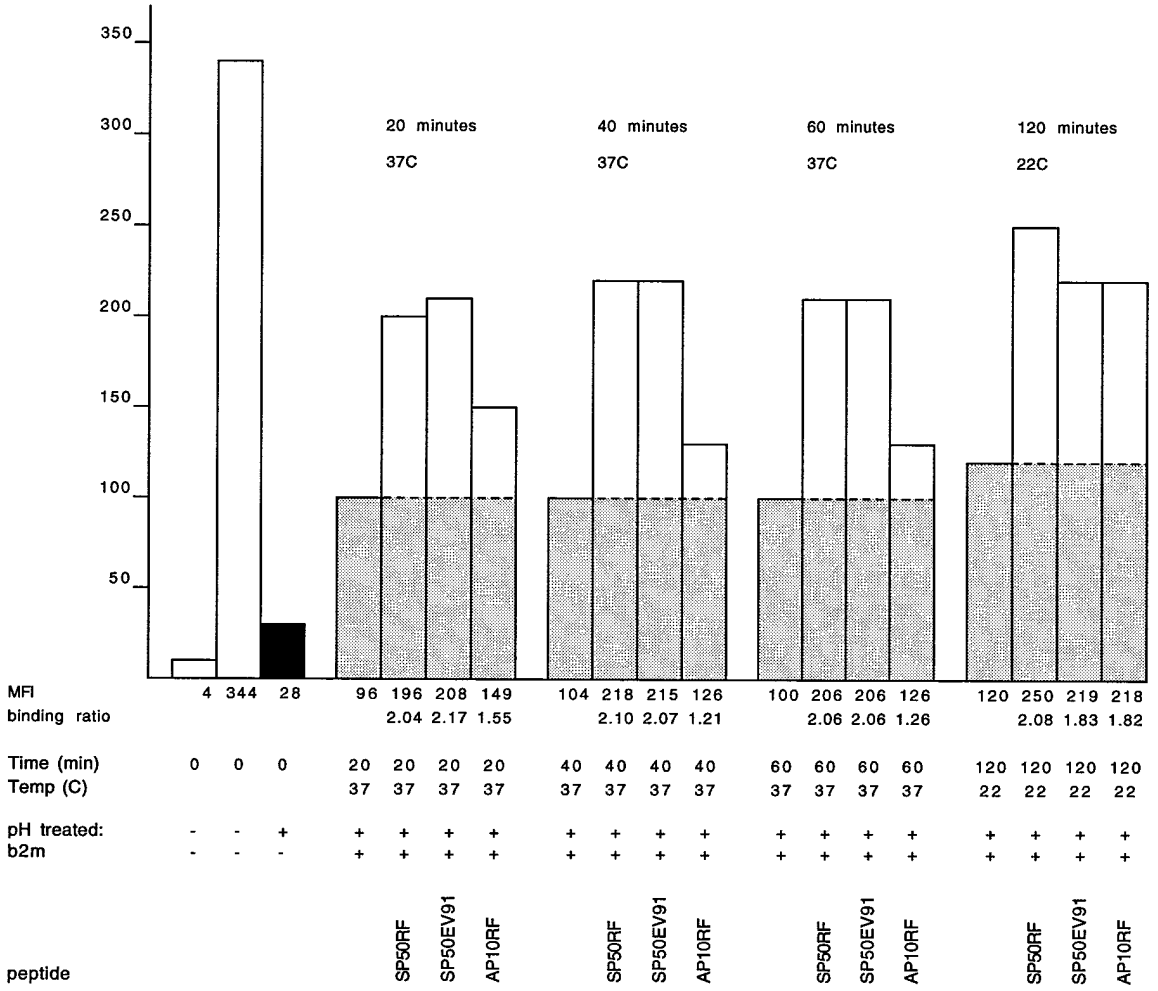
Peptides and the allele they bind to:

[uM peptide]	MAGE3 HLA-A1	FLU HLA-A2	T1 HLA-A1	T1 HLA-A2	T1SP10 MN(A) HLA-A1	T1SP10 CANOA(A) HLA-A1
1.5	0.97	1.18		1.09		
3	**1.24**	**1.24**		1.09		
6	**1.71**	**1.35**		1.05		
12.5	**1.59**	**1.77**		1.11		
25	**1.86**	**2.01**	**1.80**	**1.34**	1.08	1.05
50		**2.15**	**3.96**	**1.52**	**1.56**	0.91
100	**1.90**	**2.45**	**21.18**	**3.63**	**2.05**	**1.23**
150		**2.65**	**49.12**			**1.36**
200	**1.90**	**2.26**	**102.54**	**7.85**	**3.44**	**1.71**

Numbers represent binding ratios (MFI cells + b2m/MFI cells + b2m + peptide). Values > 1.19 (bold) represent positive binding

Figure 1

Binding of peptides to HLA-B7 occurs in <20 minutes



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SCIENCE

Toward an Understanding of the Correlates of Protective Immunity to HIV Infection

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Considerable progress has been made recently in understanding the genetic, immunologic, and virologic factors in human immunodeficiency virus (HIV)–infected individuals who either rapidly progress or do not progress to acquired immunodeficiency syndrome (AIDS). In addition, detection of HIV-specific immune responses in HIV-negative individuals who have been exposed to the virus multiple times suggests that natural immune responses to HIV may be protective in rare individuals. Understanding the correlates of protective immunity to HIV infection is critical to efforts to develop preventive HIV vaccines as well as to determine the feasibility of treating HIV infection by boosting immunity to HIV.

A spectrum of clinical courses can occur after HIV infection. Approximately 10% of HIV-infected subjects progress to AIDS within the first 2 to 3 years of HIV infection (rapid progressors) (*1*, *2*); approximately 5 to 10% of HIV-infected subjects are clinically asymptomatic after 7 to 10 years and have stable peripheral blood $CD4^+$ T cell levels (nonprogressors) (*1*, *2*); and the remaining HIV-infected subjects are projected to develop AIDS within a median time of approximately 10 years from initial infection (typical progressors). Data from the Multicenter AIDS Cohort Study suggest that 20 years after infection, 10 to 17% of HIV-infected individuals will be AIDS-free (*1*, *2*).

In this article, we consider recent progress in understanding immunologic and virologic characteristics of HIV-infected typical progressors, rapid progressors, and nonprogressors; summarize data on host genetic factors that may determine the effectiveness of immune responses to HIV; and summarize goals of future research.

Typical Progressors

In typical progressors, within weeks of HIV infection, viremia falls coincident with the induction of anti-HIV cellular and humoral immune responses (*3*). The fall in viremia correlates best with the appearance in peripheral blood of anti-HIV major histocompatibility complex (MHC) class I–restricted $CD8^+$ cytotoxic T cells (CTLs) (*3*).

During acute HIV infection there is oligoclonal expansion of V_β immunoglobulin families, predominantly restricted to $CD8^+$ T lymphocytes; within this population are contained HIV-specific CTLs (*4*). Mobilization of a restricted T cell receptor–for–antigen repertoire may be ultimately associated with a less effective immune response, thus facilitating persistence of HIV (*4*, *5*).

$CD8^+$ T cells are thought to be important in the immune response to HIV during the latent phase of HIV infection for the elimination of productively infected cells and for control of the viral load (*6*). However, HIV-specific $CD8^+$ CTLs may also be involved in the immunopathogenesis of HIV infection; they may contribute to the depletion of antigen-presenting cells either through a direct mechanism (that is, killing of the virus-expressing antigen-presenting cells) or indirectly through tissue damage after the release from CTLs of certain cytokines such as tumor necrosis factor α/β and interferon γ during the process of cytolysis (*5*, *7–10*). Nowak *et al.* have hypothesized that patients whose immune systems recognize fewer immunodominant HIV epitopes have a more stable and effective immune response to HIV than those whose CTL responses are against multiple, less dominant epitopes (*11*).

In addition to CTLs, neutralizing antibodies may be a component of the initial control of HIV replication (*12*, *13*). However, as HIV variants emerge over time, new variants frequently are not neutralized by autologous sera, and in some cases, antibodies against newly emerging HIV variants may enhance HIV replication in vitro (*12*, *13*), although the significance in vivo of enhancing antibodies is controversial (*13*). Heath *et al.* have reported that HIV virions coated with neutralizing antibody and attached to tonsillar follicular dendritic cells were still infectious for $CD4^+$ T cells (*14*). This study raised the important question of whether neutralizing antibodies can prevent dendritic cell–associated HIV infectivity in vivo. A randomized trial of passive immunotherapy of HIV-infected patients suggested that the administration of heat-inactivated plasma from HIV-infected individuals every 2 weeks for 1 year could slow the progression to AIDS in the recipients (*15*). Thus, antibodies appear to be involved in protective immunity against the progression of HIV infection, although the specificities of anti-HIV neutralizing antibodies that might be protective in patients remain unresolved.

Progression to AIDS is associated with generalized activation of the immune system, manifested by elevated serum concentrations of neopterin, soluble interleukin-2 receptor, soluble CD8, and β_2-microglobulin, and with activation of a large proportion of $CD8^+$ T cells (*4*, *7*, *8*, *16*, *17*). HIV-infected cells, circulating virions, and viral particles trapped in the follicular dendritic cell network of lymph node and spleen maintain chronic stimulation of the immune system.

Several components of generalized immune activation associated with HIV infection, such as stimulation of different T cell subsets and high levels of antibody production with specificities against a large range of epitopes of different HIV proteins, reflect the efforts of the immune system to control the replication and spread of the virus. However, as the disease progresses, both cell-mediated and humoral immune responses are severely impaired, resulting at least in part from the loss of the regulatory function of $CD4^+$ T lymphocytes and defective or increased production of either immunoregulatory or proinflammatory cytokines or both (*18*). Thus, as a consequence of the impaired regulation of both T and B cell functions, immune activation may ultimately become inappropriate and detrimental effects will predominate.

Rapid Progressors

Rapid progressors have a rapid decline in $CD4^+$ peripheral blood T cell levels, usually within 2 to 3 years after primary HIV infection (*1*, *2*). In general, rapid progressors are characterized by lower levels of antibodies to HIV proteins (*1*, *2*, *19*, *20*) and by low or absent antibodies that neutralize autologous HIV variants (*19*, *21*). High levels of antibodies that enhance the growth of autologous HIV isolates in vitro have been reported in rapid progressors (*22*). Levy *et al.* have found that noncytolytic $CD8^+$ T cell responses that suppress HIV replication are initially present and then decrease in rapid progressors (*23*). Two groups (*24*, *25*) have recently reported identification of

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$CD8^+$ T cell soluble factors that inhibit HIV replication (*23*). Baier *et al.* report that the anti-HIV $CD8^+$ T cell factor is IL-16 (*24*), whereas Cocchi and colleagues showed that $CD8^+$ cell–derived chemokines RANTES, MIP-1α, and MIP-1β synergized to suppress HIV replication (*25*). It will be critical to determine whether production of any of these cytokines is defective in rapid progressors. Others have found anti-HIV $CD8^+$ CTL activity in rapid progressors (*4, 26, 27*). Rinaldo *et al.* reported low levels of memory $CD8^+$ CTLs by precursor frequency analysis in rapid progressors compared with nonprogressors, although anti-HIV CTL effector cell activity was present in fresh peripheral blood cells from rapid progressors that was comparable with CTL activity in nonprogressors (*26*). Other characteristics of rapid progressors include elevated numbers of activated $CD8^+$ $CD38^+$ DR^+ T cells (*27*) and elevated serum markers of immune activation (*1, 2, 28*).

A uniform finding has been a high viral load in rapid progressors that does not fall dramatically after primary HIV infection (Table 1) (*28–32*). But an issue that has yet to be resolved is the amount of heterogeneity of the viral load. Both Delwart *et al.* and Wolinsky *et al.* found more homogeneity in HIV isolates in rapid progressors compared with typical progressors and nonprogressors, implying that the immune response to HIV is ineffective in rapid progressors and is incapable of driving HIV variant diversification (*33*). In contrast, Yu *et al.* found more viral heterogeneity in HIV isolates over time in rapid progressors (*34*).

Nonprogressors

Nonprogressors have high levels of $CD8^+$ $CD38^-$ CTLs (*23*), high peripheral blood $CD8^+$ MHC class I–restricted anti-HIV CTL levels that do not fall over time (*19, 35*), strong $CD8^+$ non-MHC–restricted HIV suppressor activity (*36, 37*), and high levels of antibodies to HIV (*19, 35, 38*). Several investigators have reported increased neutralizing antibodies to HIV or a wide breadth of cross-reactive neutralizing antibodies (or both) in nonprogressors (*19, 21, 35, 39*). Thus, neutralizing antibody levels may well be important for the control of HIV in nonprogressors, although the specificity of such salutary neutralizing antibodies is not known. Stable $CD4^+$ peripheral blood T cell levels are a hallmark of this group with low concentrations of serum and cellular markers of immune activation (*1,2, 27, 35*). Finally, in nonprogressors the structure and function of lymph node germinal centers are maintained and the follicular dendritic cells are preserved (*7, 8, 19*).

It has been proposed that cytolysis of HIV-infected antigen-presenting cells leads to early and severe immunosuppression and is crucial to AIDS pathogenesis (*40*). Zinkernagel and Hengartner have argued that in spite of in vitro cytolytic effects of HIV on susceptible T cell lines, HIV in vivo may not be a cytolytic virus, but rather induces profound $CD8^+$ T cell–dependent destruction of HIV-infected antigen-presenting cells and T cells (*41*). This hypothesis predicts that rare individuals will be able to eliminate HIV-infected cells with potent HIV-specific $CD8^+$ T cells (*41*). Similarly, if AIDS is primarily mediated through pathogenic CTL immune responses to HIV, then in this scenario an asymptomatic carrier state should exist in which there are high viral loads and essentially no anti-HIV $CD8^+$ T cell responses (*41*). The fact that recent data have demonstrated that the viral load is low and that anti-HIV CTL levels are generally high in nonprogressors argues against this latter hypothesis.

In contrast to rapid progressors, HIV variants in nonprogressors have been reported by some investigators to be diverse, suggesting that HIV variant heterogeneity may be a reflection of effective immune responses to HIV (*33*). Thus, in nonprogressors, it appears that immune responses are sufficently effective to maintain or at least markedly prolong the clinically latent phase of HIV infection.

There is also evidence that some nonprogressors are infected with constitutively less pathogenic or nonpathogenic HIV strains (Table 1). Thus, nonprogressors likely represent a heterogeneous group in whom host responses and the level of pathogenicity of the virus variably contribute to the state of nonprogression of HIV infection.

Multiply Exposed, HIV-Seronegative Individuals

Studies of individuals who have been exposed multiple times to HIV and are persistently HIV-seronegative have raised the possibility that, although these individuals show T cell responses to HIV proteins, a small percentage of them may be resistant to HIV, or may have been able to clear the infection without making antibodies to HIV (*37, 42, 43*).

Clues to the explanation of multiply exposed HIV-negative individuals comes from observations in rhesus macaques and chimpanzees of resistance to low doses of HIV or simian immunodeficiency virus (SIV) given intrarectally or intravaginally (*44*). Primate studies have suggested that there may be local cellular mucosal immune responses capable of protecting against low-dose mucosal HIV or SIV challenges (*44*). However, given the rapidity with which anti-HIV circulating CTLs arise in primary HIV infection of humans and yet do not usually prevent the development of AIDS (*3, 4*), and the fact that anti-HIV CTLs develop in vertically infected children without usually protecting against progression of HIV infection (*45*), complete clearance of HIV infection by HIV-specific CTLs (if it occurs at all) must be a rare event (*45*). The timing and regional location of the appearance of CTLs may be important. If CTLs develop after the initial dissemination of the virus, they may not be capable of curtailing the progression of disease, whereas if CTLs are present at the site of challenge, that is, the genital mucosa before virus dissemination as in the case of preimmunization, adequate control of infection may be achieved. Arguing against this latter point is the fact that SIVgag-immunized rhesus monkeys with no antibodies to SIV but with high levels of anti-SIVgag CTLs were not protected when challenged with intravenous SIVmnc in vivo (*46*). However, the intra-

Table 1. Characteristics of HIV in typical progressors, rapid progressors, and nonprogressors to AIDS.

Clinical course	Comments
Typical progressors	Monocytotopic homogeneous HIV strains are transmitted during primary infection (*58*).
	HIV isolates during the clinically latent stage are initially monocytotopic, nonsyncytium-inducing, slowly replicating variants (*29*).
	HIV isolates during progression to AIDS are frequently more rapidly replicating, T cell–tropic variants (*6, 29, 58*).
Rapid progressors	High viral load in primary HIV infection that generally does not fall dramatically to the levels seen with typical progressors (*28–32*).
	Rapid progressors have higher levels of unspliced HIV mRNA compared to nonprogressors or typical progressors (*31, 59*).
	Some rapid progressors may be infected with more rapidly replicating, virulent HIV strains (*29, 30*).
Nonprogressors	Viral load is generally lower in nonprogresors than in rapid progressors (*19, 29, 31, 32, 35*).
	Some, but not all, nonprogressors may be infected with constitutively less pathogenic HIV variants (*60, 61*).

venous nature of the challenge might have overcome any protection afforded by CTLs, whereas it is possible that these monkeys may have withstood a mucosal challenge.

Genetic Factors Implicated in Modulating Host Immune Responses to HIV Infection

The MHC class I and class II genes play a major role in determining the specificity of T and B cell antiviral immune responses. A number of MHC alleles as well as other host genetic factors have been described that may influence predisposition or protection against HIV infection or disease (Table 2).

There are several mechanisms whereby MHC-encoded molecules might predispose an individual to rapid or nonprogression to AIDS. First, having a certain MHC class I or class II allele could protect against HIV progression by serving as a restricting element for one or several immunodominant HIV T helper or CTL epitopes, thus promoting a salutary immune response to HIV and protection from progression to AIDS. Such a protective effect of the MHC class II E_{α}^{d} gene in the development of murine AIDS has been documented (*47*). Similarly, the lack of protective MHC alleles could predispose to developing AIDS because of a lack of salutary responses to HIV (*48*).

Second, having a certain MHC class I or class II allele could predispose an individual to pathogenic immune responses against a viral epitope in certain tissues such as the central nervous system or lungs, or against certain HIV-infected cell types such as monocyte (or macrophage) and dendritic cells. Similarly, the lack of an AIDS-promoting MHC allele would protect against pathogenic immune responses to HIV.

Third, having rare MHC class I and class II alleles could facilitate the rapid recognition of HIV-infected allogeneic cells during the early stages of HIV infection, thus promoting rejection of HIV-infected cells by means of alloreactive T cell responses (*49*). Sheppard and colleagues have shown that human sera from alloimmunized individuals neutralized HIV in vitro (*50*). Similarly, having common MHC alleles could promote less effective anti-HIV alloantigen responses and thus promote HIV infection or progression.

Fourth, human leukocyte antigen (HLA)–HIV disease associations are not absolute; thus, the data in Table 2 might reflect the association of genes linked to or within the MHC (*51*). For example, genetic markers linked to the *HLA-A1*, *CW7*, *B8*, and *DR3* haplotype, such as the complement C4 null allele (*C4AQO*) and a polymorphism in the tumor necrosis factor α promoter, have been suggested as MHC-linked gene candidates that might participate in a multigene effect on outcomes of HIV infection (*51*).

Fifth, the recent discovery that the level of MHC class I expression on virus-infected cells regulates the susceptibility of these cells to natural killer cell–mediated lysis provides a new area of investigation into the role of host MHC I genes in regulating the effectiveness of natural killer cell responses to HIV (*52*).

Finally, roles for transporter-associated with antigen-presenting (*TAP*) gene alleles have been proposed in determining the outcome after HIV infection (*48*, *53*). Data have suggested that combinations of MHC-encoded *TAP* and class I genes may synergize either in providing certain salutary anti-HIV responses or in avoiding pathogenic anti-HIV immune responses, or both.

Table 2. Genetic factors implicated in modulating host immune responses to HIV infection.

Factor	Effect	Reference
	Major histocompatibility loci-encoded genes	
B35, C4, DR1, DQ1	Associated with Kaposi's sarcoma	(*62*)
DR1	Associated with Kaposi's sarcoma	(*51, 63*)
DR2, DR5	Associated with Kaposi's sarcoma	(*63*)
DR5	Associated with Kaposi's sarcoma	(*63*)
Aw23, Bw49	Associated with Kaposi's sarcoma	(*63*)
B62	Associated with fever, skin rash in primary HIV infection	(*51*)
Aw19	Associated with HIV seropositivity in HIV multiply exposed individuals	(*49*)
A1, A24, C7, B8, DR3	Associated with rapid progression to AIDS	(*51, 64*)
*DR4, DQB1*0302*	Associated with rapid progression to AIDS	(*65*)
DR3, DQ1	Associated with rapid progression to AIDS	(*66*)
B35	Associated with rapid progression to AIDS	(*51, 67*)
TAP2.1	Promotes HIV progression to AIDS	(*48*)
DR5	Associated with thrombocytopenia and lymphadenopathy in HIV infection	(*68*)
DR5, DR6	Association of diffuse infiltrative $CD8^+$ lymphocytosis with Sjogren's-like syndrome in HIV	(*69*)
Bw4	Associated with slow decline in $CD4^+$ cell numbers	(*65*)
*B13, B27, B51, B57, DQB1*0302,0303*	Protects from progression to AIDS	(*48*)
A26, B38, TAP1.4, TAP2.3	Associated with ability to clear HIV infection in transiently infected HIV-seronegative individuals	(*53*)
A28, Bw70, Aw69, B18	Associated with protection from HIV infection	(*49*)
A32, B4, C2	Associated with long-term survival in HIV infection	(*70*)
*A11, A32, B13, C2, DQA1*0301, DQB1*0302, DRB1*0400, DRB4*0101*	Associated with long-term survival in HIV infection	(*70*)
	Other genes	
p53 tumor suppressor gene	Controls HIV replicative patterns and determinant of viral latency	(*71*)
Unknown inherited trait	Associated with PB mononuclear cell resistance to HIV infection in vitro	(*72*)

Summary and Future Directions

A pattern is emerging that many nonprogressors to AIDS have an immune response to HIV that is quantitatively and qualitatively superior to anti-HIV immune responses that occur in HIV-infected individuals who rapidly progress to AIDS. The HIV load in peripheral blood mononuclear cells varies widely from patient to patient and generally increases within individual patients as the disease progresses (*32*). Recent studies now suggest that the cellular viral load level is established early on in HIV infection and is a predictor of the subsequent clinical course, with smaller viral loads after serconversion predicting longer survival (*32*). The initial key unanswered question is whether a small viral load after seroconversion in nonprogressors is related to low pathogenecity of the infecting HIV strain, to a particularly effective anti-HIV immune response, or to both. The answer to this question may not be the same for all patients.

A second important issue that must be explored quickly is that of the role of the host genetic background in determining the rate of progression of the clinical course. Though HIV proteins are of sufficient size to contain many immunogenic epitopes, HIV proteins contain strongly immunodominant regions, as well as a myriad of regions with sequence similarities to a wide spectrum of host proteins. Thus, it is critical to determine if MHC-encoded or other host genetic factors are responsible for a qualitatively more effective anti-HIV immune response in nonprogressors. If, in fact, nonprogressors are genetically programmed to successfully

control HIV, then immune reconstitution to rebuild a "better immune system" with allogeneic bone marrow and thymus grafts becomes a theoretical possibility.

Third, the need for determining the specificity of both CTL responses and protective neutralizing antibodies in nonprogressors is extraordinarily important for understanding both the biological basis of the nonprogressor status and the design of effective HIV vaccine immunogens. It is critical to determine the specificity of serum antibodies in nonprogressors that neutralize HIV primary isolates grown in peripheral blood mononuclear cells.

Fourth, the role of viral factors in determining nonprogressor status must be better understood. Although recent data demonstrated that *nef*-deleted mutants are not a common finding in nonprogressors (*61*), some investigators have found that HIV is more difficult to isolate from nonprogressors compared with typical progressors (*19*). The key question is whether a particular virus type or strain interacts with a particular host genotype to eventuate in nonprogressor status.

Fifth, the types of anti-HIV immune responses that are generated by small- and large-inoculum HIV infection through genital mucosa are critical to understand. More sensitive and inexpensive assays of HIV viral load are needed to determine the levels of HIV infection in various tissues. Studies are needed that profile mucosal and systemic immune responses to HIV after both genital and systemic routes of HIV infection, and that determine what immune responses are protective for systemic and genital challenges in animal models such as SIV infection of rhesus macaques.

Sixth, although the use of attenuated HIV strains as a vaccine remains controversial, attenuated SIV strains have protected adult rhesus monkeys against SIV challenge (*54*), and primary infection with HIV-2 may confer some protection against HIV-1 in humans (*55*). It is important to understand the correlates of protective immunity in these settings. Of note is the fact that although live-attenuated *nef*-deleted SIV is not pathogenic in adult rhesus monkeys, it may be pathogenic in neonatal animals (*56*). The immune and other factors in neonatal and adult rhesus monkeys that lead to protection in adults and to disease in neonates are critical to understand.

Finally, the role of pathogenic compared with salutary $CD8^+$ T cell responses to HIV in determining the various clinical courses of HIV infection must be defined. The role of $CD8^+$ T cell cytokines in suppression of HIV replication in long-term nonprogressors is particularly important to study. The fact that nonprogressors have high levels of anti-HIV CTLs strongly suggests that CTL responses to HIV may be important in the control of virus replication over time. However, the question of whether qualitative differences among anti-HIV CTLs, including the possibility of pathogenic effects, has not yet been resolved. Resolving this question is critical to HIV vaccine immunogen design and to the design of novel strategies to induce protective immune responses in those patients early in the clinical course of progression to AIDS. It has been hypothesized that CTL immune responses may be more effective if they are targeted at a major immunodominant epitope of HIV rather than at several less dominant regions (*11*). If this were true, it is possible that successful immunotherapy of a HIV-infected patient might boost the CTL response to a single conserved epitope, making it immunodominant by increasing the frequency of reacting T cells and inducing a more stable and effective CTL response (*11*). Such a trial of peptide-based immunotherapy has just begun in HIV-infected patients (*57*).

We are clearly entering a new era of understanding the pathogenesis of HIV infection and of appreciation of the novelty and complexity of the cellular and molecular mechanisms of HIV-host interactions. This new knowledge has reinforced the conviction that to develop effective anti-HIV drugs and vaccines, viral pathogenesis and the correlates of protective immunity must be understood.

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HIV series

HIV vaccines: where we are and where we are going

Barton F Haynes

As the HIV-1 epidemic continues to spread world wide, the need for an effective vaccine remains urgent. Efforts to develop such a vaccine have been hampered by three main factors: (a) the extraordinary ability of the virus to mutate; (b) inability of most known specificities of anti-HIV antibodies to neutralise HIV primary isolates consistently; and (c) lack of understanding of the correlates of protective immunity to HIV infection. In view of the complex biology of HIV-host interactions, the most fruitful avenue may be development of multivalent HIV immunogens tailored to HIV isolates in specific geographical locations.

Recent progress in HIV basic research has led to a better understanding of the pathogenesis of AIDS. Moreover, we now know that not all HIV-infected individuals develop AIDS. This new knowledge has refocused HIV vaccine development efforts, and has forced us to rethink current HIV vaccine strategies.[1] I will discuss three key questions relevant to HIV vaccine development:

- What type of immunogen is needed for an effective HIV vaccine?
- What do we have now?
- What do we need to accomplish in future studies?

What type of immunogen is needed for an effective HIV vaccine?

One of the main hindrances to HIV vaccine development has been our lack of understanding of the correlates of protective immunity to the virus.[1] Recently, investigators have realised that, although most HIV-infected persons develop AIDS, about 10–15% remain AIDS free after 10 years of infection; these individuals are called non-progressors to AIDS.[2,3] Of those HIV-infected individuals who do develop AIDS, about 10% progress within the first 2–3 years of infection—rapid progressors to AIDS.[2,3]

Characterisation of anti-HIV immune responses in non-progressors and rapid progressors has shown that rapid progressors generally have lower concentrations of antibodies to HIV proteins,[2,4,5] and low or absent antibodies that neutralise autologous HIV isolates.[4,5] Anti-HIV CD8 cytotoxic T-lymphocyte (CTL) activity is present in peripheral blood T cells of rapid progressors,[6] although one study showed low concentrations of memory CD8 CTL by precursor frequency analysis in this group by comparison with non-progressors (Rinaldo C, unpublished). Plasma concentrations of HIV virions are generally higher in rapid progressors than in non-progressors, and rapidly replicating HIV strains are isolated more frequently from rapid progressors,[7–9] either as a consequence of immunodeficiency and selection of more virulent HIV variants, or because more virulent HIV variants infect rapid progressors.[10] In conjunction with data showing that the fall in plasma viraemia in primary HIV infection correlates with the presence of CD8 anti-HIV CTL activity,[11] these results suggest that anti-HIV CD8 CTL that kill HIV-infected cells and antibodies that broadly neutralise HIV primary isolates might be protective anti-HIV immune responses in uninfected individuals who are subsequently exposed to HIV.[1,12]

The definition of a successful preventive HIV immunogen is controversial. Protective anti-HIV immune responses may prevent HIV infection completely, may allow only transient infection, leading to clearance of virus, or may merely limit the extent of HIV infection, but in so doing prevent the development of AIDS. One suggestion is that clearance of HIV occasionally occurs after both maternal-fetal HIV transmission[13,14] and sexual transmission of HIV.[15] Consequently, if protective anti-HIV immune responses could be induced by an immunogen in an HIV-uninfected person, protection might be achieved via early termination of HIV infection.

What are protective anti-HIV cellular immune responses?

Less effective anti-HIV CD8 CTL responses may be oligoclonal with respect to T-cell receptor (TCR) Vβ usage and targeted at several non-immunodominant HIV CTL epitopes, whereas more effective anti-HIV CTL responses may be polyclonal and targeted at fewer immunodominant epitopes.[6,15,16] These observations, together with data suggesting that the inheritance of certain HLA-encoded or other host genes may be associated with either rapid progression or non-progression to AIDS,[1] indicate that host gene expression may determine the quality and/or quantity of host anti-HIV immune responses. This could occur via HLA or HLA-related genes (*a*) by determining which HIV epitopes are recognised by CTL or T helper cells; (*b*) by determining which TCR Vα and Vβ regions are used in the CTL and T helper cell anti-HIV responses; or (*c*) by other events such as regulation of cytokine expression that may determine the quality of anti-HIV immune responses.

Levy et al[17] have described potent non-HLA-restricted CD8 T cell anti-HIV activity that suppresses the ability of HIV to replicate. This CD8 "HIV suppressor" activity, which is initially present in rapid progressors and declines with the onset of AIDS,[17] may be partly mediated by cytokines such as interleukin (IL)-16,[18] and by the chemokines, RANTES, MIP-1α, and MIP-1β.[19] Feng and colleagues[20] lately reported a novel host molecule, fusin, which is required for T-cell tropic HIV to infect CD4 T cells; this molecule shows 37% homology with a known chemokine receptor, the IL-8 receptor.

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Immunogen responses	Tested in non-human primates	HIV immunogen tested in human beings	Induces antiretroviral CD4 T cell proliferative responses	Induces antiretroviral CD8 CTL	Induces neutralising antibodies	
					Against laboratory-adapted retrovirus strains	Against retrovirus primary isolates
HIV subunits in various adjuvants						
Recombinant gp120 envelope	Yes	Yes	Yes	Rare	Yes	No
gag core proteins[a]	Yes	Yes[a]	Yes	Yes	No	No
Envelope gp120+ envelope V3 peptides	Yes	Yes[b]	Yes	Yes	Yes	No
Envelope and *gag* in pseudovirions	Yes	No	Yes	Yes	Yes	No
HIV proteins in live vectors[c]						
Envelope (gp120 or gp160)+*gag* p24 in vaccina	Yes	Yes	Yes	Yes	Weak	No
Envelope +/− *gag* p24 in canarypox	Yes	Yes	Yes	Yes	Weak	No
Envelope +/− *gag* p24 in BCG	Yes	No	Yes	Yes	No	No
Whole-virus vaccines						
Whole killed HIV or SIV	Yes	Yes[b]	Yes	Rare	Yes[d]	No
Attenuated live HIV or SIV[e]	Yes	No	?[f]	Yes[g]	Yes	?[f]
Others[h]						
Envelope and other retroviral proteins in DNA vaccines	Yes	Yes	?[f]	Yes[i]	Yes	No
Mixtures or sequential immunisations of HIV proteins in live vectors and HIV envelope subunits	Yes	Yes	Yes	Yes	Yes	No

[a]*gag* p24 immunogens of SIV have been extensively tested in rhesus monkeys; these immunogens have been formulated in various adjuvants such as liposomes and oil-based adjuvants. *gag* p17 peptide immunogens have been tested in human beings. Several formulations of SIV *gag* p24 prime and boost CD8 anti-SIV CTL.

[b]C4-V3 hybrid peptides and whole killed HIV are being tested in HIV-seropositive individuals. Formulations of multiple HIV peptides are also being tested in HIV-seronegative individuals.

[c]Other live vectors carrying HIV proteins are also under various stages of development—eg, *Salmonella* spp, Semliki Forest virus, rhinovirus, and poliovirus. +/−="with or without".

[d]Neutralising activity induced by whole killed SIV in rhesus monkey serum was found to be targeted to human HLA class II DR molecules present in the whole killed SIV immunogen grown in HLA class II DR human T cells.

[e]Attenuated live SIV and HIV currently being tested either have *nef* gene deleted or mutated, or multiple deletions in several regulatory genes.

[f]?=not known or currently under study.

[g]CTL against SIV-infected cells have been found in animals protected from SIV infection by *nef*-deleted SIV (P Johnson, unpublished).

[h]In addition to the vaccines listed, various HIV immunogens are being tested via systemic and mucosal routes with several adjuvants and/or immune modulators designed to modulate cellular and/or humoral anti-HIV immune responses. These adjuvants and/or immune modulators include CD30 ligand, IL-1, IL-10, IL-12, and IL-15.

[i]N Letvin, unpublished.

Table: **Experimental retroviral immunogens under evaluation**

These discoveries regarding HIV-host interactions have led to new ways of thinking about protective host responses to HIV infection, and have provided new insight into the types of immune cell responses that may need to be induced by an HIV immunogen. Thus, for induction of CD8 "HIV suppressor" cells, CD8 CTL, and CD4 T helper cells by an HIV immunogen, one probably needs immunogens that induce these anti-HIV responses to a sufficient number of HIV variants so that most variants in a geographical area will be recognised.

What are protective anti-HIV antibody responses?

Sullivan et al[10] lately showed that anti-recombinant (r) gp120 envelope antibodies raised in animals or in human volunteers neutralise HIV grown in laboratory-adapted T-cell lines but not primary isolates of the virus grown in peripheral blood mononuclear cells. This observation raises important questions about the roles of various specificities of neutralising antibodies in protection against HIV. The predominant types of anti-HIV neutralising antibodies raised against rgp120 are antibodies against the third variable (V3) region of gp120, as well as antibodies against the conformationally determined CD4 binding site centred around the fourth constant (C4) region of gp120.[10] Sullivan et al[10] found that both the V3 and the CD4 binding site neutralising determinants are inaccessible to antibody binding to the envelope of HIV primary isolates, although these antibodies do bind to and neutralise laboratory-adapted HIV strains. Although laboratory-adapted variants are pathogenic and have caused AIDS in man after laboratory accidents,[21] the relevance of these variants in vivo in community-acquired infections is unknown. Serum concentrations of antibodies against the V3 gp120 region and of antibodies that neutralise laboratory-adapted HIV strains do not protect individuals from developing AIDS,[22] nor do anti-V3 antibodies seem protective against maternal-fetal HIV transmission.[23]

Thus, for induction by HIV immunogens of neutralising antibodies to prevent HIV infection, we probably need HIV immunogens capable of inducing anti-HIV antibodies that neutralise both HIV laboratory-adapted isolates and HIV primary isolates grown in peripheral blood mononuclear cells.[24]

Can HIV variation be overcome by immunogen design?

A key obstacle to vaccine development is the extraordinary variability of HIV and the rapidity and extent of HIV mutation.[25] Recent data in patients treated with antiretroviral drugs have shown that, after initiation of treatment, HIV variants emerge rapidly, and can be isolated from peripheral blood by 3 weeks.[26,27] Moreover, as many as 10^9 new HIV virions are produced in an infected individual per day, and the half-life of HIV variants is about 2 days.[26,27]

Myers and colleagues[28] analysed HIV sequences world wide and divided HIV isolates into groups or clades, providing a basis for evalulating the evolutionary relation of individual HIV isolates to each other. The degree of variation in HIV protein regions that contain CTL and T helper epitopes has also been analysed by Korber et al,[29] and sequence variation documented in many CTL and T helper epitopes among HIV isolates.

A new level of HIV variation complexity was recently reported by Hahn et al,[30] who found frequent recombination of HIV among clades. These researchers

suggest that as many as 10% of HIV isolates are mosaics of recombination; if so, vaccines based on only one clade will not protect immunised individuals from mosaic isolates. However, for preventive HIV vaccine development, the degree of HIV variation that must be dealt with is unknown, since the viruses isolated early during HIV infection are relatively homogeneous in sequence.[31]

What do we have now?

The table summarises some of the general types of HIV or simian immunodeficiency virus (SIV) immunogens being tested in animals or in man, and the types of immune responses that these immunogens induce. The most encouraging results in retrovirus protection trials so far have come from immunising rhesus monkeys with *nef*-deleted attenuated live SIV strains: the resulting infection by the attenuated SIV strain provided full protection from challenge with highly virulent SIV 2 years later.[32] Shiramizu and colleagues[33] have reported a common site of integration of HIV in T-cell tumours that occur in HIV-infected patients, suggesting that HIV may be oncogenic. Baba et al[34] showed that *nef*-deleted attenuated SIV strains, whilst non-pathogenic for adult rhesus monkeys, induced fatal disease in newborn monkeys. In view of these data, there is considerable concern about development and use of a live-attenuated vaccine in human beings.[12] Moreover, for immunisation with attenuated HIV strains as well as with any of the non-HIV live vectors that contain HIV proteins, the problem of HIV variability remains, possibly necessitating the use of a multivalent immunogen irrespective of HIV immunogen type. Thus, the large number of HIV variants available for transmission and the possible immunodominant nature of what may be protective anti-HIV T-cell responses have suggested the need for HLA-based HIV subunit vaccines.[35–40]

What are HLA-based HIV vaccines?

To design an HLA-based HIV vaccine several variables must be taken into account:

- HLA molecules that are expressed in a population or cohort to be immunised,[40]
- CTL or T helper epitopes present in an immunogen and their respective HLA-restricting elements,
- HIV variants present in the geographical location of the cohort to be vaccinated.

Thus, HLA-based vaccines for induction of anti-HIV T-cell immunity would be multivalent mixtures of immunogens reflecting the most common HIV variants in a particular location, and containing sufficient immunogenic CTL and T helper epitopes capable of binding to the HLA molecules expressed on antigen-presenting cells of individuals of the cohort to be vaccinated. Such a mixture of immunogens could range from a mixture of non-HIV vectors expressing HIV proteins, to mixtures of HIV recombinant proteins and/or synthetic peptides (table).[35–38,40,41]

The implications of such a vaccine design are: (*a*) depending on the immunogen, not all members of a cohort may have an immunogenic HIV epitope present to bind to their particular HLA antigens; (*b*) not all relevant HIV variants would probably be represented in an immunogen; (*c*) vaccines may need to be designed for specific geographical locations, and perhaps even for specific ethnic groups within them; and (*d*) such vaccine formulations would almost certainly be less than 100% effective in the vaccinated cohorts.[1]

Panel: **Steps in development of an HLA-based subunit HIV vaccine for induction of anti-HIV CTL in African-Americans in the USA**

1. Analysis of common HLA types in cohort to be immunised.
   Example: For African-Americans in the USA, 80% will express HLA A2, A3, A28, A30, B7, or B8.
2. Analysis of the HLA class I HIV CTL epitopes restricted by these common HLA types in African-Americans in the cohort to be immunised.
   Example: For HLA-A2, aa77-85, from p17 *gag*; for HLA-A3, aa73-82 from *nef*; for HLA-A28, aa583-592, from gp41; for HLA-A30, aa844-863, from gp41; for HLA-B7, aa302-312, from gp120; for HLA-B8, aa586-593 from gp41 (CTL epitopes and their restricting elements from ref 29).
3. Analysis of the HIV clade variants in the geographical locations of the cohort to be immunised.
   Example: The most common clade in the USA and western Europe is clade B. For clade B, epitope aa77-85 from p17, of 26 isolates analysed in the Los Alamos database, there are eight variants, necessitating inclusion of eight peptides for this sequence. A similar analysis for the other five epitopes would require an additional 54 peptides, for a total of 62.
4. T-cell help can be obtained for anti-HIV CTL induction by peptides by synthesising immunodominant T helper determinants N terminal to the CTL epitopes or by inclusion of T helper determinants in larger subunits of HIV immunogens*.[35,46]
   Example: Th_1-X_1, TH_2-H_2, Th_3-X_3, TH_N-X_N, and, where Th=immunodominant T-helper epitopes and X=HLA class I CTL epitopes.

*Specific CTL epitopes would have to be included because of the restricted nature of CTL-epitope presentation by HLA molecules.[35,36,47] It is likely that only the most common immunodominant T-helper epitopes would have to be included for T cell help for CTL generation, without matching T-helper epitopes to HLA class II types in the population to be immunised, since many HIV T helper epitopes are presented by multiple HLA class II types,[36,47] and some retrovirus CTL epitopes may also have T-helper-cell stimulating activity.[36,47]

The datasets that are needed to develop HLA-based AIDS vaccines are now becoming available:

- Compilation of CD8 CTL and CD4 T helper epitopes in HIV proteins[29,42]
- Listing of the HLA restricting antigens that present HIV CTL and T helper epitopes[29]
- Compilation of HIV variants present in specific geographical locations[28,29]
- Listing of HLA types for some ethnic groups in geographical locations.[43]

The panel shows an example of a theoretical HLA-based HIV vaccine design for CTL induction for African-Americans. Steps in development are analyses of (*a*) the most common HLA types present in the cohort to be immunised; (*b*) HLA class I CTL epitopes restricted by the common HLA types in the cohort; and (*c*) HIV variants in the particular geographical location. Obviously, the most useful HIV preventive immunogens will be those designed for all members of a cohort to be immunised in a geographical area, irrespective of ethnic background. Theoretically, such immunogens could be designed by expanding the number of HLA types used in the analysis and by choosing HIV CTL immunogenic epitopes presented by several disparate HLA molecules.[29,38] Ward et al[39] have suggested that, since several HIV CTL epitopes are presented by more than one allotype of HLA class I molecules, by using nine CTL epitopes (as well as variant peptides that reflect HIV mutations at these sites) 95·4%

of African-Americans, 97·5% of Caucasian Americans, 99·4% of Native Americans, and 97·2% of Thais would theoretically respond to such an HIV immunogen.

There are considerable limitations and concerns of such an analysis (panel). For example, we do not know whether the degree of variability seen in existing databases reflects the true degree of HIV variability in all patients, or whether all variants of each HLA type will equally present each peptide to T cells. Another unknown factor is whether each HIV CTL epitope variant will be a potent agonist and trigger T cells to a salutary anti-HIV immune response; there are well-documented examples of mutations in HIV T-cell epitopes that modify peptide presentation.[16,36]

If subunits of HIV proteins larger than individual synthetic HIV immunogenic peptides are used in HIV immunogens (eg, whole proteins containing multiple immunogenic epitopes), such as might be expressed in non-HIV live vectors or in HIV pseudovirions, there would be less concern with "matching" the common HLA antigens with CTL and T-helper epitopes in the immunogens. However, with large immunogen molecules, one would need to construct multivalent immunogens capable of providing immunity against many HIV variants.

For an HLA-based preventive immunogen that induced both anti-HIV T-cell responses and anti-HIV neutralising antibodies, one would have to add HIV β-cell immunogens that induce broadly neutralising antibodies for HIV primary isolates to a multivalent HIV T-cell immunogen.

What do we need to accomplish in future studies?

Although studies in multiply exposed but uninfected individuals with anti-HIV CTL and no anti-HIV antibody responses[13–15] suggested that anti-HIV CTL alone might sometimes protect against HIV infections, rhesus monkeys with only anti-SIV CTL present and challenged intravenously with SIV have not so far been protected from SIV infection (N Letvin, unpublished). Studies are needed to determine whether genital mucosal SIV challenge would have resulted in SIV infection in this setting.

The work of Sullivan et al[10] implies that envelope oligomers of HIV primary isolates may be appropriate immunogens for induction of anti-HIV neutralising antibodies against primary HIV isolates grown in peripheral blood mononuclear cells. Future studies should focus on the envelope of HIV primary isolates as the target of neutralising antibodies. If HIV envelope oligomers are successful in inducing antibodies that neutralise HIV primary isolates, the neutralising antibody specificity may be variant specific and, if so, the issue of HIV variability would still need to be addressed.

We also need immunogens and immunisation strategies for inducing genital and gastrointestinal mucosal T and B cell immunity to HIV. Studies are under way that suggest inhaled HIV subunit immunogens coupled with cholera toxin or other adjuvants may be useful in this respect.[44] Studies targeted at defining the presence and effectiveness of mucosal antiretroviral T and B cell responses following infection or vaccination with retroviral immunogens are critical to successful vaccine development.

For an HIV vaccine development effort to be targeted at the appropriate immune responses, the correlates of protective immunity must be understood in the context of (*a*) human non-progression to AIDS; (*b*) attenuated *nef*-deleted SIV protection of rhesus monkeys from SIV challenge;[32] and (*c*) HIV-2 infected human beings protected from HIV-1 infection.[45] The extreme variability of HIV, the possible need to consider complex HLA-based HIV immunogen design, and the probable need for immunogens that induce antibodies that neutralise HIV primary isolates still provide high hurdles to be overcome before a safe and effective HIV vaccine can be implemented.

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Review article

Pathophysiology of itching

M W Greaves, P D Wall

Itching is the predominant symptom of skin disease but it is ill-understood and a challenge for future research. Even the major nerve pathways for itch, and its relationship to pain are debatable. In inflamed skin, histamine plays a major role and its mode of release from mast cells in, for example, chronic urticaria is now better appreciated. Tachykinins including substance P and cytokines including interleukin-2 are evidently important peripherally. Opioid μ-receptor-dependent processes activate inhibitory circuits in the central nervous system and regulate the extent of intensity and quality of perceived itch. It is proposed that stimulation of large areas of skin such as by scratching, generates inhibitory activity which suppresses itch excitation. Therapeutic intervention based upon understanding these regulatory processes is a real prospect.

Itch is a skin sensation leading to a desire to scratch. The sensation is distinct from touch and pain in its nature, persistence, and localisation. Tissue damage caused by scratching may lead to acute conditions such as scratch prurigo (itchy weals from scratching)[1] or to the chronic lichenified patches of prurigo nodularis. Itch is the dominant symptom of skin disease. Hardly any acute or chronic dermatoses are itch free. There have been few sustained attempts to study and characterise itching due, at least in part, to lack of satisfactory animal models. In consequence there is no specific treatment available for itch. But the situation is changing.

What causes itch in inflamed skin?

Itching inflamed skin has been searched for soluble compounds which might be responsible for the itch. As more is known about inflammation, the list of candidates lengthens (table). Itching, however, is not necessarily caused by pharmacologically active mediators; itching in dry, cracked, and scaly skin is not associated with any known soluble mediators and may be caused by mechanical or osmotic mechanisms.

Histamine

In 1927, Lewis proposed that "H-substances" caused itching in inflammatory skin diseases.[2] Lewis' "H-substances", histamine, causes itching if injected superficially into the skin, but a burning pain if injected more deeply. Two main subclasses of histamine receptors have been defined, H_1 and H_2. Experiments with H_1 and H_2 agonists and antagonists have established that both subclasses of receptor are expressed in human skin,[3,4] and that histamine-induced itching involves H_1 but not H_2 receptors.[5] Other H receptors (H_3[6] and Hic[7]) have not convincingly been shown to be in skin and there is no evidence that they are involved in itching. Although histamine provokes itching, it is possible that other mediators, released indirectly by the actions of histamine, also play a part.[8] The main sources of histamine in skin are mast cells which both synthesise and store histamine in their glycosaminoglycan granules. Release of histamine may occur via cross linking of the membrane high-affinity immunoglobulin E receptors (FcεRIα) by specific antigen, or by anti-FcεRIα autoantibodies.[9,10] It may also occur independently of FcεRIα when evoked by complement C5a and tachykinins, including the neuropeptide, substance P.

Histamine is recoverable in biologically relevant amounts from inflamed skin in itchy dermatoses, and H_1-receptor antihistamines are effective in relief of itching in many inflammatory dermatoses including most types of urticaria. In atopic eczema, antihistamines are widely used for suppression of itching although the extent to which this is attributable to the side-effect of central sedation rather than local histamine antagonism in the skin is unclear.[11]

Prostaglandins and related lipids

Prostaglandin E_2 is found in increased concentrations in tissue fluid from many inflammatory dermatoses including psoriasis,[12] contact allergic dermatitis,[13] and ultraviolet B-irradiated inflamed skin.[14] Prostaglandin E_2 does not itself cause itching. However, if abraded skin is pretreated with low concentrations of prostaglandin E, the threshold concentration of histamine above which itching is

Amines	**Proteins/peptides**
Histamine	Kallikrein
Histamine liberators	Cytokines
morphine	interleukin-2
codeine	Proteases
compound 48/80	trypsin
Serotonin (5-hydroxy tryptamine)	papain
	mucunain
Lipids	Tachykinins
Prostaglandins	substance P
Platelet activating factor	CGRP*
	VIP†
	Opioid peptides
	β-endorphins
	leu-encephalin
	met-encephalin

*Calcitonin gene-related peptide. †Vasoactive intestinal peptide.

Table: **Main classes of mediators causing itch in inflamed skin**

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Mucosal Immunity to HIV-1

Systemic and Vaginal Antibody Responses After Intranasal Immunization with the HIV-1 C4/V3 Peptide T1SP10 MN(A)[1]

Herman F. Staats,[2] W. Garrett Nichols, and Thomas J. Palker

To optimize mucosal immune responses to the HIV-1 peptide vaccine candidate T1SP10 MN(A), we intranasally immunized BALB/c and C57BL/6 mice with C4/V3 HIV-1 peptide together with the mucosal adjuvant cholera toxin (CT). Four doses over a 4-wk period resulted in peak serum anti-peptide IgG titers of >1:160,000 in BALB/c mice and >1:520,000 in C57BL/6 mice, and significant levels (>1:30,000) persisted in both strains of mice for longer than 6 mo. Furthermore, intranasal immunization with peptide and CT induced serum IgG reactivity to HIV-1 gp120 and HIV-1_{MN} neutralizing responses. The primary anti-peptide IgG subclass was IgG1, suggesting a predominant Th2-type response. In addition to elevated serum anti-peptide Ab responses, intranasal immunization with T1SP10 MN(A) and CT induced both vaginal anti-peptide IgG and IgA responses, which persisted for 91 days in both strains of mice. Vaginal anti-HIV IgA was frequently associated with secretory component, suggesting transepithelial transport of IgA into vaginal secretions. Cervical lymph nodes contained the highest relative concentration of anti-T1SP10 MN(A) IgG-producing cells, while the spleen was the next major site of anti-T1SP10 MN(A) IgG-producing cells. Ag-specific proliferative responses were also detected in cervical lymph node and spleen cell populations after intranasal immunization with T1SP10 MN(A) and CT. In addition, intranasal immunization with T1SP10 MN(A) and CT was able to induce anti-HIV cell-mediated immunity in vivo as indicated by the induction of delayed-type hypersensitivity. Therefore, intranasal immunization with hybrid HIV peptides provides a noninvasive route of immunization that induces both long-lived systemic and mucosal Ab responses as well as cell-mediated immunity to HIV. *The Journal of Immunology,* 1996, 157: 462–472.

It is estimated that by the year 2000, 30 to 40 million people worldwide will be infected with HIV, the causative agent of AIDS (1). Currently, the predominant route of HIV transmission is via sexual contact, where HIV is transmitted at the mucosal surface of the genito-urinary tract or rectum (2). Recent evidence has suggested that HIV transmission may also occur after oral exposure to HIV (3). Because the mucosal surface is most commonly the first site of contact between the host and HIV, studies of vaccine strategies that induce both mucosal and systemic immunity are essential.

Attempts to produce a protective HIV vaccine have not been promising to date. A variety of novel approaches are currently being investigated, which include but are not limited to attenuated, recombinant bacterial vectors expressing antigenic epitopes from HIV (4), recombinant adenovirus vectors (5), recombinant vaccinia virus (6), hybrid hepatitis particles expressing a V3 loop peptide (7), DNA vaccines expressing gp120 (8), and synthetic peptides containing T and B cell epitopes of HIV as immunogens (9–18). It has been previously shown that the hybrid C4/V3 HIV peptide T1SP10 MN(A) containing T helper, CTL, and neutralizing B cell epitopes from HIV-1_{MN} gp120 induced type-specific neutralizing Ab responses (19–21) as well as class-I restricted CTL responses (22) when administered systemically with CFA. Phase 1 clinical studies are currently evaluating the safety and immunogenicity of T1SP10 peptide immunogens in HIV-1 seropositive individuals.

Although a variety of HIV vaccine immunization protocols have been shown to induce serum neutralizing Ab responses after systemic vaccine administration, immunization via a systemic route rarely induces secretory IgA (S-IgA)[3] immune responses (23, 24, and this study). The development of HIV vaccines that induce S-IgA responses may be important for protection against sexual transmission of HIV-1, since S-IgA exhibits unique effector functions that are well suited to protect the mucosal surfaces. Mucosal immunization at one anatomical location has been shown to induce detectable mucosal immune responses at the site of immunization as well as distant mucosal effector tissues, an observation that has led to the term "common mucosal immunologic system" (CMIS) (25). Recent reports have provided evidence that the female reproductive tract has characteristics of mucosal effector tissues including IgA-producing cells, secretory component (SC), and J chain (26). S-IgA has also been identified in the male urethral epithelium, suggesting that this is also a mucosal effector tissue (27). Therefore, immunization strategies that involve the CMIS

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[3] Abbreviations used in this paper: S-IgA, secretory immunoglobulin A; SC, secretory component; CMIS, common mucosal immunologic system; CT, cholera toxin; CLN, cervical lymph node; SIV, simian immunodeficiency virus; aa, amino acid; ASC, antibody-secreting cells; PP, Peyer's patches; MLN, mesenteric lymph nodes; DTH, delayed-type hypersensitivity; pIgR, polymeric immunoglobulin receptor; ELISPOT, enzyme-linked immunospot.

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may lead to mucosal immune responses in the female and male reproductive tract that may prevent sexual transmission of HIV.

The lack of a good animal model for mucosal transmission of HIV hinders the development of a protective vaccine for HIV. Simian immunodeficiency virus (SIV) infection in rhesus macaques has a similar clinical presentation as HIV infection in humans and therefore has been used as a model for HIV. Several studies using the SIV system have provided evidence that combined systemic and mucosal immunization with a hybrid p27-Ty virus-like particle conjugated to the B subunit of cholera toxin (CT-B) was able to induce anti-SIV Ab responses in systemic compartments (serum) as well as the reproductive tracts of female (28) and male (24) rhesus macaques. Others have used microencapsulated SIV as the Ag to evaluate mucosal immunization protocols for the induction of anti-SIV mucosal immune responses. Rhesus macaques intramuscularly immunized with microencapsulated SIV and then boosted via the i.m., oral, or intratracheal route were intravaginally challenged with SIV on two separate occasions (29). All animals immunized by the i.m. route only became infected with SIV while two of three animals boosted by the intratracheal route became infected after two repeated intravaginal challenges. Oral boosting of i.m. primed macaques resulted in the highest level of protection, with only one of three animals infected after two intravaginal challenges. These data suggest that a combination of systemic and mucosal immunity will likely be necessary for optimal protection against HIV infection at mucosal surfaces. In the present study, we have exploited the CMIS for the induction of anti-HIV secretory immunity in the female reproductive tract by intranasal immunization with the C4/V3 HIV peptide T1SP10 MN(A) and the mucosal adjuvant, CT.

Materials and Methods

Animals

Female C57BL/6 and BALB/c mice, weighing 16 to 18 g, were purchased from the Frederick Cancer Research and Developmental Center, National Cancer Institute, Frederick, Maryland. Animals were housed in filter-top cages and provided food and water ad libitum. All procedures for use and care of mice were approved by Duke University's Institutional Animal Care and Use Committee.

Peptide

The sequence of T1SP10 MN(A) is as follows: KQIINMWQEVGKAMY ACTRPNYNKRKRIHIGPGRAFYTTK. The T1SP10 MN(A) peptide contains, at the amino terminus, sequences from HIV $gp120_{MN}$ between amino acids (aa) 428–443 (T1, KQIINMWQEVGKAMYA), previously shown to evoke $CD4^+$ Th cell responses in mice and humans (9, 13). This T1 sequence has been linked to a carboxyl-terminal sequence derived from the third variable (V3) domain of HIV-1_{MN} gp120 (aa 320–324), which contains both a principal neutralizing determinant (SP10, aa 302–319) and a site designated as (A) (aa 320–324; FYTTK), which is recognized by $CD8^+$ CTL in BALB/c mice (18-22). The T1SP10 MN(A) peptide was synthesized on an Applied Biosystems (Foster City, CA) 431A peptide synthesizer using the F-moc (9-fluorenylmethyloxycarbonyl) method and lyophilized. Peptide was solubilized in water with 25% acetic acid and dialyzed extensively against 25% acetic acid in distilled water. The peptide solution was then dialyzed two times against two liters of distilled water. The insoluble material was removed by filtration across a 0.45-μm filter, and the soluble material was lyophilized and stored at 4°C until used.

Immunization

Mice were intranasally immunized with the indicated amount of T1SP10 MN(A) with or without 1 μg of the mucosal adjuvant CT (List Biological Laboratories, Inc., Campbell, CA). Ag preparations were diluted to the appropriate concentration in sterile PBS, and 5 μl of the Ag mixture was introduced into each nostril while mice were under isoflurane anesthesia (IsoFlo, USP; Solvay Animal Health, Inc., Mendota Heights, MN). Mice were immunized on day 0, 7, 14, and 28. Two groups of BALB/c and C57BL/6 mice were boosted on day 196. For s.c. immunizations, mice were immunized with 50 μg T1SP10 MN(A) in CFA on day 0 and boosted with 50 μg T1SP10 MN(A) in IFA on day 22 or 24 after the primary immunization.

Sample collection

Blood was collected from the retro-orbital plexus using a heparinized Natelson capillary tube (Baxter Healthcare Corporation, McGaw Park, IL) while mice were under isoflurane anesthesia. Fresh fecal samples were collected and extracted using PBS with 0.1% sodium azide. One milliliter of PBS-azide was added for every 100 mg of feces; the samples were then vortexed for 20 to 30 min and centrifuged in a microcentrifuge for 10 min. Salivary secretions were obtained by injecting mice i.p. with 3 μg carbamylcholine chloride (Carbochol; Sigma Chemical Co., St. Louis, MO) to induce salivary secretions. Vaginal wash samples were collected by washing the vaginal cavity with 100 μl of sterile PBS while mice were under isoflurane anesthesia. This procedure resulted in an approximate 10-fold dilution of vaginal samples (data not shown). To determine this dilution factor, 100 μl of sterile PBS containing 1% BSA and [^{3}H]thymidine was used to perform the intravaginal wash procedure in eight C57BL/6 mice. After centrifugation, aliquots of the [^{3}H]thymidine-containing vaginal wash samples from independent mice were counted in a 2000CA TRICARB liquid scintillation analyzer (Packard, Downes Grove, IL). A dilution factor of 10 was estimated by comparing cpm values between vaginal wash samples and pre-wash samples. Vaginal and salivary samples were centrifuged for 10 min in a microcentrifuge to remove particulate matter. All samples were stored at −20°C until assayed for anti-T1SP10 MN(A) Abs.

Enzyme-linked immunosorbent assay

ELISA was used to determine the presence of anti-T1SP10 MN(A) Abs in serum, fecal, and vaginal wash samples. For the T1SP10 MN(A) ELISA, T1SP10 MN(A) peptide was suspended in CBC buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at a concentration of 40 μg/ml and plated to 96-well microtiter plates (Costar 3590, Cambridge, MA) at 50 μl/well. HIV-1 gp120 was coated onto 96-well microtiter plates at 1 to 2 μg/ml in PBS. After overnight incubation at 4°C, the contents of the wells was discarded and blocking buffer (CBC with 3% nonfat dry milk) was added at 200 μl/well. After incubating at room temperature for 2 h, plates were stored at −20°C until used. ELISA plates were washed four times with ELISA wash buffer (PBS, 0.05% Tween-20, 0.1% sodium azide) before the addition of samples. All samples were diluted in serum diluent (PBS, 2.5% BSA, 2.5% nonfat dry milk, 5% normal goat serum, 0.1% sodium azide, 0.05% Tween-20) and added to ELISA plates at 100 μl/well (serum and fecal) or 50 μl/well (vaginal and salivary). After overnight incubation at 4°C, plates were washed four times with ELISA wash buffer before the detection Ab was added. Alkaline phosphatase-conjugated goat anti-mouse IgG, IgA, IgM, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates (SBA), Birmingham, AL) was diluted 1:1000 (PBS, 0.05% Tween-20, 0.5% BSA) and used as the detection Ab (100 μl/well). After incubation at room temperature for three h, plates were washed four times with ELISA wash buffer and reacted with the alkaline phosphatase substrate *p*-nitrophenyl phosphate. After a 10-min incubation, plates were read at 405 nm on a Titertek Multiscan Plus plate reader (ICN Biomedicals, Costa Mesa, CA). For the detection of anti-T1SP10 MN(A) Abs that contained SC, rabbit anti-rat SC (generously provided by Dr. Brian Underdown, McMaster University Medical Centre, Hamilton, Ontario, Canada), was used at a dilution of 1:1500 and was detected with an alkaline phosphatase-conjugated goat anti-rabbit Ig (SBA). For Ag-specific ELISAs, sample dilutions were considered positive if the optical density recorded for that dilution was at least twofold higher than the OD recorded for a naive sample at the same dilution. For calculation of the average end point titer of anti-T1SP10 MN(A) Ab responses, the mean log of the end point dilutions was determined and used to calculate the average end point titer. Vaginal total IgG and IgA concentrations were determined with an ELISA performed according to manufacturers instructions (SBA).

Cell isolation

Lymphocytes were removed from cervical lymph nodes (CLN), spleen (SP), Peyer's patches (PP), and mesenteric lymph nodes (MLN). For CLN, SP, and MLN, tissues were teased apart using sterile forceps and passed through a sterile screen to obtain single-cell suspensions. Histopaque 1083 (Sigma Chemical Co., St. Louis, MO) was used to remove RBC from SP cells. For isolation of mononuclear cells from PP, techniques previously described were followed (30).

Immunoglobulin ELISPOT

To test for the presence of anti-T1SP10 MN(A) IgG-, IgA-, or IgM-producing cells, an Ag-specific ELISPOT assay was performed as described (31). T1SP10 MN(A) was diluted to 3 μg/ml in PBS and used to coat the plates (100 μl/well). Plates were blocked with DMEM with 2% BSA and 2% normal goat serum. Cells were incubated in a 37°C, 10% CO_2 in air, humidified environment overnight. Anti-T1SP10 MN(A) IgG, IgA, or IgM was detected with horseradish peroxidase-conjugated anti-mouse IgG, IgA, or IgM, respectively (SBA). 3-Amino-9-ethylcarbazole (AEC) (Pierce, Rockford, IL) was used as the substrate.

Proliferation assays

For proliferation assays, CLN, MLN, PP, and SP single-cell suspensions were adjusted to 2×10^6 cells/ml. Equal volumes of cells and complete media or complete media with T1SP10 MN(A) at 200, 20, or 2 μg/ml were mixed to give a final concentration of 1×10^6 cells/ml in media alone or media with peptide at 100, 10, or 1 μg/ml. Cells (100 μl) were added to round-bottom 96-well microtiter plates in triplicate and incubated in a 37°C, 10% CO_2 in air, humidified environment for 5 days. Twelve hours before harvesting, 1.0 μCi [^{3}H]thymidine (New England Nuclear Research Products, Boston, MA) was added to each well. Cells were harvested onto glass filters using a PHD 228 sample harvester (Cambridge Technology Inc., Watertown, MA). Incorporation was determined by placing the filters in ScintiVerse BD scintillation fluid (Fisher, Pittsburgh, PA) and counting with a 2000CA TRI-CARB liquid scintillation analyzer (Packard).

Delayed-type hypersensitivity

For the measurement of DTH responses, an ear swelling assay was employed as previously described (32), except that 12.5 μg HPLC-purified T1SP10 MN(A) was injected into the right ear in 10 μl of sterile PBS while 10 μl sterile PBS was injected into the left ear as control. Ear swelling was measured 24 h after injection with a dial thickness gauge (Mitutoyo code #7326, Mitutoyo/MTI Corp., Elk Grove Village, IL). Ag-specific ear swelling was calculated by subtracting the ear swelling of the PBS-injected ear from the swelling of the T1SP10 MN(A) injected ear.

HIV-1_{MN} neutralization assay

The assay used to detect neutralization has been described (33, 34). The criterion for neutralization by test serum was a 10-fold reduction in infectious titers of HIV-1_{MN} compared with the virus titer in the presence of normal mouse serum. Briefly, heat-inactivated (56°C, 30 min) immune serum at dilutions of 1:50 or 1:150 was incubated in duplicate with serial fourfold dilutions of virus for 1 h before addition of the virus/antiserum mixture to AA5 cells. Cells were cultured in 96-well microtiter plates by addition of fresh medium every other day. On day 7 postinfection, supernatants from each well were tested for the presence of reverse transcriptase activity as a criterion for successful infection in each well. Infectious titer for each treatment group was calculated using the Reed and Muench formula.

Statistical analysis

The Student *t* test was used to determine the significance of differences between groups.

Results

Serum anti-T1SP10 MN(A) Ab responses after intranasal immunization with T1SP10 MN(A)

To determine the dose of T1SP10 MN(A) required to induce high-titered anti-T1SP10 MN(A) Ab responses, C57BL/6 mice were intranasally immunized with 10, 25, or 50 μg T1SP10 MN(A) with 1 μg of CT on day 0, 7, 14, and 28. Mice were immunized with 50 μg of T1SP10 MN(A) peptide only (no CT) on the same schedule to determine the effect of CT. All peptide doses tested were able to induce detectable serum anti-T1SP10 MN(A) IgG responses in a dose-dependent fashion (Table I). Intranasal immunization with 50 μg of T1SP10 MN(A) and 1 μg of CT induced the highest serum anti-peptide responses at all times tested and was therefore used throughout the remaining experiments. For comparison, 10 μg of HIV-1_{IIIB} gp120 with 1 μg of CT was intranasally administered to mice on day 0, 7, 14, and 28. Intranasal immunization with recombinant gp120 was also able to induce serum anti-gp120 IgG responses (Table I).

Table I. *Serum IgG titers (reciprocal) to immunizing Ag at weekly intervals after intranasal immunization with indicated amount of T1SP10MN(A) or gp120$_{IIIB}$ with or without 1 μg CT as a mucosal adjuvant*[a]

Group	Day 7	Day 14	Day 21	Day 28	Day 35
1 μg CT used as adjuvant with:					
50 μg T1SP10MN(A)	<64	8,192	65,536	65,536	524,288
25 μg T1SP10MN(A)	<64	1,024	32,768	32,768	262,144
10 μg T1SP10MN(A)	<64	<64	256	512	2,048
10 μg HIV-1_{IIIB} gp120	<64	<64	4,096	4,096	8,192
No adjuvant used with:					
50 μg T1SP10MN(A)	<64	2,048	4,096	8,192	32,768

[a] C57BL/6 mice were immunized on day 0, 7, 14, and 28.

Intranasal immunization with 50 μg T1SP10 MN(A) and 1 μg of CT on day 0, 7, 14, and 28 induced high serum titers of anti-T1SP10 MN(A) IgG in both BALB/c and C57BL/6 mice. BALB/c mice had detectable serum anti-T1SP10 MN(A) IgG responses on day 14 with a titer of 1:74 (mean titer of five independent groups). Anti-T1SP10 MN(A) serum IgG titers in BALB/c mice reached their peak at day 35 with a titer of 1:161,368 and persisted at high levels through day 91 when a titer of 1:92,681 was detected (Fig. 1). Serum anti-peptide IgG titers of 1:32,768 were detectable at day 196. Boosting of BALB/c mice on day 196 with 50 μg of T1SP10 MN(A) and 1 μg of CT was not associated with any detectable increase in serum anti-peptide IgG responses (Fig. 1). C57BL/6 mice had detectable IgG responses on day 7 with a titer of 1:56 (mean titer of six independent groups) (Fig. 1). By day 14, C57BL/6 mice had a serum anti-peptide IgG titer of 1:9,153 which increased to 1:116,502 on day 21. Serum IgG titers reached peak levels by 35 days in C57BL/6 mice with a titer of 1:524,288. Serum anti-T1SP10 MN(A) IgG responses persisted at elevated levels in C57BL/6 mice with an IgG titer of 1:262,144 detected 91 days after the primary immunization. Two groups of C57BL/6 mice were monitored 196 days after the primary immunization and found to have serum anti-peptide IgG titers of 1:46,340. These two groups where then boosted intranasally with 50 μg of T1SP10 MN(A) and 1 μg of CT (day 196) and monitored at 2-wk intervals for serum anti-peptide IgG responses. After boosting, serum anti-T1SP10 MN(A) titers of 1:370,727 and 1:262,144 were detected on day 210 and 224, respectively. No anti-T1SP10 MN(A) IgA responses were detected in the serum of BALB/c mice at any time tested. In one of six C57BL/6 mice groups, serum anti-T1SP10 MN(A) IgA Ab titers of 1:64, 1:512, and 1:64 were detected on day 28, 35, and 91, respectively (data not shown). The serum anti-T1SP10 MN(A) IgG titers induced by intranasal immunization with T1SP10 MN(A) and CT were comparable to peak titers induced by s.c. immunization with T1SP10 MN(A) in CFA (Table II).

To further characterize the immune response induced after intranasal immunization with T1SP10 MN(A) and CT, serum anti-T1SP10 MN(A) IgG subclass profiles were determined (Table III). For all times examined in BALB/c mice, anti-T1SP10 MN(A) IgG1 was the predominant Ag-specific IgG subclass detected. Anti-T1SP10 MN(A) IgG1 and IgG2b were comparable at all times tested in C57BL/6 mice. Anti-peptide IgG2a responses were also detected in both strains of mice by day 21.

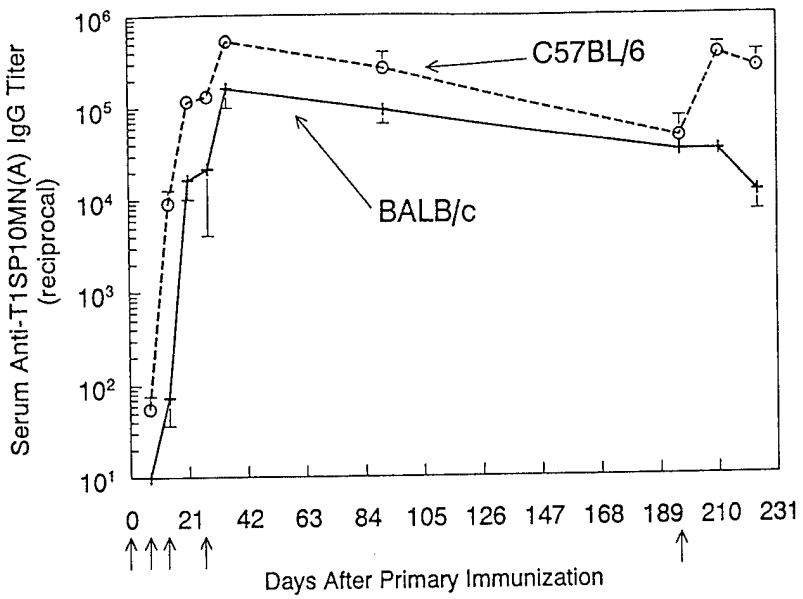

FIGURE 1. Serum anti-T1SP10 MN(A) IgG titers in BALB/c (——) and C57BL/6 (- - - -) mice after intranasal immunization with 50 μg T1SP10 MN(A) and 1 μg CT in 10 μl PBS on day 0, 7, 14, 28, and 196. Serum was assayed for the presence of anti-T1SP10 MN(A) IgG by ELISA. Results are reported as the reciprocal of last dilution giving a positive ELISA response. Arrows on the *x*-axis indicate day of immunization. Data are mean titer of five and six independent groups with three to five mice per group for BALB/c and C57BL/6 mice, respectively, for values through day 35. For values after day 35, data represent two independent groups for both BALB/c and C57BL/6 mice.

Table II. *Peak serum and vaginal anti-T1SP10MN(A) IgG and IgA titers after s.c. immunization with 50 μg T1SP10MN(A) in CFA*[a]

Mouse Strain	Serum IgG	Serum IgA	Vaginal IgG	Vaginal IgA
BALB/c	262,144	<64	64	<2
C57BL/6	131,072	<64	64	<2

[a] Titers expressed as reciprocal of highest dilution giving a positive response (three mice per group).

Table III. *Serum anti-T1SP10MN(A) IgG subclass titers (reciprocal) 21, 28, 35, and 91 days after intranasal immunization with 50 μg T1SP10MN(A) and 1 μg CT*

Group/Days	IgG1	IgG2a	IgG2b	IgG3
BALB/c				
+21	16,384	512	2,048	64
+28	32,768	512	512	<64
+35	65,536	8,192	8,192	256
+91	65,536	16,384	8,192	<64
C57BL/6				
+21	131,072	8,192	65,536	1,024
+28	131,072	8,192	65,536	128
+35	262,144	16,384	131,072	2,048
+91	262,144	8,192	262,144	2,048

Serum anti-HIV-1 neutralizing activity

Serum collected from intranasally immunized mice was tested for reactivity to native gp120 and in vitro HIV-1_{MN} neutralizing activity. Intranasal immunization with T1SP10 MN(A) and CT induced serum IgG reactive with HIV-1 $gp120_{MN}$, $gp120_{SF2}$, and $gp120_{IIIB}$ (Table IV). Anti-T1SP10 MN(A) serum contained low but detectable HIV-1_{MN} neutralizing Abs in one of two experiments (Table V). The toxic effect of naive mouse serum in the neutralization assay inhibited our ability to detect low-titered neutralizing Ab responses in experiment two (data not shown).

Mucosal anti-T1SP10 MN(A) Ab responses after intranasal immunization with T1SP10 MN(A)

Fecal samples, vaginal wash samples, and salivary secretions were tested for the presence of anti-T1SP10 MN(A) Abs to determine whether intranasal immunization with T1SP10 MN(A) and CT induced mucosal Ab responses to T1SP10 MN(A). Salivary secretions from BALB/c and C57BL/6 mice contained low levels of both anti-T1SP10 MN(A) IgG and IgA, with IgG being the predominant class of anti-T1SP10 MN(A) Ig detected at all times tested (data not shown). Anti-T1SP10 MN(A) IgA was only occasionally detected in fecal extracts of intranasally immunized C57BL/6 mice at a titer of 1:8 on day 21 and 35 and 1:16 on day 91 (one of six groups positive; data not shown). Although fecal anti-T1SP10 MN(A) IgA responses were detectable at various

Table IV. *Anti-T1SP10MN(A), $gp120_{MN}$, $gp120_{IIIB}$, and $gp120_{SF2}$ end point titers for serum from BALB/c or C57BL/6 mice immunized intranasally with T1SP10MN(A) and CT on day 0, 7, 14, and 28*[a]

Group	T1SP10MN(A)	$gp120_{MN}$	$gp120_{IIIB}$	$gp120_{SF2}$
BALB/c				
Expt. 1	8,192	2,048	1,024	8,192
Expt. 2	16,384	1,024	256	2,048
Expt. 3	16,384	65,536	2,048	16,384
C57BL/6				
Expt. 1	65,536	65,536	8,192	16,384
Expt. 2	131,072	65,536	65,536	16,384
Expt. 3	16,384	32,768	16,384	16,384

[a] Day 35 serum from different experiments was tested for reactivity to the specifiecd Ag. Titers reported as the reciprocal of the last dilution resulting in a positive reading.

Table V. *Neutralization activity of serum after intranasal immunization of C57BL/6 or BALB/c mice with T1SP10MN(A) and CT on day 0, 7, 14, and 28*

Group	No. of Days After Primary Immunization Serum Collected	Anti-T1SP10MN(A) IgG ELISA Titer	Serum Dilution Used for Neutralization Assay	HIV-1_{MN} $TCID_{50}$[a]
BALB/c	+35	1:32,768	1:50	≤750
			1:150	3,000
C57BL/6	+35	1:32,768	1:50	3,000
			1:150	6,000
Naive mouse serum		<1:64	1:50	12,000
			1:150	24,000

[a] HIV-1_{MN} 50% tissue culture infective dose ($TCID_{50}$) at the completion of the neutralization assay.

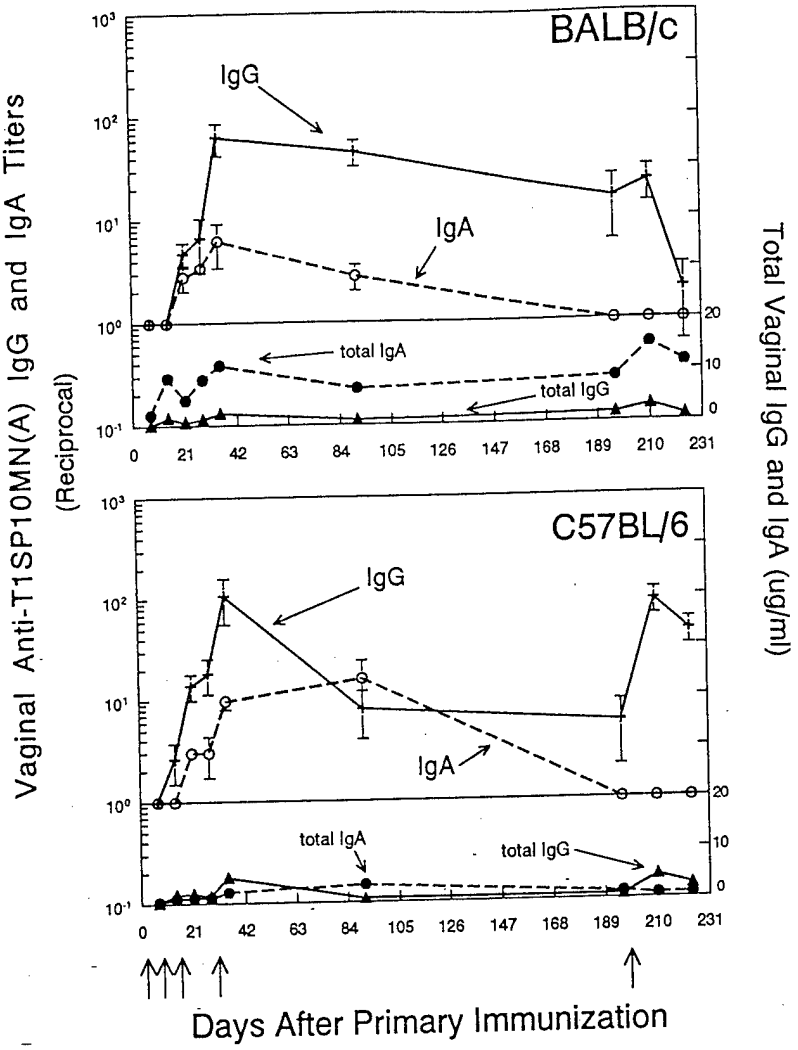

FIGURE 2. Vaginal anti-T1SP10 MN(A) IgG (——) and IgA (- - - -) titers (left axis) and total vaginal IgG (——) and IgA (- - - -) (right axis) after intranasal immunization of BALB/c or C57BL/6 mice with 50 μg T1SP10 MN(A) and 1 μg CT in 10 μl PBS. Anti-T1SP10 MN(A) results are reported as the reciprocal of the last dilution giving a positive response in the ELISA. Arrows on the x-axis indicate day of immunization. The procedure used to collect vaginal secretions results in the dilution of vaginal secretions by a factor of 10 (data not shown) and therefore may dilute low responses beyond the level of detection. Thus, Ab titers in undiluted vaginal secretions are approximately 10-fold higher than shown.

times in C57BL/6 mice, fecal IgA responses were not detected in BALB/c mice at any time tested (data not shown).

Vaginal secretions from both mouse strains tested contained anti-T1SP10 MN(A) IgG as well as IgA (Fig. 2). The procedure used to collect vaginal samples diluted vaginal secretions by a factor of 10 (data not shown); therefore, low anti-T1SP10 MN(A) IgA responses may be present but diluted to undetectable levels by the collection procedure. Consequently, Ab titers in undiluted vaginal secretions are estimated to be 10-fold higher than shown in Figure 2. BALB/c mice had detectable vaginal anti-T1SP10 MN(A) responses by day 21 with an IgG titer of 1:5 and an IgA titer of 1:3 (Fig. 2). BALB/c vaginal anti-T1SP10 MN(A) responses peaked at day 35 with an IgG titer of 1:64 and an IgA titer of 1:6. Vaginal IgG responses remained detectable throughout the study (day 224), while IgA responses were not detectable after day 91 in BALB/c mice. C57BL/6 mice also had vaginal anti-T1SP10 MN(A) IgG Ab responses which were first detectable at 14 days and reached a peak at day 35 with an IgG titer of 1:108 (Fig. 2). Vaginal anti-T1SP10 MN(A) IgA responses in C57BL/6 mice were first detectable 21 days after primary immunization and reached a peak titer of 1:16 on day 91 (Fig. 2). In the two groups of C57BL/6 mice monitored through day 196, vaginal anti-T1SP10 MN(A) IgG was detectable at a titer of 1:6. After boosting at day 196, vaginal anti-peptide IgG titers increased to 1:90 and 1:45 on day 210 and 224, respectively. Anti-T1SP10 MN(A) IgA responses were not detectable after day 91. C57BL/6 and BALB/c mice immunized s.c. with T1SP10 MN(A) (50 μg) and CFA attained high levels of vaginal anti-T1SP10 MN(A) IgG (1:64 titer at day 42) but no IgA was detected (Table II), suggesting that the nasal route of immunization, CT, or both, was responsible for the induction of vaginal anti-T1SP10 MN(A) IgA responses.

Vaginal and fecal samples from various times after immunization were tested for the presence of anti-T1SP10 MN(A) responses that were detectable with a cross-reactive anti-rat SC polyclonal Ab. Vaginal samples collected from both strains of mice with vaginal anti-T1SP10 MN(A) IgA titers >1:4 were positive for anti-T1SP10 MN(A) detectable with anti-SC (Table VI). Fecal samples that contained anti-T1SP10 MN(A) IgA were positive for anti-T1SP10 MN(A) detectable with anti-SC (data not shown).

Anti-T1SP10 MN(A) Ab-secreting cells after intranasal immunization with T1SP10 MN(A) and cholera toxin

A T1SP10 MN(A) Ig ELISPOT assay was performed with mononuclear cells from various anatomic sites to determine the location(s) in the host that contained anti-T1SP10 MN(A) secreting cells. From two independent experiments 33 days after the initial immunization (5 days after the last boost), superficial CLN, SP, MLN, and PP cells were isolated and assayed for the presence of anti-T1SP10 MN(A) Ab-secreting cells (ASC). Although the relative frequency of Ab-secreting cells was different between the strains of mice, the majority of anti-T1SP10 MN(A) Ab-secreting cells were detected in the CLN followed by the SP. BALB/c CLN

Table VI. *Anti-T1SP10MN(A) IgG, IgA, SC containing Ab titers (reciprocal) in serum, and vaginal secretions of mice intranasally immunized with 50 μg T1SP10MN(A) and 1 μg CT on day 0, 7, 14, and 28*[a]

Group/Day	Serum IgG	Serum IgA	Vaginal IgA	Serum SC	Vaginal SC
BALB/c					
+28	16,384	<256	8	<256	+
+28	32,768	<256	4	<256	+
+28	32,768	<256	4	<256	+
+35	131,072	<256	2	<256	−
+91	65,536	<256	<4	<256	−
+91	131,072	<256	4	<256	−
C57BL/6					
+28	131,072	<256	<2	<256	−
+28	131,072	<256	2	<256	−
+28	131,072	<256	8	<256	+
+35	262,144	<256	4	<256	−
+91	524,288	<256	32	<256	+
+91	131,072	<256	8	<256	+

[a] For vaginal samples tested for anti-T1SP10MN(A) Ab responses containing SC, samples were tested undiluted and recorded as positive (+) or negative (−). Each time point represents an individual group of mice containing samples pooled from three to five mice.

cells contained 93 anti-T1SP10 MN(A) IgG ASC/100,000 cells, while only 6 IgA ASC/100,000 cells were detected (Fig. 3). In the spleen, BALB/c mice had 7 IgG ASC/100,000 cells with no detectable IgA ASC (Fig. 3). Compared with BALB/c mice, C57BL/6 mice had a higher frequency of Ag-specific Ab-producing cells in the CLN with 510 IgG ASC and 15 IgA ASC/100,000 CLN cells (Fig. 3). SC populations from C57BL/6 mice contained 52 IgG ASC/100,000 and 4 IgA ASC/100,000 SP cells. A very low frequency of IgG anti-T1SP10 MN(A) Ab-secreting cells was detected in the MLN of C57BL/6 (4 ASC/100,000 cells), while none were detected in the MLN of BALB/c mice (data not presented). No anti-T1SP10 MN(A) Ab-secreting cells were detected in the PP of BALB/c or C57BL/6 mice.

Lymphocyte proliferation after intranasal immunization with T1SP10 MN(A) and CT

CLN, SP, MLN, and PP cells were also tested for in vitro proliferative responses to various doses of T1SP10 MN(A) (100, 10, or 1 μg/ml) 33 days after the primary immunization. Only cells isolated from the CLN and SP responded to T1SP10 MN(A) in vitro. CLN cells from BALB/c mice had undetectable Ag-specific proliferative responses when stimulated with T1SP10 MN(A) in vitro, while CLN cells from C57BL/6 mice had detectable but low Ag-specific proliferative responses (Fig. 4). In contrast, SP cells from both BALB/c and C57BL/6 mice had high proliferative responses when stimulated with T1SP10 MN(A) in vitro (Fig. 4). CLN or SP cells from either strain of mice did not proliferate when stimulated with equivalent amounts of an unrelated HTLV-1 peptide (data not shown).

Ag-specific delayed-type hypersensitivity responses induced after intranasal immunization with T1SP10 MN(A) and CT

To determine whether Ag-specific cell-mediated immune responses could be detected in vivo, DTH responses were determined using an ear swelling assay 35 to 45 days after primary intranasal immunization. Intranasal immunization with T1SP10 MN(A) and CT induced significantly increased DTH responses in both mouse strains evaluated. BALB/c mice developed DTH responses of 45.6×10^{-4} and 38×10^{-4} inches when immunized by the intranasal and subcutaneous routes, respectively (Fig. 5). C57BL/6 mice immunized by the intranasal route had a DTH response of 24.3×10^{-4}, inches whereas animals immunized s.c. had a DTH response of 40.5×10^{-4} inches (Fig. 5).

Discussion

Our attempt to induce mucosal as well as systemic immunity to HIV-1 has relied on the use of the hybrid C4/V3 HIV-1 peptide T1SP10 MN(A) as the vaccine immunogen. The 40-amino acid C4/V3 synthetic peptide contains a T helper (T1), a neutralizing B cell (SP10 MN), as well as a CTL(A) epitope from gp120 of HIV-1 (19–22). T1SP10 MN(A) has been shown to induce type-specific serum-neutralizing Ab responses when systemically administered with CFA in a variety of animal species (18–21). This immunization protocol has also been shown to induce class I-restricted CTL responses in mice (22). Although T1SP10 MN(A) has been shown to induce neutralizing Ab as well as CTL responses when administered systemically, mucosal immune responses are rarely, if ever, induced by systemic immunization. To induce mucosal immune responses to HIV, we immunized mice with the C4/V3 hybrid peptide T1SP10 MN(A) and the mucosal adjuvant CT by the gastric, vaginal, and intranasal routes. Only the intranasal route of immunization induced any detectable immune responses to T1SP10 MN(A). Attempts to induce mucosal and systemic responses to T1SP10 MN(A) by gastric or vaginal routes failed even when multiple immunizations of up to 500 to 1000 μg of T1SP10 MN(A) with CT were used (data not shown). The failure of gastric and vaginal immunization protocols to induce immunity to T1SP10 MN(A) may be due to peptide sensitivity to the gastric environment and the lack of organized mucosal inductive tissues in the vaginal cavity, respectively.

We have provided the first evidence that intranasal immunization with HIV-1 synthetic peptides plus CT induced both high serum IgG responses as well as vaginal IgG and IgA responses. When serum anti-T1SP10 MN(A) IgG titers were compared between mice immunized with T1SP10 MN(A) intranasally or s.c., intranasal immunization induced serum IgG titers comparable to titers detected after systemic immunization. Intranasal immunization also induced vaginal anti-T1SP10 MN(A) IgG and IgA responses. However, s.c. immunized mice only had detectable vaginal anti-T1SP10 MN(A) IgG responses. Therefore, intranasal immunization with T1SP10 MN(A) is as effective as systemic immunization for the induction of serum IgG responses but has the advantage of inducing vaginal IgA in addition to IgG responses. Another major advantage of intranasal immunization is that this route of immunization is noninvasive and thus eliminates the use of needles. This will be an important consideration for large-scale immunization protocols initiated in third-world countries.

The use of CT as an adjuvant was required to induce high titered serum anti-peptide IgG titers as well as vaginal and fecal IgA responses. When C57BL/6 mice were intranasally immunized with 50 μg of T1SP10 MN(A) on day 0, 7, 14, and 28, the serum anti-peptide IgG titer reached 1:32,768 by day 35. However, when 1 μg of CT was coadministered with the peptide, the serum anti-peptide IgG titer reached 1:524,288 by day 35, a 16-fold increase over the titer reached by immunizing with peptide alone. Additionally, C57BL/6 mice immunized with T1SP10 MN(A) and CT maintained serum anti-peptide IgG titers of 1:262,144 91 days after primary immunization (63 days after the last boost) and 1:46,340 196 days after primary immunization (168 days after the last boost). Intranasal boosting of C57BL/6 mice on day 196 increased the serum anti-T1SP10 MN(A) IgG titer to 1:370,727 on day 210 (an 8-fold increase over day 196) and this remained at elevated levels of 1:262,144 on day 224. In contrast, intranasal boosting of BALB/c mice with T1SP10 MN(A) and CT on day

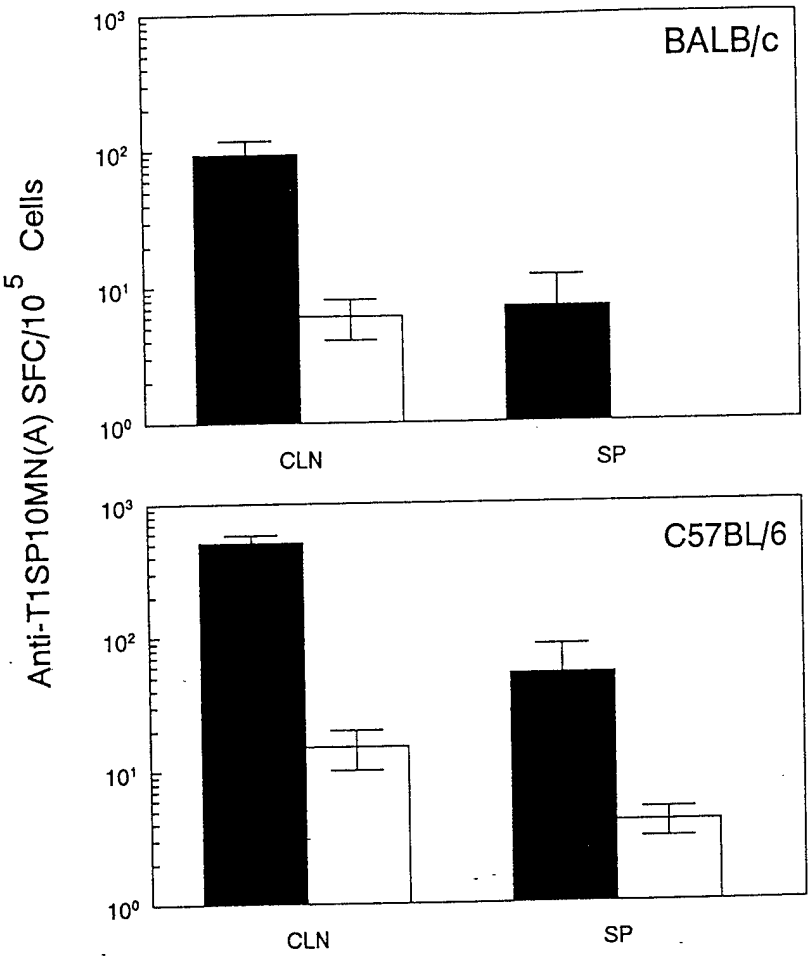

FIGURE 3. Anti-T1SP10 MN(A) IgG (solid boxes) and IgA (open boxes) spot-forming cells (SFC) detected in the cervical lymph node (CLN) and spleen (SP) with the ELISPOT technique after intranasal immunization of BALB/c or C57BL/6 mice with 50 μg T1SP10 MN(A) and 1 μg CT in 10 μl PBS on day 0, 7, 14, and 28. At day 33, CLN and SP cells were removed and assayed for the frequency of anti-T1SP10 MN(A) SFCs per total mononuclear cells assayed.

196 was not associated with an increased serum anti-peptide IgG titer. Because BALB/c mice had detectable serum anti-peptide IgG titers only after several intranasal immunizations (Fig. 1, Day 7 and 14, BALB/c vs C57BL/6); it is likely that BALB/c mice may require repeated boosts to observe an anamnestic serum anti-peptide IgG response in this model. Taken collectively, the data suggest that we have induced long-lived Ag-specific anamnestic responses by intranasal immunization with T1SP10 MN(A) and CT.

The mechanism by which CT acts as a mucosal adjuvant is not clear. Several reports suggest that the adjuvant action of CT may be related to its ability to prime T cells to the coadministered Ag (35, 36), increase permeability to luminal Ags (37), and/or stimulate production of the costimulatory cytokines IL-1 and IL-6 by epithelial cells (38). Due to its association with the development of immediate hypersensitivity responses, the use of CT as an adjuvant in humans seems unlikely (39). However, the development of nontoxic mutants of CT (40) and the related heat-labile enterotoxin of *Eschericia coli* (41) could provide molecules that are able to function as mucosal adjuvants but do not have the toxic side effects of CT. The use of CT as a mucosal adjuvant in this study was not associated with any visible adverse effects.

Serum anti-T1SP10 MN(A) IgG subclass profiles were determined for both strains of mice intranasally immunized with T1SP10 MN(A) and CT as a means of characterizing the immune response to T1SP10 MN(A) as Th1- or Th2-like. Murine T cells bearing the $CD4^+$-cell surface molecule may be classified as Th1 or Th2, based on cytokine secretion profiles of Ag-stimulated cells (42, 43). Th1 cells produce IL-2, IFN-γ, and lymphotoxin in response to specific Ag, whereas Th2 cells do not produce these lymphokines. When stimulated with specific Ag, Th2 cells produce IL-4, IL-5, and IL-6, but Th1 cells do not produce these molecules. Th1-type responses are associated with DTH, a measure of cell-mediated immunity, as well as IFN-γ-dependent, Ag-specific Ab responses of the IgG2a isotype. Th2-type responses are characterized by IL-4-dependent, Ag-specific IgG1 and IgE but weak or no DTH responses. For both strains of mice, Ag-specific IgG1 responses predominated at all examination times, suggesting that a Th2-like response had been induced. In support of this observation, others have shown that mucosal immunization with soluble proteins plus CT as a mucosal adjuvant induced Th2-like responses, as determined by Ag-specific cytokine secretion (44) and IgG subclass profile (45). Although Ag-specific serum IgG subclass profiles suggested that a Th2-type response had been induced, intranasal immunization with T1SP10 MN(A) and CT also induced significant DTH responses and elevated anti-peptide IgG2a responses, characteristics of a Th1-type immune response. Therefore, it appears that intranasal immunization with T1SP10 MN(A) and CT induced an immune response with both Th1 and Th2 characteristics. Although the immune-protective correlates to HIV infection are unknown, detailed analysis of the type of immune responses induced by vaccination will be beneficial for future vaccine development.

Although intranasal immunization with T1SP10 MN(A) and CT induced high serum IgG titers, serum anti-T1SP10 MN(A) IgA was not detected in BALB/c mice and was detected in only one of six groups of C57BL/6 mice. Since mice were immunized via a

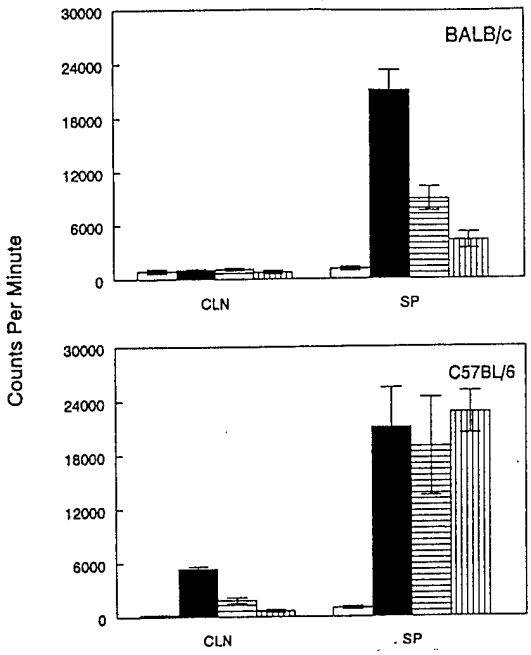

FIGURE 4. T1SP10 MN(A) specific proliferative responses in CLN and SP cells isolated from BALB/c and C57BL/6 mice after intranasal immunization mice with 50 μg T1SP10 MN(A) and 1 μg CT in 10 μl PBS on day 0, 7, 14, and 28. At day 33, CLN and SP cells were isolated and tested for in vitro proliferative responses to T1SP10 MN(A) at 100 μg/ml (solid bars), 10 μg/ml (bars with horizontal lines), 1 μg/ml (bars with vertical lines), or media only (open bars).

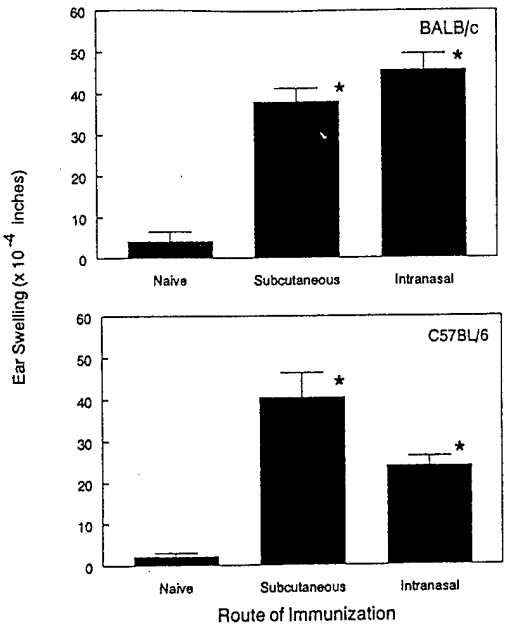

FIGURE 5. DTH responses to T1SP10 MN(A) in BALB/c and C57BL/6 mice 35 to 45 days after intranasal immunization with 50 μg T1SP10 MN(A) and 1 μg CT in 10 μl PBS on day 0, 7, 14, and 28 ($^*p <$ 0.0001 as compared with naive mice). For the intranasal groups, data represent the mean ear swelling response for three independent experiments with three to six mice per experiment.

mucosal route, we anticipated the induction of serum IgA responses. Others have shown that intranasal immunization with influenza HA (46), *Chlamydia trachomatis* (47), *Streptococcus mutans* Ag I/II (48), or recombinant *S. mutans* Pac (49) induced Ag specific serum IgA in addition to IgG responses. However, intranasal immunization with the filamentous hemagglutinin from *Bordetella pertussis* did not induce the production of serum anti-filamentous hemagglutinin IgA even though serum IgG responses were induced (50). Induction of serum IgA responses to intranasally administered Ag therefore varies with the immunizing Ag. However, anti-T1SP10 MN(A) IgA spot-forming cells (Ab secreting cells) were detected in the CLN of both mouse strains used and the spleen of C57BL/6 mice after intranasal immunization with T1SP10 MN(A) and CT even though serum anti-peptide IgA responses were not detected. It is possible that high serum IgG responses competed for Ag in the ELISA and inhibited our ability to detect marginal serum IgA responses. Alternatively, the anti-T1SP10 MN(A) IgA spot-forming cells detected in the CLN may have been transiently migrating through the CLN to mucosal effector sites. This latter hypothesis seems more likely, since anti-T1SP10 MN(A) IgA responses were detected in vaginal secretions in the absence of serum IgA responses. Immunization of mice with recombinant vaccinia virus vectors expressing the capsid protein from human *Papillomavirus* also resulted in the induction of Ag-specific vaginal IgA responses in the absence of serum IgA responses (51).

Vaginal anti-T1SP10 MN(A) IgG and IgA responses remained detectable through day 91 in both strains of mice. C57BL/6 mice had a vaginal IgG titer of 1:8 with an IgA titer of 1:16, while BALB/c mice had vaginal IgG titers of 1:45 and an IgA titer of 1:3. BALB/c mice had low but detectable vaginal IgG responses through the last day of observation, day 224. C57BL/6 monitored on day 196 had detectable anti-T1SP10 MN(A) IgG titers of 1:6, which increased to 1:90 on day 210 after intranasal boosting on day 196. No vaginal anti-T1SP10 MN(A) IgA responses were detected on day 196 or later. It may be that repeated booster immunizations are required to maintain vaginal IgA responses. It is important to emphasize that the procedure used to collect vaginal samples dilutes vaginal secretions by a factor of 10 (data not shown); therefore, the amount of Ab in the actual vaginal secretion is higher than the measured level, and low levels of vaginal anti-T1SP10 MN(A) may be diluted to an undetectable range by this procedure.

Polymeric IgA produced in mucosal effector tissues may be transported to external secretions by epithelial cells expressing the polymeric Ig receptor (pIgR) (52). Polymeric IgA interacts with pIgR at the basolateral surface of pIgR$^+$ epithelial cells, becomes internalized, is transported through the cell, and after enzymatic cleavage of the pIgR, is released onto the mucosal surface as S-IgA. The extracellular region of pIgR that remains associated with S-IgA is known as secretory component (SC) (52). Since serum IgA responses were not detected with the ELISA (except the one group of C57BL/6), it is possible that vaginal anti-T1SP10 MN(A) IgA was locally produced. Indeed, vaginal anti-T1SP10 MN(A) was found to be positive for SC, suggesting that vaginal anti-T1SP10 MN(A) IgA was polymeric in nature and had been actively transported across the mucosal epithelium of the female reproductive tract. Only vaginal washes from intranasally immunized mice containing higher titers of anti-T1SP10 MN(A) IgA reacted with the anti-SC reagent. The low sensitivity of the anti-SC may have inhibited our ability to detect anti-T1SP10 MN(A) SC$^+$ Abs in samples that contained low titers of anti-T1SP10 MN(A) IgA. Vaginal washes from naive mice were not positive for anti-peptide SC Abs, indicating that we were not detecting free SC.

Recent studies have shown that the human female reproductive tract contains the necessary components to be classified as an effector tissue of the mucosal immune system. The fallopian tube, endocervix, ectocervix, and vagina contained IgA, J chain (a component of pIgA), and SC, which are all necessary for locally produced pIgA to be transported to the mucosal surface (26). The male urethral epithelium has recently been shown to contain S-IgA (27). Therefore, both the male and the female reproductive tracts may be able to serve as effector tissues of the mucosal immune system. By taking advantage of the common mucosal immune system, intranasal immunization with T1SP10 MN(A) may induce mucosal immunity in the local environment of both the male and female reproductive tracts, where secretory anti-HIV immune responses could possibly prevent sexually transmitted HIV infection.

The intranasal route of immunization has been successfully used to prevent infection of the female reproductive tract in mice. In a mouse model of salpingitis, intranasal immunization with *C. trachomatis* induced serum anti-*C. trachomatis* IgG, IgM, and IgA Ab responses and vaginal IgG and IgA responses (53). After challenge in the ovarian bursa with *C. trachomatis*, the intranasally-immunized mice shed no *C. trachomatis* from the vagina, while sham-immunized mice had *C. trachomatis*-positive vaginal cultures. Only 12% of the sham-immunized, *C. trachomatis*-challenged mice were fertile after mating, while 80% of the intranasally-immunized mice were fertile after mating. In another study, intranasal immunization with *Mycoplasma pulmonis* rendered mice resistant to intravaginal infection with *M. pulmonis* (54). Unfortunately, this study did not report the immune responses induced by intranasal immunization with *M. pulmonis*. Although the role mucosal immune responses played in these studies was not investigated and the protective correlates were not determined, these reports support the potential of intranasal immunization to prevent infection of the female reproductive tract.

The importance of Ag-specific S-IgA transport across epithelial surfaces to external secretions should be considered when vaccines are being designed to inhibit the spread of sexually-transmitted HIV-1. Passive transfer studies in mice using Ag-specific monoclonal IgA have provided evidence that Ag-specific IgA alone was able to protect against intranasal infection with influenza (55), intestinal infection with *Vibrio cholerae* (56) or *Salmonella typhimurium* (57) as well as gastric infection with *Helicobacter felis* (58). Ag-specific IgA presumably forms immune complexes with the infectious organism and thereby inhibits the interaction of the infectious agent with the host, a protective mechanism known as immune exclusion. Induction of HIV-specific S-IgA responses in the reproductive and gastrointestinal tracts may thereby provide a means to prevent sexually transmitted HIV infections. A macromolecular muticomponent peptide HIV-1 vaccine candidate, VC1, was recently shown to induce HIV-1-neutralizing fecal IgA responses when administered by the oral route with CT, suggesting that anti-HIV IgA may be able to block infection at mucosal surfaces (59). However, serum anti-HIV-1 IgA from HIV-1-infected individuals has been shown to possess both neutralizing (60, 61) and enhancing (62) activity when assayed in vitro. The enhancing effect of anti-HIV-1 IgA was mediated by Fcα receptors and was not significant in the presence of nonenhancing anti-HIV-1 IgG, suggesting that anti-HIV-1 IgG blocked the enhancing effect of anti-HIV-1 IgA (62). Unfortunately, the HIV-1 epitope recognized by the enhancing IgA was not identified. Further studies are required to determine whether mucosal anti-HIV-1 IgA induced by mucosal vaccines enhances or blocks infection at mucosal surfaces and to identify HIV-1 epitopes that block rather than enhance infection.

HIV-1 has the ability to mutate from the initial infectious virus to a multitude of quasi-species in vivo (63) and changes in the V3 region may allow a virus infection that was once neutralizable by specific Ab to become enhanced by specific Ab (64). Although synthetic peptide immunogens have been used to induce protective immunity against a variety of pathogens or their toxic products, including foot-and-mouth disease virus (65), shiga toxin (66), lymphocytic choriomeningitis virus (67), *S. mutans* (68), and group A streptococci (69), it is not clear whether HIV V3 synthetic peptides will be able to induce protective immune responses against HIV infection, given its ability to mutate. We provide the first evidence that intranasal immunization with HIV-1 peptides plus CT as an adjuvant is able to induce Ag-specific serum and vaginal IgG, vaginal IgA, and systemic DTH responses. The serum anti-T1SP10 MN(A) Ab responses induced by this immunization protocol remained elevated for up to 6 mo, while vaginal IgG and IgA responses were detectable through 91 days after the initial immunization. The induction of mucosal immune responses in the male and female reproductive tracts will likely prove to be a critical component of a protective HIV vaccine, regardless of which Ag is used.

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Identification of a Synthetic Peptide that Mimics a HIVgp120 Envelope Conformational Determinant Exposed Following Ligation of gp120 by CD4

By

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Abstract

CD4 ligation of HIV envelope gp120 results in conformational changes in gp120 that lead to exposure of the gp41 fusogenic domain and fusion with the host cell membrane. One determinant at or near the CD4 binding site exposed on gp120 subsequent to CD4 binding is defined by two human mabs termed 17b and 48d. These mabs do not block CD4 binding to gp120; rather, their binding to gp120 is upregulated following CD4 binding. To determine if synthetic peptide mimetopes could be found that reflect conformational determinants on the surface of gp120, synthetic gp120 peptides from ten divergent HIV isolates were screened for their ability to bind to 17b and 48d in ELISA assays. Although mab 48d binds to HIV IIIB recombinant gp120 protein, in our studies 48d selectively bound only to the HIV Can0A V3 peptide and not to HIV IIIBV3 peptide, whereas mab 17b bound none of the peptides tested. Mab 48d bound to the HIV Can0A V3 peptide both in solid phase ELISA and in solution in a competitive ELISA, but could not bind to HIV Can0A V3 peptide bound to human T cells. The HIV Can0A V3 peptide induced anti-HIV antibodies in rhesus monkeys that neutralized the laboratory-adapted HIV MN strain but did not induce antibodies that neutralized HIV IIIB/LAI, HIV SF-2 or HIV RF isolates, nor neutralized HIV primary isolates. These data suggested that the primary sequence of the HIV Can0A V3 loop exists in a conformer that mimics a non-V3 determinant of native gp120 exposed subsequent to CD4 binding on the surface of gp120 of laboratory-adapted HIV strains. Structural studies of the Can0A V3 peptide and/or the 48d mab may provide important information regarding the nature of gp120 conformational changes that occur following gp120 ligation by CD4.

Introduction

The interaction of the Human Immunodeficiency Virus (HIV) surface glycoprotein, gp120, with T cell or monocyte CD4 is a critical event that initiates entry of HIV into host immune cells. Envelope glycoprotein gp120 is therefore an important target for antibodies aimed at interfering with HIV infection. Neutralizing regions of gp120 that have been extensively studied to date include a conformationally-determined CD4 binding site that includes the fourth constant (C4) region (1,2), and third variable (V3) loop region neutralizing determinant of gp120 (3-5). Following the binding of HIV to host cell surface CD4, exposure of gp120 epitopes occurs subsequent to a CD4-induced conformational change in gp120 (6,7). Newly exposed fusogenic components of gp41 trigger virus-cell membrane fusion in a pH-independent manner (6,7). Deletions in the C4 region, particularly of tryptophan at amino acid 427, impair the binding of gp120 to CD4 (7). Select anti-gp120 C4 monoclonal antibodies (mabs) block gp120-CD4 binding (8,9). Amino acid changes in gp120 outside of the C4 region also affect gp120-CD4 binding, including amino acid substitutions in the V3 loop (10).

Recent data suggest that the V3 loop may be physically located in close proximity to the CD4 binding site of gp120 (10), and may participate in virus-host cell fusion (11,12). Amino acid changes in the C4 region of gp120 have been reported to increase mab binding to the amino-terminal side of the V3 loop (10,13), and amino acid changes at the base of the V3 loop can increase the binding of mabs to the C4 region of gp120 (10).

The human anti-HIV envelope mabs 17b and 48d were derived from Epstein-Barr virus-transformed B cells from HIV-infected patients and bind to gp120 near the CD4 binding site (14). These mabs do not block the binding of anti-CD4 binding site mabs such as 15e, but the binding of mabs 17b and 48d to gp120 is blocked by anti-CD4 binding site mabs (14). Amino acid changes in areas shown to be important for CD4 binding result in decreased recognition and neutralization of native gp120 by 17b and 48d (14).

Importantly, soluble CD4 binding to gp120 is not blocked by the mabs 17b and 48d. Rather, the binding of these mabs to gp120 is increased following gp120-CD4 binding (14). Thus, these results suggest that the 17b- and 48d-defined epitopes are exposed on gp120 following CD4 binding.

Because gp120 epitopes exposed following CD4-gp120 binding may be important for virus-cell membrane fusion, the epitopes of gp120 to which mabs 17b and 48d bind are of interest. Our laboratory has synthesized several HIV gp120 hybrid peptides that are comprised of sequences from the C4 and V3 regions of gp120. Our study was designed to use mabs 17b and 48d to examine the possibility that C4 and/or V3 synthetic peptides could mimic the 17b- or 48d-defined epitopes on HIV. We found that mab 48d selectively bound to the gp120 V3 loop peptide derived from the HIV Can0A isolate.

Materials and Methods

Peptide Synthesis. The sequences of synthetic peptides used in this study are shown in Table 1. These include sequences derived from the V3 loop of divergent HIV strains, as well as peptides containing these V3 loop sequences and a helper T cell epitope from the C4 region of gp120. All sequences are taken from the Los Alamos Database (15). Peptide synthesis was performed using F-moc chemistry using an Applied Biosystems 431A peptide synthesizer (Foster City, CA). Peptides were purified by HPLC and molecular mass was determined by electrospray mass spectrometry performed by Dr. Robert D. Stevens, Department of Pediatrics, Division of Genetics and Metabolism, Duke University Medical Center, on a TRIO 2000 VG Biotech, Fisons quadrupole system (VG Biotech, Altrincham, UK). Acetonitrile/water (1:1) containing 1% formic acid was used as the sample diluent and carrier stream, and calibration was performed using horse heart myoglobin. Peptide solutions (10 ul, 200 pM) were injected into the carrier stream and were scanned from 600 to 1000 m/z. The results were analyzed and data printed using the

software provided by the instrument manufacturer (VG Biotech, Altrincham, U.K.).

ELISA assays. ELISA plates were coated with 2ug of peptide antigen per microtiter well in 50 ul/well of binding buffer (0.015 M Na_2C0_3, 0.035 M $NaHCO_3$, 0.02% NaN_3 in distilled water, pH 9.6) and incubated overnight at 4°. Plates were blocked for 2 hr with blocking buffer (3% bovine serum albumin [BSA] in binding buffer) and washed three times with wash buffer (0.05% Tween 20 in phosphate-buffered saline [PBS]). The primary antibody was diluted in serum solvent (95 ml of wash buffer, 5 g of BSA, 2 ml of normal goat serum) and added (60 ul/well) for 1 hr at 20°. Wells were washed three times with wash buffer. Alkaline phosphatase-conjugated anti-human IgG was diluted in serum solvent and added (100 ul/well) for 1 hr at 20°. The wells were then washed as described above. Next 100 ul/well of p-nitrophenyl phosphate in substrate buffer (0.015 M Na_2C0_3, 0.035 M $NaHCO_3$, 0.02% $MgCl_2$) was added and incubated 1 hr at 20° in the dark. Absorbance was read at 405nM on an ELISA reader (Anthros; Denley Instruments Co., Durham, NC). Data are expressed as optical density (OD) at 400nM or as the rarion of experimental (E) OD of 48d mab binding to control (C) mab binding (E/C ratio).

Addition of Soluble Peptide. ELISA assays were performed as described above. Prior to the addition of the primary antibody to the ELISA plate, the antibody was preincubated for 1 hr at 20° in a separate ELISA plate with 20ug of soluble peptide dissolved in serum solvent (95 ml of wash buffer, 5 g of BSA, 2 ml of normal goat serum). Following this incubation, the mixture of antibody and peptide was transferred to the peptide-coated ELISA plate and the ELISA continued.

8M Urea Treatment. ELISAs were performed as described above. Following the first experiment, the ELISA plate was washed three times with wash buffer. If the plate was not to be used immediately, blocking buffer was added to wells and the plate frozen at -20°

and thawed when needed. Then 200 ul of stripping solution (8M urea, 2% sodium dodecyl sulfate [SDS], 2% b-mercapto-ethanol [BME]) was added to each well and incubated for 2 hr at 37°. Plates were washed ten times with wash buffer following the incubation. The plates were then blocked again with blocking buffer and frozen at -20°. On the day of the experiment, the plates were thawed, washed x3 with wash buffer, and a second ELISA performed as described above.

Flow Cytometry. HSB acute lymphoblastic leukemia T (HSB T-ALL) cells were incubated with 2.24 x 10^{-8} M (100ug) C4-V3 Can0A peptide in 50 ul 0.9% saline for 45 minutes at 4°, and washed twice with PBS. Cells were incubated with primary antibody for 30 minutes at 4° and washed as described above. Finally, cells were incubated with goat anti-human IgG-FITC (Organon Teknika Corp., West Chester, PA) for 30 minutes at 4° and washed. All samples were analyzed by flow cytometry methods using an EPICS Profile cytometer (Coulter Electronics, Inc., Hialeah, FL).

Induction of Neutralizing Antibodies. Anti-HIV MN and anti-HIV Can0A antibodies were induced in rhesus monkeys as previously described (16). The ability of rhesus monkey serum to inhibit HIV syncytium formation induced by HIV-infected CEM T cells has been previously described (17), as has the assay of antibody levels against recombinant gp120 from HIV SF-2 (16).

Results

Binding of Monoclonal Antibody 48d to HIV Can0A Peptides. The ability of the human monoclonal antibody (mab) 48d to bind to C4-V3 peptides from divergent HIV isolates was determined using solid phase ELISA. Figure 1A shows the results of these assays (n for each peptide is shown above each bar). Comparison was made between binding of mabs 48d and DUHP20 (a human paraprotein mab used here as a negative control).

Statistical analysis was performed on groups with N of two or greater. Mab 48d reacted strongly with the C4-V3 Can0A peptide (mean ± SEM O.D., 0.970 ± 0.096; $p < 0.01$) and weakly with the C4-V3 MN peptide (O.D. 0.316 ± 0.115; $p = 0.06$). 48d did not react with any of the other C4-V3 peptides tested. A similar pattern of reactivity was seen when the binding ability of 48d to V3 peptides was tested (Figure 1B). 48d again reacted well with the V3 Can0A peptide (O.D. 0.708 ± 0.064; $p < 0.001$), minimally with the V3 MN peptide peptide, and did not react with other V3 peptides tested. The mab 48d therefore selectively strongly bound to the V3 portion of the HIV Can0A peptides.

The three human mabs 48d, 17b and 15e were assessed for their ability to bind the C4-V3 Can0A peptide in a concentration-dependent manner in solid phase ELISA (Figure 2). Reactivity of mab 48d with the C4-V3 Can0A peptide increased with increasing concentrations of antibody, whereas mabs 17b and 15e remained nonreactive at all concentrations of antibody used.

Next, 20ug of soluble V3 Can0A peptide was added to subsaturating amounts of mab 48d at the time of 48d binding to V3 Can0A peptide on ELISA plates. The soluble V3 Can0A peptide (n=2) or soluble C4-V3 Can0A peptide (n=3) significantly inhibited the binding of mab 48d to the plate-bound peptide (Figure 3, $p = 0.043$). Thus, the mab 48d selectively bound the V3 Can0A peptide both in solid phase ELISA and in solution in competitive ELISA.

To determine the site of binding on the C4-V3 peptide of mab 48d, the binding of 48d to the C4-V3 Can0A peptide was compared to its binding to the V3 Can0A peptide. Solid-phase ELISA assays were performed using equimolar amounts of C4-V3, V3 and C4 peptides as well as a mixture of C4 peptide mixed with V3 Can0A peptide. A dilution curve of mab 48d was compared to mab 17b and the human DUHP20 control antibody. Mab 48d reacted with the C4-V3 Can0A peptide, the V3 Can0A peptide, and the C4 + V3 Can0A peptide mixture (Figure 4). Binding of 48d to the C4-V3 Can0A peptide was significantly lower compared to binding of 48d to V3 Can0A ($p = 0.02$) or to binding of

48d to the mixture of the C4 and V3 Can0A peptides ($p = 0.04$). There was no significant difference between the binding of 48d to the V3 Can0A peptide and to the mixture of C4 and V3 Can0A peptides. Thus, monoclonal antibody 48d had specificity for the HIV Can0A V3 loop peptide.

Effect of 8M Urea Treatment on Monoclonal Antibody 48d Binding to V3 Can0A Peptide. Mab 48d, the anti-V3 mab 1727, and a rhesus monkey polyclonal anti-GPGRAF serum (18987) (16) were assayed for reactivity with the C4-V3 Can0A peptide both before and after treatment of the peptide on the plate with the denaturing agent 8M urea. Treatment of the G4-V3 Can0A peptide prior to antibody binding with 8M urea significantly decreased the ability of mab 48d to bind to the C4-V3 Can0A peptide ($p = .0006$), but had no significant effect on the binding of the other anti-V3 monoclonal or polyclonal antibodies that reacted with the C4-V3 Can0A peptide (Figure 5).

Lack of Binding of Monoclonal Antibody 48d to C4-V3 Can0A Peptide Bound to Cells. HSB T-ALL cells were incubated with 100μg C4-V3 Can0A peptide, washed, and then incubated with human mabs 48d, 17b or an anti-V3 human mab, 19b. As shown in Figure 6, mab 19b reacted strongly with the C4-V3 Can0A peptide bound to the surface of HSB cells. In contrast to mab 48d binding the C4-V3 Can0A peptide in solution and in solid phase ELISA, mab 48d did not react with the cell-bound C4-V3 peptide. Mab 17b, which does not react with the C4-V3 Can0A peptide, was used as a negative control, and had the same binding pattern as the human mab DUHP20 negative control.

Ability of the HIV C4-V3 Can0A Peptide to Induce Anti-HIV MN Neutralizing Antibodies. The C4-V3 Can0A peptide was used to immunize rhesus monkeys to determine the ability of the HIV Can0A peptide to induce antibodies against native HIV, and compared with anti-HIV C4-V3 MN antisera. Like anti-HIV C4-V3 MN antisera (16), anti-HIV C4-V3

Can0A peptide antibodies neutralized HIV MN grown in CEM cells, but did not neutralize the laboratory-adapted HIV strains IIIB/LAI, RF or SF-2. In two of three rhesus monkeys immunized with C4-V3 Can0A peptide, anti-HIV Can0A antisera neutralized HIV MN with a mean geometric titer of 4365 (titer is reciprocal of serum dilution which reduced infectious virus titer by 10 fold), but did not neutralize HIV IIIB/LAI, SF-2 or RF in CEM T cells (geometric mean titer of <20). Similarly, neither rhesus anti-HIV MN C4-V3 nor anti-HIV Can0A C4-V3 antisera neutralized any of the HIV primary isolates grown and assayed in PBMC with V3 loop sequences that were near identical to that of HIV MN (primary isolates 301712, 301724, 301715, 301716 and DH012). Thus, the specificity of anti-HIV Can0A antisera was not broadly cross-reactive, but rather was similar in reactivity spectrum to rhesus anti-HIV MN V3 antisera (16). The HIV Can0A isolate itself was not available for neutralization studies.

Discussion

In this paper we have shown that the human anti-gp120 mab 48d selectively bound to the V3 loop sequences of HIV isolate Can0A, and to a lesser extent to the HIV MNV3 peptide, but not to the V3 loop sequences of other HIV isolates. The binding of 48d to the V3 loop of HIV Can0A was observed when the peptide was in solution as well as when the peptide was in solid phase ELISA. These observations are of interest because the HIV gp120 epitope defined by mab 48d is exposed on HIV-positive cellular gp120 following gp120 binding to soluble CD4 (14), and the epitope to which mab 48d binds has not been conclusively mapped.

Mabs 17b and 48d are derived from different HIV-1-infected individuals. The two antibodies compete with each other and with anti-CD4 binding site antibodies for gp120 binding (14). Both mabs have been reported to neutralize a number of divergent HIV-1 isolates (14), leading to the hope that the epitopes for these mabs may be relatively

conserved among some HIV-1 isolates. Regions of gp120 that have been postulated to serve as epitopes for these mabs include the disulfide linkage between C3 and C4, and conserved regions at the base of the stem-loop structures of V1 and V2, C2 and C5 (14).

A key issue for this paper is whether the 48d mab is solely a V3 region mab. Sodroski and colleagues have analyzed a series of HIV HXB2 env mutant molecules expressed in cos cells for reactivity with mabs 48d and 17b (18). Deletion of the entire gp120 V3 loop abrogated the ability of mab 48d to immunoprecipitate gp120; however addition of sCD4 to V3-deleted gp120 resulted in restoration of mab 48d immunoprecipitation of gp120 (18). In a gp120 ELISA assay, mab 48d did not bind well to V3-less gp120 either in the presence or absence of sCD4, whereas mab 17b did bind the same env mutant protein (18). Finally, Sodroski and colleagues demonstrated that a large number of env mutation abrogated mab 48d binding to gp120 suggesting the 48d epitope to be conformational (18). Even though mab 48d binds to the HIVIIIB clone HXB2 envelop protein (14,18), in our study 48d did not bind to the HIVIIIB V3 loop peptide. Moreover, we and others have found that mab 48d strongly neutralizes the HIVMN laboratory-adapted HIV strain, but only weakly bound ($p=.06$) the HIVMN V3 loop peptide (Figure 1). Thus, taken together, these data strongly suggest that mab 48d binds to a complex conformational determinant that is affected by, but does not directly include, the V3 loop. Therefore, the conformers of the HIV Can0A V3 loop peptide likely mimic the 48d-defined gp120 conformational determinant because of the higher order structures formed by the HIV Can0A V3 loop peptide. That the primary amino acid sequence of the HIV Can0A peptide that bound mab 48d was from the HIV V3 region is likely coincidental.

In the ELISA assays, it is interesting that treatment of the HIV Can0A C4-V3 peptide with 8M urea prevented mab 48d binding to the peptide while not affecting the binding of other known antibodies to gp120 V3 region linear determinants (Figure 5). The decrease in binding of 48d following 8M urea treatment suggested but did not prove that mab 48d recognized conformationally determined structural elements of the HIV Can0A V3

peptide that were present only before the treatment with urea. Alternatively, these data could simply reflect the possibility that the affinity of the 48d mab for the Can0 C4-V3 peptide is less than that of the 1727 and 18987 anti-V3 antibodies.

It was also interesting that the C4-V3 Can0A peptide induced neutralizing antibodies against HIV MN but not against HIV IIIB/LAI or HIV RF, since the only V3 peptides other than HIV Can0A reactive with 48d was the HIV MN C4-V3 and V3 peptides. It was disappointing that C4-V3 Can0A-induced antibodies did not neutralize other disparate HIV isolates, nor neutralized HIV primary isolates grown and assayed in peripheral blood mononuclear cells. Thus, the HIV Can0A V3 peptide epitope reactive with-mab 48d is not likely well-expressed on the surface of HIV primary isolates. In studies of others, mab 48d has inconsistently neutralized primary isolates (19). Thus, the notion that the 48d/HIV Can0A V3-defined gp120 epitope is widely conserved among a large number of HIV isolates was not supported by these data. Interestingly, Moore et al. found that mab 48d bound to a large panel of HIV gp120 proteins in a similar pattern to anti-HIV MN murine mabs (20). Vu and colleagues have recently identified a structural basis for the similar specificity of HIV MN and Can0A-induced anti-V3 antibodies (21). They have shown that the NMR-derived preferred conformers of HIV Can0A and HIV MN V3 loop regions in HIV C4-V3 peptides are very similar in secondary structure, though disparate in primary amino acid sequence (21).

It is interesting to speculate that the 48d-defined conformers on the HIV Can0A V3 peptide may be involved in interactions between gp120 and the target host cell during HIV infection of cells. Mab 48d bound the C4-V3 Can0A peptide both in solid phase ELISA and in solution in a competitive ELISA. This binding was lost, however, when the peptide was bound to HSB T-ALL cells. When the HIV Can0A peptide was bound to the cell, it is possible that the 48d-defined epitope on the C4-V3 Can0A was covered. Alternatively, this epitope on the V3 loop may play a functional role, perhaps by interacting with CD4 or another host cell surface molecule and strengthening the initial interaction between virus

and cell, such as the recently described HIV coreceptor, fusin (22). It is also possible that the decreased binding of mab 48d following peptide binding to cells was caused by a conformational change in the peptide, altering the site on the peptide that is recognized by mab 48d. Structural studies of mab 48d-Can0A V3 peptide complexes may provide important information regarding the nature of gp120 changes that occur following gp120 ligation of CD4.

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Figure Legends

Figure 1. Binding of Monoclonal Antibody 48d to C4-V3 and V3 HIV Peptides: Figure shows reactivity of 5ug monoclonal antibody 48d with 2ug C4-V3 peptides (Figure 1A) or V3 peptides (Figure 1B) from divergent HIV isolates bound to ELISA plate [number (n) of experiments performed for each peptide is shown above each bar]. Human monoclonal antibody DUHP20 was used as a negative control. Monoclonal antibody 48d selectively bound to C4-V3 Can0A ($p=0.002$) and V3 Can0A ($p<0.001$) peptides, and bound weakly to the C4-V3 MN ($p=0.06$) and V3 MN peptides. Data represent the OD at 405nM for mab 48d or control DUHP20 mab binding in ELISA assay.

Figure 2. Binding of Monoclonal Antibodies 17b, 48d and 15e to the C4-V3 Can0A Peptide in Solid Phase ELISA Assay: Figure shows reactivity of increasing amounts of monoclonal antibody to 2ug of C4-V3 Can0A peptide bound to ELISA plate. Monoclonal antibody 48d reacted well with plate bound C4-V3 Can0A, whereas monoclonal antibodies 17b and 15e did not react. Data representative of seven experiments.

Figure 3. Soluble V3 Can0A Peptide Can Block Monoclonal Antibody 48d Binding to Plate Bound V3 Can0A Peptide: 20ug of soluble C4-V3 Can0A peptide ($n=3$) or soluble V3 Can0A peptide ($n=2$) was added to subsaturating amounts of monoclonal antibody 48d at the time of monoclonal antibody 48d binding to Can0A peptide on the ELISA plate. The soluble peptide inhibited the binding of 48d to the plate bound Can0A peptide ($p=0.043$).

Figure 4. Monoclonal Antibody 48d Binds to the V3 Sequence of the C4-V3 HIV Can0A Peptide: 4.5×10^{-10} M of the C4-V3 Can0A peptide, (2ug) the C4 peptide alone, the V3 Can0A peptide alone, and a 4.5×10^{-10} M mixture of each of the C4 and V3 Can0A peptides were each plated in microtiter wells, and a dilution curve of monoclonal antibody 48d compared to 17b and a human paraprotein control antibody were studied in ELISA assay.

Monoclonal antibody 48d reacted with the C4-V3 Can0A peptide, the V3 Can0A peptide and the C4 plus V3 Can0A peptide mixture. Binding of 48d to equal molar amounts of the C4-V3 Can0A peptide was significantly lower compared to binding of 48d to the V3 Can0A peptide ($p=0.021$) or to the equimolar mixture of C4 plus V3 Can0A ($p=0.037$). Each data point represents the mean of eight replicates from a total of four experiments.

Figure 5. Effect of Urea Treatment on Monoclonal Antibody 48d Binding to V3 Can0A Peptide: Monoclonal antibody 48d, the anti-V3 monoclonal antibody (1727), and a rhesus monkey polyclonal anti-GPGRAF serum (18987) (16) were assayed for reactivity to 2μg C4-V3 Can0A peptide in solid phase ELISA, both before and after treatment of the peptide on the plate with 8M urea. Following treatment for 1 hr with 8M urea, the plates were washed and the reactivity of the peptide on the plate with the three different antibodies determined. 8M urea treatment of the C4-V3 Can0A peptide significantly decreased the ability of monoclonal antibody 48d to bind to the peptide ($n=8$; $p<.0001$), but had no effect on binding of the anti-V3 monoclonal antibody 1727 ($n=3$) or polyclonal rhesus antiserum 18987 ($n=2$).

Figure 6. Monoclonal Antibody 19b (Anti-V3 Human Monoclonal Antibody) Strongly Reacted with the C4-V3 Can0A Peptide Bound to T Cells Whereas Monoclonal Antibody 48d Did Not Recognize C4-V3 Can0A Peptide Bound to T Cells: HSB T-ALL cells were incubated with 100ug/ml of C4-V3 Can0A peptide, washed, and incubated with 10ug of either monoclonal antibody 17b, 48d or 19b. Monoclonal antibody 19b reacted strongly with the C4-V3 Can0A peptide bound to HSB cells, whereas monoclonal antibody 48d did not react. The activity of 17b monoclonal antibody (which does not react with the C4-V3 Can0A peptide in ELISA assay) was used as a negative control, and had the same binding pattern as the DUHP20 human paraprotein negative control. Data are representative of 3 experiments.

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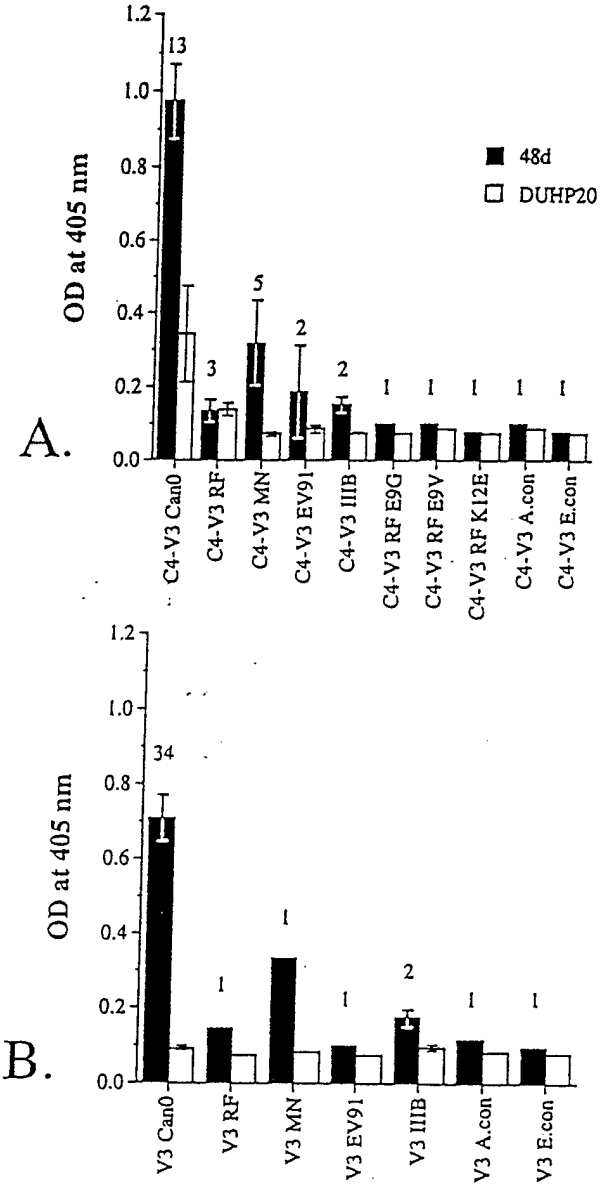
Table 1
Synthetic Peptide Sequences

C4-V3 Can0A	K Q I I N M W Q E V G K A M Y A T R P H N N T R K S I H M G P G K A F Y T T G
C4-V3 MN	K Q I I N M W Q E V G K A M Y A T R P N Y N K R K R I H I G P G R A F Y T T K
C4-V3 IIIB/LAI	K Q I I N M W Q E V G K A M Y A C T R P N N N T R K S I R I Q R G P G R A F V T I
C4-V3 EV91	K Q I I N M W Q E V G K A M Y A T R P G N N T R K S I P I G P G R A F I A T S
C4-V3 A.Con	K Q I I N M W Q E V G K A M Y A T R P N N N T R K S V H I G P G Q A F Y A T G D I
C4-V3 E.Con	K Q I I N M W Q E V G K A M Y A T R P S N N T R T S I T I G P G Q V F Y R T G D I
C4-V3 RF	K Q I I N M W Q E V G K A M Y A T R P N N N T R K S I T K G P G R V I Y A T G
C4-V3 RF E9G	K Q I I N M W Q G V G K A M Y A T R P N N N T R K S I T K G P G R V I Y A T G
C4-V3 RF E9V	K Q I I N M W Q V V G K A M Y A T R P N N N T R K S I T K G P G R V I Y A T G
C4-V3 RF K12E	K Q I I N M W Q E V G E A M Y A T R P N N N T R K S I T K G P G R V I Y A T G
V3 Can0A	T R P H N N T R K S I H M G P G K A F Y T T G
V3 MN	T R P N Y N K R K R I H I G P G R A F Y T T K
V3 IIIB/LAI	C T R P N N N T R K S I R I Q R G P G R A F V T I
V3 EV91	T R P G N N T R K S I P I G P G R A F I A T S
V3 A.con	T R P N N N T R K S V H I G P G Q A F Y A T G D I
V3 E.con	T R P S N N T R T S I T I G P G Q V F Y R T G D I
V3 RF	T R P N N N T R K S I T K G P G R V I Y A T G

Sequences taken from Human Retroviruses and AIDS, 1995. A compilation and analysis of nucleic acid and amino acid sequences. G. Myers, B. Korber, S. Wain-Hobson, R.F. Smith and G.N. Pavlekis, (eds). Published by Theoretical Biology and Biophysics Group, T-10, Mail Stop K710, Los Alamos National Laboratory, Los Alamos, NM 87545.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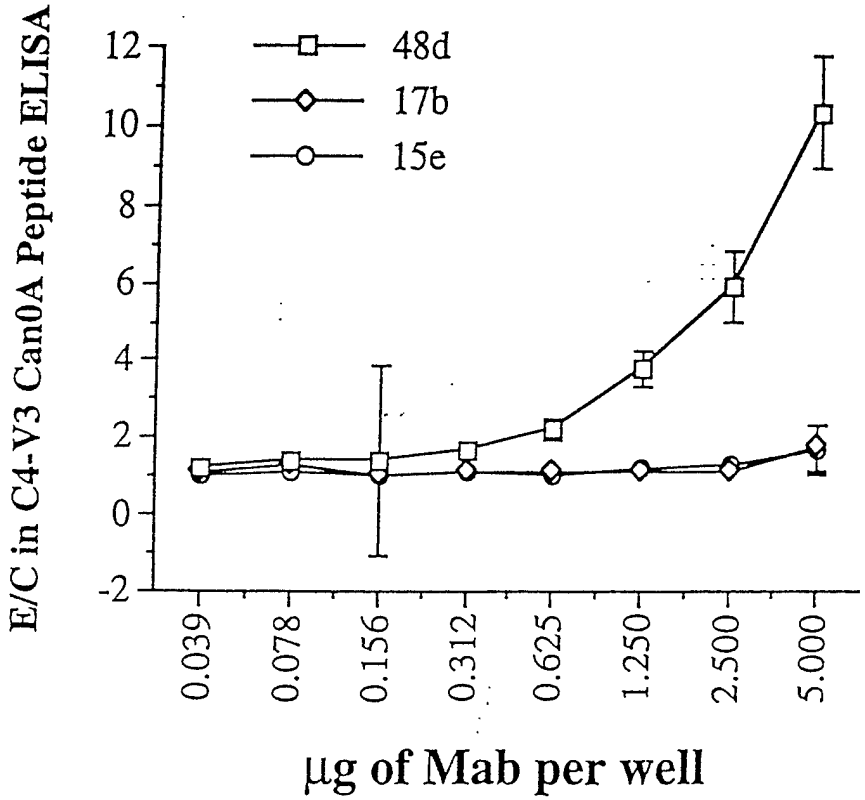
1.0
0.8
0.6
0.4
0.2
0.0
48d
DUHP20
13
3
5
2
2
1
1
1
1
1
C4-V3 Can0
C4-V3 RF
C4-V3 MN
C4-V3 EV91
C4-V3 IIIB
C4-V3 RF E9G
C4-V3 RF E9V
C4-V3 RF K12E
C4-V3 A.con
C4-V3 E.con
B.
OD at 405 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
34
1
1
1
2
1
1
V3 Can0
V3 RF
V3 MN
V3 EV91
V3 IIIB
V3 A.con
V3 E.con

Figure 1


E/C in C4-V3 Can0A Peptide ELISA
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10
8
6
4
2
0
-2
48d
17b
15e
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0.156
0.312
0.625
1.250
2.500
5.000
µg of Mab per well

Figure 2

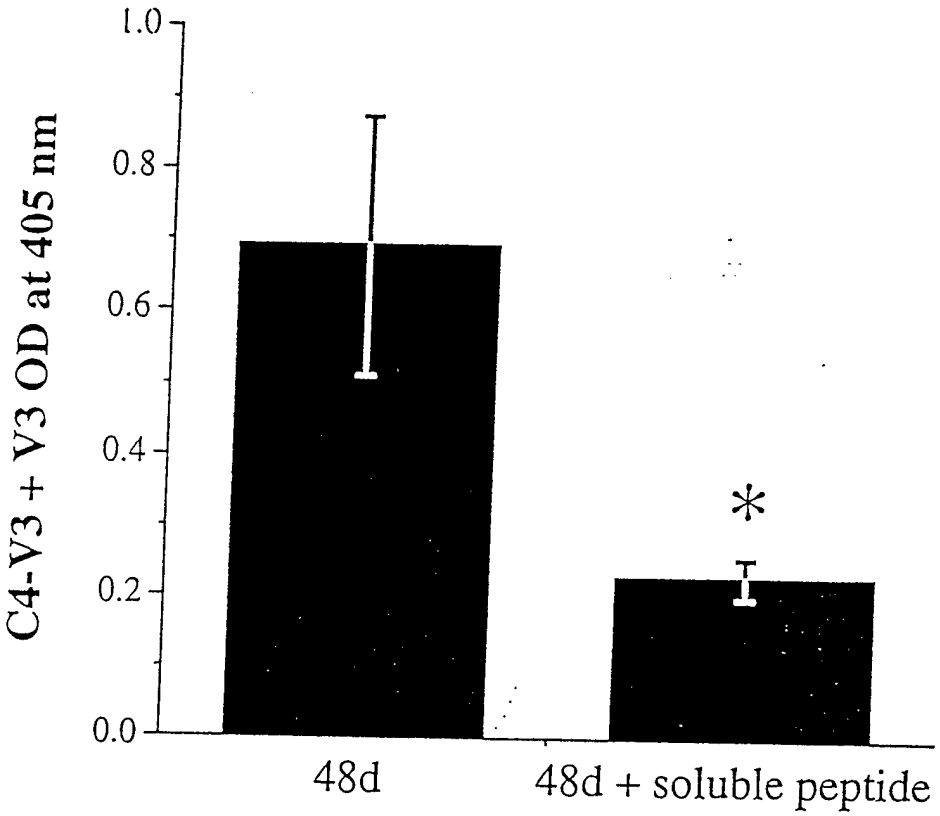
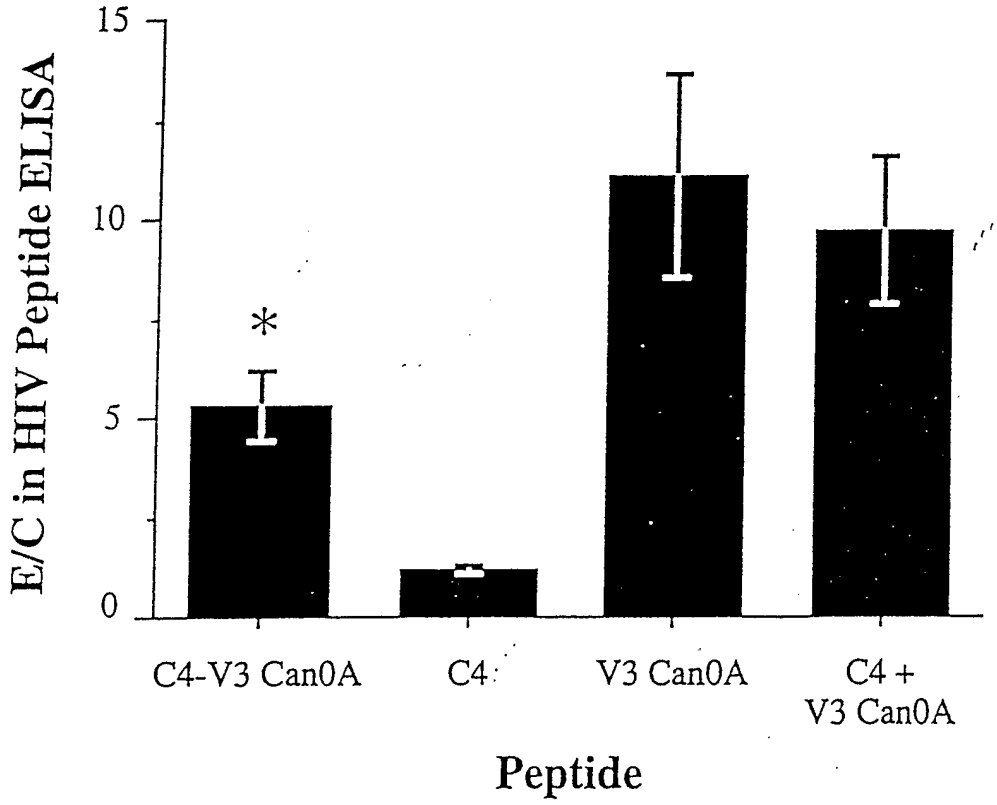


Figure 3


E/C in HIV Peptide ELISA
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10
5
0
*
C4-V3 Can0A
C4
V3 Can0A
C4 +
V3 Can0A
Peptide

Figure 4

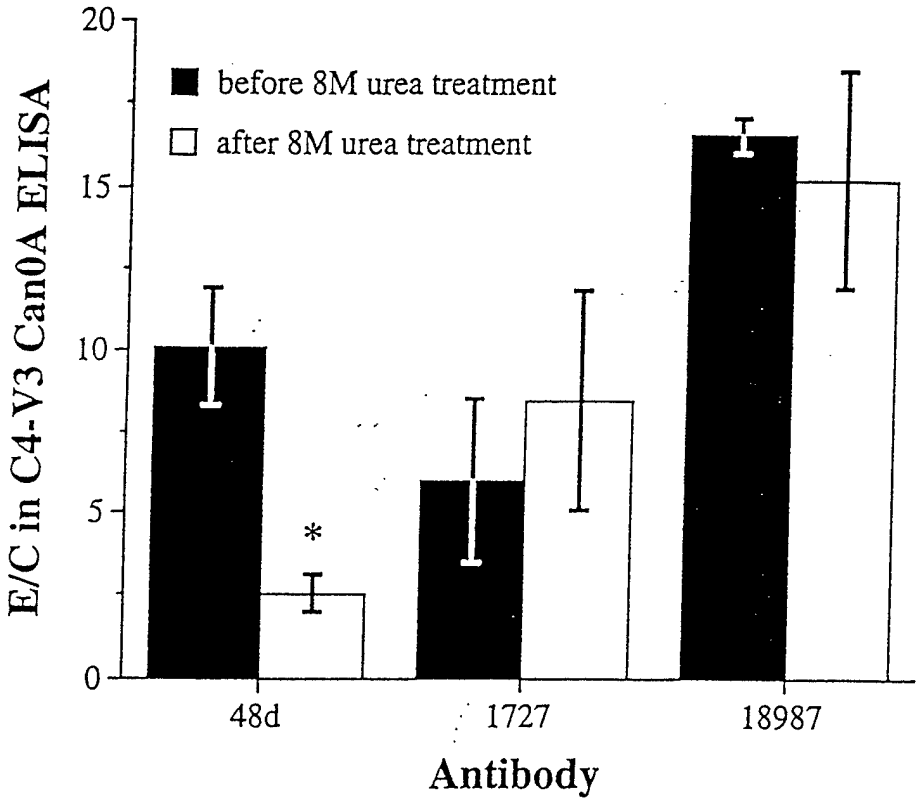
20
15
10
5
0
E/C in C4-V3 Can0A ELISA
before 8M urea treatment
after 8M urea treatment
*
48d
1727
18987
Antibody

Figure 5

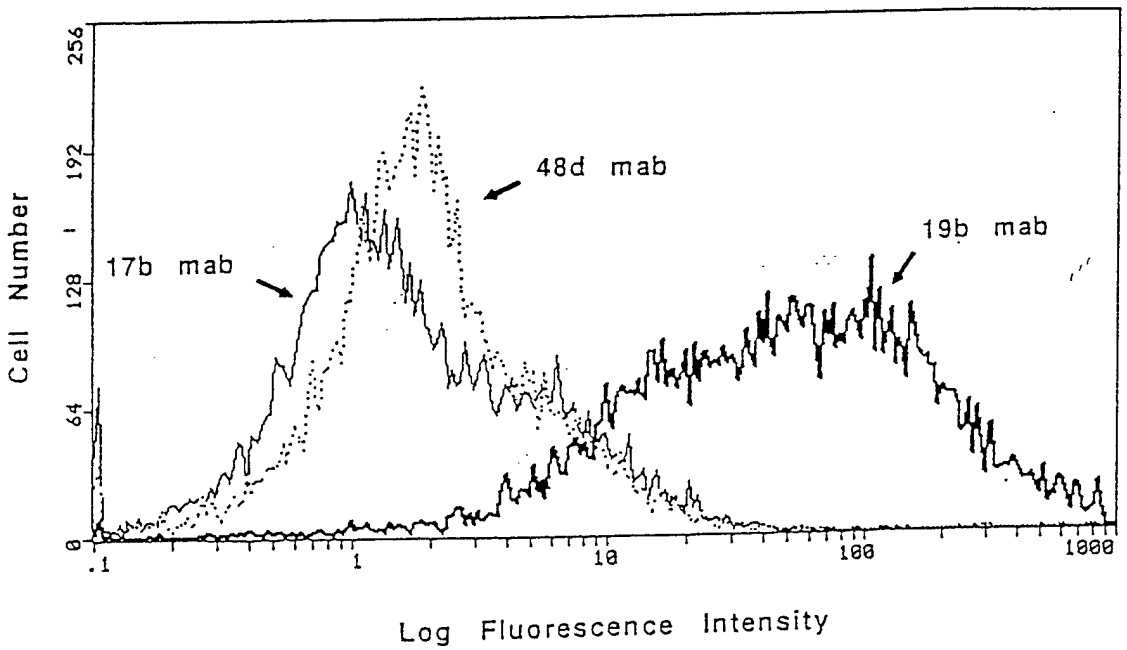
Cell Number
256
192
128
64
0
.1
1
10
100
1000
17b mab
48d mab
19b mab
Log Fluorescence Intensity

Figure 6

The V3 Domain of SIVmac251 gp120 Contains a Linear Neutralizing Epitope

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Running Head: V3 neutralization of SIVmac251

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ABSTRACT

Antisera to 21 synthetic peptides containing hydrophilic sequences of simian immunodeficiency virus strain mac251 (SIVmac251) gp120 and gp32 were tested for the ability to neutralize SIVmac251. Goat antisera raised to peptides SP-1 and SP-1V containing the carboxy-terminal portion of the V3 domain of SIVmac251 gp120 between amino acids (a.a.) 327-339 inhibited syncytium formation (90% inhibition at a 1/1024 dilution) and cell killing of CEMx174 cells by SIVmac251 (50% inhibition of cell killing at a dilution of 1/5832), SIVDeltaB670 (1/568) and SIVsmH4 (1/740). Neutralizing antibodies to SIVmac251, SIVDeltaB670 and SIVsmH4 could be adsorbed by peptides containing a neutralizing V3 sequence of SIVmac251 gp120 (GLVFHSQPIND, amino acids 329-339) but not by peptides lacking this sequence. This V3 neutralizing region corresponds to a homologous V3 neutralizing site within HIV-2 gp120 reported by Björling et al. 1991, 1994). Antibodies in 20 of 31 sera obtained from rhesus macaques infected with SIVmac251 reacted with a peptide containing the entire V3 sequence of SIVmac251 gp120; whereas no sera contained antibodies reacting with the V3 neutralizing site between amino acids 329-339. Low levels of antibody-mediated recognition and subsequent lack of selective pressure against this linear V3 neutralizing site might in part explain why sequences within this region do not vary during the course of SIV infection.

INTRODUCTION

Human immunodeficiency virus types 1 and 2 are lentiviruses that are etiologically linked to AIDS (Barré-Sinoussi et al. 1983; Levy et al., 1984). Simian immunodeficiency viruses (SIV) are non-human primate lentiviruses with biological characteristics and genomic organization similar to both HIV-1 and HIV-2 (Daniel et al., 1985; Letvin et al., 1985; Murphey-Corb et al., 1986; Chakrabarti et al., 1987; Franchini et al., 1987; Hirsch et al., 1989). Infection of rhesus monkeys with some SIVs results in an AIDS-like disease characterized by destruction of CD4+ T-cells and the onset of opportunistic infections (Daniel et al., 1985; Letvin et al., 1985; Murphey-Corb et al., 1986). Strains of HIV-2 have the closest genetic similarity to SIVs infecting rhesus macaques (SIVmac) and sooty mangabeys (SIVsm) (Kornfeld et al., 1987; Gardner and Luciw, 1988; Hirsch et al., 1989; Gao et al. 1992). Comparison of nucleotide sequences within the pol and env genes and the long terminal repeat regions of HIV-2 indicates that HIV-2, SIVmac and SIVsm can be grouped together phylogenetically even though they are isolated from different species (Chakrabarti et al., 1987; Franchini et al., 1987; Hirsch et al., 1989; Gao et al. 1992).

There are substantial functional and structural similarities among the envelope glycoproteins of HIV-1, HIV-2 and SIV. The envelope genes of HIV-1, HIV-2 and SIV (Veronese et al., 1989) encode polyprotein precursors that, upon proteolytic processing,

give rise to gp120 external envelope glycoprotein and gp41/32 transmembrane glycoproteins. The envelope glycoproteins of HIV-1 and SIV have 40% amino acid sequence identity; whereas HIV-2 and SIV are approximately 75% identical (Chakrabarti et al., 1987; Franchini et al., 1987; Hirsch et al., 1989; Myers et al., 1993). Based on the non-random clusters of sequence changes found in various HIV-1 and HIV-2 isolates, it is possible to divide the amino acid sequences of HIV-1 and HIV-2 gp120 into five constant and variable (V) domains (Myers et al., 1992). While SIV gp120 also has V1, V2, V4 and V5 regions homologous to those found in HIV-1 and HIV-2 gp120, the putative V3 domain does not vary during the course of SIV infection (Burns and Desrosiers, 1991; Johnson et al., 1991; Overbaugh et al., 1991; Almond et al., 1992). However, for both SIV and HIV-1, amino acid changes within V3 are associated with altered cell and tissue tropism (Shioda et al., 1991; Cann et al., 1992; Hwang et al., 1992; Kodama et al., 1993; Campbell and Hirsch, 1994; Hirsch et al., 1994; Kirchhoff et al., 1994). The V3 domain of HIV-1 gp120 is bounded by cross-linked cysteines and contains dominant type specific neutralizing sites (Palker et al., 1988; Rusche et al., 1988; Goudsmit et al., 1988, Kinney-Thomas et al., 1988). Similarly, Björling et al. (1991, 1994) have reported two linear neutralizing sites within and adjacent to the C-terminus of the V3 region of HIV-2 gp120.

Despite antigenic similarities of gp120 from SIVmac/sm and HIV-2 (Robert-Guroff et al., 1992) as well as the identification of

linear neutralizing sites within V3 of HIV-2 gp120 (Björling et al., 1991, 1994) and the phylogenetic grouping of SIVmac/sm with HIV-2 (Chakrabarti et al., 1987; Franchini et al., 1987; Hirsch et al., 1989), it has not been possible to demonstrate that the V3 domain of gp120 from SIVmac strain 251 contains a linear neutralizing site (Robert-Guroff et al., 1992; Javaherian et al., 1992). Previous studies have shown that, in contrast to the linear neutralizing sites within HIV-1 V3, the principal neutralizing determinant (PND) of SIVmac251 is conformational (Haigwood et al., 1992; Javaherian et al., 1992, 1994). Here we provide corroborative data regarding the conformational nature of the PND of SIVmac251 but also report confirmation in 3 independent laboratories (T.P., A.L., D.M.) that anti-peptide antisera raised to a linear determinant within the C-terminal region of SIVmac gp120 V3 can neutralize SIVmac251 as well as related viruses.

MATERIALS AND METHODS

Peptides

Deduced amino acid sequences of SIVmac251 envelope glycoproteins are from Franchini et al. (1987) and the Los Alamos Database (Myers et al., 1992). Within the putative V3 domain, reported sequences from these sources differ in two envelope amino acids (a.a. 337, 338); thus, V3 peptides SP-1 and SP-1V were synthesized containing sequences from Franchini et al. (1987) and the Los Alamos database (Myers et al., 1993), respectively (Table

1). Hydrophilic amino acid sequences of SIVmac251 envelope were identified by computer analysis using the method of Kyte and Doolittle (1982). Peptides SP-1, 1V, 2-7, 17, 18, V5 as well as peptides with the prefix DP were synthesized at the Duke University Center for AIDS Research Peptide Synthesis Core Facility on an Applied Biosystems Inc. (ABI) 431A Peptide Synthesizer using T-boc or F-moc protecting groups. Peptides SP-8 to SP-16 were synthesized at the Howard Hughes Medical Institute Durham Biopolymer Laboratory on an ABI 431A Peptide Synthesizer using F-moc protecting groups. Peptides RG-1, RG-2 and alanine-substituted peptides A1-A11 in which each amino acid (a.a.) within the V3 sequence GLVFHSQPIND (a.a. 329-339) was sequentially replaced with alanine were synthesized at Research Genetics, Inc., Huntsville, AL. Peptides coupled to carrier proteins and used for immunization were evaluated by analytical reverse phase HPLC; these peptides were judged to be 55-70% pure, depending on the length of the peptide. Peptides used for adsorption of antisera were estimated to be at least 75% pure by HPLC.

Antisera and antibodies

Synthetic peptides with reduced cysteines were covalently linked to either tetanus toxoid (TT, kindly provided by Dr. Frank McCarthy, Wyeth Laboratories) or keyhole limpet hemocyanin (KLH, Calbiochem, San Diego, CA) at a molar ratio of at least 30:1 (peptide to carrier protein) with m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, Sigma Chemical Co., St. Louis, MO)

as previously described (Green et al., 1982). Goats (N = 36) were immunized with peptide conjugates (5 mg) emulsified in complete (day 0) and incomplete (days 7, 14, 28) Freund's adjuvants injected subcutaneously in 4 quadrilateral dorsal sites. Additional booster immunizations every two weeks for 2-4 months were also administered if anti-peptide antibody titers were less than 1/1000 in ELISA. Guinea pigs (N = 12) were also immunized s.c. with peptide (SP-1, 1V, SP-16)-KLH conjugates in Freund's complete and incomplete adjuvants or with cyclized peptide SP-4 for production of antisera at Hazelton Research Products, Inc., Denver, PA. Additional antisera were raised to DP-314-KLH in C57BL/6 mice (N = 6) either by intranasal administration of 50 ug of peptide-conjugate with cholera toxin on days 0, 7, 14 and 28 (Staats et al., 1996) or by s.c. immunization with complete (day 0) and incomplete Freund's adjuvants (days 7, 14 and 28) as previously described (Palker et al., 1992). Pre-immune and immune sera collected 7-10 days after booster immunizations were stored at -70°C until use.

Sera from 31 macaques experimentally infected with SIVmac251 were provided by Dr. Norman Letvin, Beth Israel Hospital and Harvard University. Polyclonal antiserum to recombinant gp120 from the SIV molecular clone 1A11 was provided by P. Luciw, University of California, Davis, CA and K. Steimer, Chiron Corp., Emeryville, CA. Monoclonal antibodies (mab) KK8, KK45, KK46 and KK65 to SIVmac251 gp120 (Kent et al., 1991, 1992) as well as SIVmac239 gp130 and HIV-1IIIB/LAI gp120 were obtained from the NIH AIDS

Research and Reference Reagent Program operated by McKesson BioServices Corp., Rockville, MD. Monoclonal antibodies to HIV-2 gp120 were provided by Dr. James Hoxie, University of Pennsylvania, Philadelphia, PA.

Viruses and cell lines

The HuT-78 and CEMx174 human T-cell lines were obtained from Dr. Barton Haynes, Duke University Medical Center and Dr. James Hoxie, University of Pennsylvania, Philadelphia, PA, respectively. Cells were grown at 37 °C in a humidified chamber containing 5% CO2 at 1-5 x 10^5/ml in RPMI-1640 media containing 100 U/ml penicillin, 100 µg/ml streptomycin and 20% heat-inactivated (56 °C, 30 min.) fetal bovine serum that had been screened for mycoplasma contamination (Sigma Chemical Co., St. Louis, MO). SIVmac251 was obtained from the NIH AIDS Research and Reference Reagent Program operated by McKesson BioServices Corp., Rockville, MD. Virus pools were prepared in CEMx174 cells, titered for syncytia forming units and infectious units as previously described (Langlois et al., 1991) and stored in aliquots at -70 °C.

Neutralization assays

A syncytium inhibition assay for detecting antibodies that neutralized SIVmac251 was performed as previously described (Langlois et al., 1991). Briefly, 30 µl of pooled viral supernatants containing 100 syncytia-forming units were added to wells of a 96-well microtiter plate (Costar half area wells, cat.

#3696) containing 30 µl of two-fold serial dilutions of heat-inactivated (56 °C, 30 min.) pre-immune or immune serum. After incubating the microtiter plate at 37 °C for 30 min., HUT-78 or CEMx174 cells (3×10^3 in 30 µl) were added and plates were further incubated in a humidified chamber at 37 °C for 3-4 days. The number of syncytia were then counted using an ocular grid. The neutralizing titer was expressed as the reciprocal serum dilution that inhibited syncytium formation by 90% as estimated by plotting the number of syncytia versus serum dilution. High titered neutralizing antiserum from a rhesus monkey infected with SIVmac251 was included in each serum panel as a positive control. To demonstrate specificity, neutralizing anti-peptide antibodies were adsorbed by incubating equal volumes of antisera with either cognate or negative control peptides (1 mg/ml) for 30 min. at 23 °C prior to use in neutralization assays. Adsorption of anti-peptide antisera was also performed in a similar manner with SIVmac239 gp130 and HIV-1IIIB/LAI gp120 (10-50 µg).

Neutralizing antibodies to SIVmac251, SIVDeltaB670 and SIVsmH4 were also detected with a modified cell viability assay previously used to measure neutralization of HIV-1 in MT-2 cells (Montefiori et al., 1988). For the assay, cell free virus (50 µl containing 0.5-1 ng p27) was added to multiple dilutions of test sera (100 µl) in triplicate wells of 96-well microdilution plates and incubated at 37°C for 30 min before adding CEMx174 cells (10^5 cells in 100 µl added per well). Cell densities were reduced and media replaced after 3 days of incubation. Infection leads to

extensive syncytium formation and virus-induced cell killing in approximately 6 days when neutralizing antibodies are not present. Neutralization was measured by staining viable cells with Finter's neutral red in poly-L-lysine coated plates. Neutral red uptake by CEMx174 cells was linear from 3.1×10^4 to 5×10^5 viable cells/well, corresponding to A_{540} values of 0.25 to 1.6. Percent protection was calculated by taking the difference in absorption (A_{540}) between test wells (cells + serum sample + virus) and virus control wells (cells + virus) and dividing by the difference in absorption between cell control wells (cells only) and virus control wells. Assays were harvested when virus-induced cell killing in virus control wells was greater than 70% but less than 100%. Neutralizing titers are given as the reciprocal of the plasma dilution required to protect at least 50% of cells from virus-induced killing. To evaluate the specificity of neutralizing antibodies in the cell viability assay, anti-peptide antisera were pre-adsorbed with cognate or negative control peptides as described for the syncytium inhibition assay.

ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to measure anti-peptide antibody titers in sera from immunized and SIV-infected animals. For the assay, 50 µl of peptide or protein in CBC buffer (15mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at a concentration of 40 µg/well was added to wells of a 96 well microtiter plate (Costar #3590, Cambridge, MA) and incubated overnight at 4 °C.

Wells were then emptied and incubated with 200 µl of blocking buffer (CBC buffer with 3% bovine serum albumin and 0.1% sodium azide) at 23 °C for 2 hr and then stored at -20 °C until used. ELISA plates were washed three times with 200 µl/well of ELISA wash buffer (0.15 M NaCl with 0.05% Tween 20) prior to addition of serum samples. Samples diluted in serum diluent [PBS, 0.05% Tween 20, 5% normal goat serum, 5% (w/v) bovine serum albumin] were added to ELISA wells (100 µl/well) and incubated for 90 min at 23 °C or overnight at 4 °C. Wells were washed three times with 200 µl/well of ELISA wash buffer, further incubated for 60 min at 23 °C with 100 µl/well of alkaline phosphatase conjugated antiserum to goat, guinea pig or human IgG (Cappel, West Chester, PA) diluted 1/7500 in serum dilution buffer lacking 5% goat serum. ELISA microtiter plates were then washed three times with wash buffer and then reacted for 45 min at 23 °C with 100 µl of the alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) at 1 mg/ml in buffer containing 50 mM carbonate buffer, pH 9.8 and 1 mM $MgCl_2$. Optical density readings were evaluated on a Titertek Multiskan II ELISA plate reader (Flow Laboratories) at 405nm. Samples were considered positive if the optical density reading was at least two-fold higher than the optical density reading obtained with pre-immune or normal serum samples at the same dilution.

RESULTS

Neutralization of SIVmac251, SIV Delta B670 and SIVsmH4

Twenty-one peptides (Figure 1, Table 1) containing hydrophilic amino acid sequences of SIVmac251 gp120 and gp41/36 were synthesized, linked either to tetanus toxoid or keyhole limpet hemocyanin (KLH) and used to immunize 36 goats, 12 guinea pigs [SP-1, 1V, 16 linked to KLH and cyclized SP-4 (no carrier)] and 6 mice (DP-314-KLH). Antibody titers in ELISA against the immunizing peptide ranged from 1/50 to 1/25,600 in goats (Table 1), 1/25,600 to 1/102,400 in guinea pigs (data not shown) and 1/32,768 to 1/65,536 in mice. Antisera and pre-immune sera were tested for the presence of neutralizing antibodies in two laboratories with a syncytium inhibition assay [T.P., A.L.(anti-V3 antisera)] and also with a cell viability assay (D.M., anti-V3 antisera). Only antisera raised to peptides containing sequences of SIVmac251 V3 [amino acids (a.a.) 327-346] neutralized SIVmac251 (Table 1). Neutralizing titers in various goat sera ranged from 1/20-1/1024 for 90% inhibition of syncytium formation and 1/20-1/5832 in the cell viability assay (50% inhibition of cell killing). Antisera to SP-1V also neutralized SIVDeltaB670 and SIVsmH4 (50% inhibition of cell killing at 1/568 and 1/740, respectively). For comparison, neutralizing titers in sera from macaques chronically infected with SIVmac251 ranged from 10^{-3} to 10^{-5} in both inhibition of syncytium formation and cell killing assays. Neutralizing antibodies were detected in antisera raised both to V3 peptides SP-1 and SP-1V in which sequences vary at

amino acids 337 and 338 (Table 1). Antisera raised in guinea pigs against cyclized and linear V3 peptides contained lower levels of neutralizing antibodies (1/20, data not shown) than those detected in sera from goats immunized with V3 peptides. Induction of neutralizing antibodies in goats by immunization with either SP-1V or SP-1 linked to KLH was highly variable and required from 5-10 immunizations per animal. Of 11 goats immunized with peptides containing sequences of SIVmac251 V3, only 3 (#18, 125, 246) produced neutralizing antibody titers greater than 1/100. Collectively, these data indicate that the linear neutralizing V3 site within SIVmac251 gp120 V3 is weakly immunogenic in both goats and guinea pigs. In addition, mab BC11, DC9, BB12, DF11, DA6 and FH12 raised to HIV-2 gp120 reacted strongly with SIV peptide SP-6 (a.a. 76-99) in ELISA but did not neutralize SIVmac251 (data not shown).

To evaluate the specificity of neutralizing anti-V3 antibodies, goat antisera to SP-1 and SP-1V (a.a. 327-346) were adsorbed with peptides prior to testing in neutralization assays. Adsorption of anti-SP-1V #18 antiserum with either SP-1 or SP-1V abolished neutralization of SIVmac251 in both syncytium formation and cell viability assays; whereas adsorption with equivalent amounts of peptide SP-5 containing sequence outside of V3 had no effect (Figures 2A, 3). Adsorption with SIVmac239 gp130 but not with HIV-1IIIB/LAI gp120 also removed all neutralizing activity from goat #246 antiserum raised to SP-1V (data not shown). Adsorption experiments were also performed with peptides DP-146 (a.a. 337-

346) and DP-147 (a.a.329-339) containing overlapping C-terminal or N-terminal V3 sequences of SP-1V. Peptide DP-147 but not DP-146 adsorbed neutralizing antibodies from anti-SP-1V antisera in a dose-dependent manner when tested in syncytium inhibition assay (Figures 2B, 3). These results indicated the presence of a linear neutralizing site between a.a. 327-339.

With the cell viability neutralization assay, similar results were obtained in that peptides SP-1, SP-1V and DP-147 completely adsorbed neutralizing antibodies in goat anti-SP-1V antiserum #246 (Figure 4). Adsorption of anti-SP-1V antiserum #246 with SP-1 or SP-1V also completely adsorbed neutralizing antibodies to SIVDeltaB670 (pre-adsorption neutralizing titer = 1/568, post-adsorption titer <1/10) and SIVsmH4 (pre-adsorption neutralizing titer = 1/740, post-adsorption titer <1/10); whereas adsorption with negative control peptide SP-13 did not. To delineate more precisely the linear neutralizing site within V3 of SIVmac251, we performed adsorption experiments with truncated peptides and with V3 peptides in which sequential alanine substitutions were introduced into amino acids 329 to 339 (Figure 4). Peptides DP-150, 151, 152, and 153 containing SIV V3 sequences within or including a.a. 331-339 did not adsorb neutralizing anti-V3 antibodies raised to peptide SP-1V (a.a.327-342). Neutralizing antibodies were also not adsorbed by peptides DP-171 (a.a. 329-337) or DP-176 (a.a. 329-338). However, peptide DP-154 (a.a. 329-339) completely adsorbed neutralizing antibody activity in anti-SP-1V antisera (Figure 4). Adsorption of anti-SP-1V goat

#246 antiserum with peptides containing sequential alanine substitutions revealed that replacement of amino acids F^{332}, H^{333}, S^{334}, Q^{335}, P^{336} or D^{339} with alanine decreased the ability of peptides to adsorb neutralizing anti-V3 antibodies by greater than 50%. Also, further mapping of the sites recognized by the non-neutralizing monoclonal antibodies (mab) KK45 and KK46 to the V3 region of SIVmac251 gp120 (Kent et al., 1991, 1992) indicated that these mab bound to regions outside of the minimal neutralizing site (a.a.329-339) defined in this study (Table 2). In ELISA, mab KK45 and KK46 bound to both SP-1V and DP-147 (a.a. 327-339) but not to DP-154 (a.a. 329-339) containing the minimal linear neutralizing site (Table 2). In contrast, goat anti-SP-1V antisera #125 and #18 both neutralized SIVmac251 in syncytium inhibition assay and contained antibodies that bound strongly to DP-154 in ELISA (titer = 1/12800). Results allow the mapping of a minimal neutralizing region of SIVmac251 to amino acids 329-339 (DP-154: GLVFHSQPIND, a.a. 329-339) and further demonstrate that mab KK45 and KK46 binding to an epitope displaced by 2 amino acids from this neutralizing site (DP-147: MSGLVFHSQPIND, a.a. 327-339) does not result in neutralization of SIVmac251.

<u>V3 peptides do not adsorb neutralizing antibodies raised to recombinant or native SIV gp120.</u>

Antiserum to recombinant SIV 1A11 gp120 and antisera from rhesus macaques infected with SIVmac251 were adsorbed with V3 peptides SP-1 and SP-1V or non-SIV peptide and then tested for neutralizing antibodies against SIVmac251 in syncytium inhibition

and cell killing assays. Following adsorption with an excess of V3 peptides, there were no detectable decreases in neutralizing antibody titers in either anti-SIV 1A11 gp120 antiserum (pre- and post-adsorption titers of neutralizing antibodies = 1/3200) or sera from SIVmac251-infected macaques (N=6) even though anti-V3 antibodies were detectable in anti-SIV 1A11 gp120 antiserum by ELISA and despite that recombinant SIV 1A11 gp120 can adsorb anti-SP-1V neutralizing antibodies (not shown). Data are consistent with the notion that other linear or conformational sites comprise the immunodominant neutralizing regions of SIV gp120.

The minimal V3 neutralization site of SIVmac251 is weakly immunogenic in macaques infected with SIVmac251

Sera from 31 rhesus macaques experimentally infected with SIVmac251 were tested for antibodies to linear determinants from the V3 region of SIVmac251 gp120 (Figure 5). Twenty (65%) of these sera contained antibodies that reacted with peptide SP-4 containing the entire sequence of the V3 domain. Seven sera (23%) contained antibodies reactive with peptide SP-1V (a.a. 327-346) used in the present study to raise V3-specific neutralizing antibodies to SIVmac251. However, only 4 sera (13%) contained antibodies to DP-147 (a.a. 327-339) and none of these sera contained antibodies to DP-154 (a.a. 329-339), a peptide containing the minimal neutralizing region within V3. Moreover, DP-154 bound to ELISA microtiter wells as well as DP-147 and SP-1V as determined with goat anti-SP-1V antisera #18 and #125 (not

shown), indicating that lack of rhesus antibody binding to DP-154 was not due to differential peptide binding to ELISA microtiter wells. Results are consistent with the hypothesis that the minimal neutralizing site within V3 of SIVmac251 gp120 is weakly immunogenic in macaques infected with SIVmac251.

DISCUSSION

While linear V3 neutralizing regions of HIV-1 and HIV-2 have been identified, homologous neutralizing sites within the V3 domain of SIV gp120 have not. Moreover, sequences within the V3 regions of HIV-1 and HIV-2 vary throughout the course of infection (Zagury et al., 1988; Holmes et al., 1992; Boeri et al., 1992) in contrast to the lack of genetic drift in SIV V3 (Almond et al., 1992, Burns and Desrosiers, 1991; Johnson et al., 1991; Overbaugh et al., 1991). Lack of variation within SIV V3 could indicate that immune responses to V3 do not provide sufficient selective pressure <u>in vivo</u> to result in sequence variation. However, it is also possible for retroviruses to evade immune surveillance by acquiring critical yet immunologically silent regions that are infrequently recognized as foreign by the host (Beretta et al., 1987). Regions of retroviral proteins that are weakly immunogenic or even tolerogenic (Cianciolo et al., 1985; Golding et al., 1989; Haynes et al. 1993) might allow escape from suppression of viral replication by immune effector responses and thereby contribute to viral pathogenesis. In the present study, we evaluated the

immunogenicity of SIVmac251 V3 peptides in three animal species as well as the neutralizing activity of anti-V3 antibodies in an effort to understand why linear V3 sequences of SIV gp120 do not appear to evoke neutralizing antibody responses despite sequence homology to neutralizing regions of HIV-2 gp120.

Our study defined a linear neutralizing region of SIVmac251 gp120 between amino acids 329-339 that is weakly immunogenic both in immunized animals and in macaques experimentally infected with SIVmac251 (Figure 5). Of 11 goats and 9 guinea pigs immunized with peptides containing V3 sequences of SIVmac251 gp120, only 3 goats produced neutralizing antibody responses with titers greater than 1/100 in syncytium inhibition or cell killing assays. However, neutralizing titers in these goat sera were substantial (1/1024 for 90% reduction of syncytium formation or 1/5832 for 50% inhibition of cell killing, Table 1), V3-specific (Figures 2,3; Table 2) and cross-neutralizing when tested against SIVDeltaB670 and SIVsmH4. Moreover, of 54 antisera raised to various SIV envelope peptides containing V3 and non-V3 sequences (Figure 1, Table 1), we found that only V3 antisera neutralized SIVmac251. Our results contrast with reports of others (Robert-Guroff et al., 1992; Javaherian et al., 1992) who were unable to demonstrate linear neutralizing sites within the V3 of SIV gp120. However, these negative results could be due to the weak immunogenicity of the linear V3 neutralizing site defined in our study. Lack of a detectable neutralizing antibody response to a previously defined linear neutralizing site in V4 of SIVmac251

(Torres et al. 1993) could be attributed to low levels (1/800, Table 1) of anti-V4 antibodies generated in goats immunized with SP-16 (a.a. 410-436). We were not able to demonstrate neutralizing antibodies to a previously reported linear neutralizing site between a.a. 171-188 (Benichou et al., 1992) with antisera to SP-9 (a.a. 176-196) possibly due to deletion of a.a. 171-175 from the sequence of SP-9.

We performed adsorption experiments with SIV synthetic peptides and recombinant SIVmac239 gp130 to evaluate the specificity of neutralizing antibodies raised to SIV V3 peptides. Recombinant gp130, peptides SP-1 (a.a. 327-342, Franchini et al., 1987) and SP-1V (a.a. 327-346, Myers et al., 1992) could completely adsorb anti-SP-1V neutralizing antibodies (Figures 2,3) despite sequence differences in peptides at a.a. 337 and 338 (SP-1: LT; SP-1V: IN). Further adsorption experiments revealed the presence of a minimal neutralizing region within SIVmac251 envelope amino acids 329-339 (GLVFHSQPIND, DP-154) that has homology to one (peptide A4-38, a.a. 311-330: SGRRFHSQKIINKKPRQAWC, Björling et al., 1991) of two linear neutralizing sites within the V3 region of HIV-2 gp120 (Björling et al., 1994). Using peptides in which amino acids were sequentially replaced with alanine, we identified amino acids F^{332}, H^{333}, S^{334}, Q^{335}, P^{336} and D^{339} as being important for adsorption of neutralizing antibody responses. These results are very similar to those of Björling et al. (1994) who identified the homologous HIV-2 gp120 V3 amino acids F315, H316, S317, Q318 and K319 as critical targets for antibody

binding in experiments with hyperimmune anti-HIV-2 V3 antisera and deletion sets of HIV-2 V3 peptides containing amino acids 311-330. In our study, but not in that of Björling et al. (1994), D^{339} appeared to be important for binding of neutralizing antibodies, since either replacement of D^{339} with A or deletion of D^{339} from sequences in peptides DP-171 (GLVFHSQPI, a.a. 329-337) or DP-176 (GLVFHSQPIN, a.a. 329-338) abrogated the ability of these peptides to adsorb neutralizing anti-V3 antibodies (Table 2, Figure 4). The requirement for neutralizing antibodies to recognize SIV V3 a.a. 329-339 (DP-154: GLVFHSQPIND) was further highlighted by mapping studies performed with non-neutralizing mab KK45 and KK46 against the V3 domain of SIVmac251. Both mab bound SP-1 [(C)MSGLVFHSQPLTDRPK, a.a.327-342), SP-1V (MSGLVFHSQPINDRPKQAWC, a.a.327-346) and DP-147 (MSGLVFHSQPIND, a.a. 327-339) in ELISA; however, neither mab bound DP-154 (GLVFHSQPIND, a.a.329-339) which can completely adsorb anti-SP-1V neutralizing antibodies (Table 2, Figures 2-4). In contrast, neutralizing goat #125 and #18 antisera to SP-1V bound strongly to SP-1, SP-1V, DP-147 and DP-154 in ELISA (titer against DP-154 = 1/12800). Collectively, our data map a linear neutralizing site of SIVmac251 to the C-terminal region of gp120 V3 (a.a.329-339) and define amino acids F^{332}, H^{333}, S^{334}, Q^{335}, P^{336} and D^{339} as important recognition elements for neutralizing antibodies. A comparison of HIV-2 and SIV V3 sequences from the Los Alamos database (Myers et al., 1993) reveals that the central FHSQP motif is highly conserved and thus might function as a neutralizing site for many HIV-2 and SIVmac/sm isolates.

Previous studies have shown that conformational sites within a 46 kDa V3/V4 fragment between a.a. 249-505 comprise the PND of SIVmac251 gp120 (Javaherian et al., 1992, 1994). This 46 kDa V3-V4 portion of SIVmac251 gp120 could adsorb the majority of neutralizing antibodies in sera from guinea pigs immunized with gp120 or in sera from macaques infected with SIVmac251; whereas V3 peptides could not. In the present study, we were also unable to adsorb neutralizing antibodies in goat antiserum to SIV 1A11 gp130 or in sera from macaques infected with SIVmac251. Thus, our results corroborate previous studies regarding the conformational nature of the PND of SIVmac251. In contrast to the immunogenicity of this conformational PND, we found that the linear C-terminal V3 neutralizing site between a.a. 329-339 is not recognized by antibodies in sera from any of 31 rhesus macaques infected with SIVmac251. Others have also reported that the C-terminal region of SIV gp120 V3 is weakly immunogenic in rhesus macaques when compared to regions within or adjacent to the N-terminus of V3 (Siegel et al., 1992; Benichou et al., 1993; McBride et al., 1993; Sammuelsson et al., 1993; Torres et al., 1993; Tanchou et al., 1995). Miller et al. (1992) have also reported a low antigenic index prediction for the C-terminus of SIV V3. The observation that this linear neutralizing site is poorly immunogenic and highly conserved, in contrast to the highly immunogenic and variable neutralizing V3 regions of HIV-1 and HIV-2, raises the possibility that SIV has evolved an alternate strategy of escaping V3-directed immune surveillance

other than antigenic variation. This strategy would involve the incorporation of V3 neutralizing sequences that are poorly immunogenic and therefore resistant to immune selective pressure. This process of immune evasion could be facilitated by the adoption of V3 neutralizing sequences that are either homologous to those of the host (Reiher et al., 1986; Berreta et al., 1987) or that are directly tolerogenic (Cianciolo et al., 1985; Haynes et al. 1993). Additional analysis of cellular and immune responses to the linear neutralizing V3 site defined in this study is needed to evaluate this hypothesis.

Difficulties in generating neutralizing anti-V3 antibodies to SIV are reminiscent of those associated with the resistance of HIV-1 primary isolates to V3-targeted neutralization. Moore et al. (1995) have shown that HIV-1 primary isolates are insensitive to neutralization by V3-specific antibodies which neutralize prototypic strains. In addition, VanCott et al. (1995) recently reported that antibody depletion with autologous V3 peptides had little effect on primary isolate neutralization by sera from infected individuals. Others have shown that HIV-1 contains neutralizing epitopes in the V3 loop (Bou-Habib et al., 1994) and other regions of gp120 (Broder et al., 1994; Sullivan et al., 1995) that are poorly exposed on native virus particles. In a similar manner, difficulties in neutralizing SIV with anti-V3 antibodies could also be related to problems of antibody recognition of V3 on the surface of the virion if, for instance, most of V3 were inaccessible to antibody binding except for the

linear neutralizing site between a.a. 329-339. Our results are also consistent with this hypothesis and imply that SIV and HIV-1 primary isolates could share similar neutralizing characteristics.

In summary, we have identified a linear, conserved neutralizing site of SIVmac251 gp120 V3 that is homologous to a previously reported neutralizing region of HIV-2 gp120. This neutralizing site is weakly immunogenic in rhesus macaques, goats and guinea pigs, which likely precludes its use in vaccine strategies against SIV. Lack of sequence variability and poor immunogenicity within this V3 neutralizing region could indicate that SIV evades V3-directed immune surveillance by incorporating sequences that are "self-like" or tolerogenic.

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Table 1: Reactivity in ELISA and neutralization of SIVmac251 with antisera raised to peptides containing sequences of SIVmac251 gp120 and gp36.

Peptide	Envelope Amino Acids	Sequence[a]	Antibody titer[b] ELISA	Neutralization
SP-1	327-342	(C)MSGLVFHSQPLTDRPK	3200	320
SP-1V	327-346	MSGLVFHSQPINDRPKQAWC	12800	1024 (5832)
SP-2	313-331	CRRPGNKTVLPVTIMSGLV	< 50	–
SP-3	332-346	FHSQPLTDRPKQAWC	< 50	–
SP-4	313-346	CRRPGNKTVLPVTIMSGLVFHSQPLTDRPKQAW	25600	20
SP-5A	327-362	MSGLVFHSQPLTDRPKQAWCWFGGKWKDAIKEVKQT	3200	–
SP-5	46-67	TKNRDTWGTTQCLPDNGDYSEL	12800	–
SP-6	76-99	(C)DAWENTVTEQAIEDVWQLFETSIK	1600	–
SP-7	111-131	MRCNKSETDRWGLTKSSTTIT	6400	–
SP-8	137-153	SAPVSEKIDMVNETSSC	3200	–
SP-9	176-196	GLKRDKTKEYNETWYSTDLVC	12800	–
SP-10	196-213	CEQGNSTDNESRCYMNHC	25600	–
SP-11	220-242	ESCDKHYWDTIRFRYCAPPGYAL	25600	–
SP-12	265-289	CTRMMETQTSTWFGFNGTRAENRTY	50	–
SP-13	283-306	(C)RAENRTYIYWHGRDNRTIISLNKY	100	–
SP-14/15	349-372	(C)GGKWKDAIKEVKQTIVKHPRYTGT	800	–
SP-16	410-436	(C)LNWVEDRDVTTQRPKERHRRNYVPCHI	800	–
SP-17	434-460	CHIRQIINTWHKVGKNVYLPPREGDLT	3200	–

SP-V5	468-490	LIANIDWTDGNQTSITMSAEVAE(CG)	50	-
SP-18	508-528	(C)GLAPTDVKRYTTGGTSRNKRG	800	-
DP-314	658-678	(C)EEAQIQQEKNMYELQKLNSWD	65536	-

a The V3 sequence of peptide SP-1 from Franchini et al. (1987) differs from that reported by Myers et al. (1993) at positions 337 and 338 (underlined) of SIVmac251 gp120. Amino acids in parentheses have been added to facilitate coupling of peptide to carrier protein.

b Antibody titers shown for ELISA and neutralization assays are the highest levels achieved in 1 or more immunized animals. Peptides SP-4 and DP-314 were used to immunize guinea pigs (N=3) and C57BL/6 mice (N=6) respectively. All other peptides were used for immunization of 1 or 2 goats. Neutralization is defined as ≥ 90% inhibition of syncytium formation induced by SIVmac251 on CEMx174 cells. Neutralization titer in parentheses: ≥ 50% inhibition of cell killing by SIVmac251. - = no neutralization. Antiserum to SP-1V also neutralized SIVDeltaB670 (50% inhibition of cell killing = 1/568) and SIVsmH4 (50% inhibition of cell killing = 1/740).

Table 2. Adsorption/Peptide Binding of Anti-SIVmac251 V3 Polyclonal and Monoclonal Antibodies

Adsorption of Goat Anti-SP-1V	Binding of Monoclonal Antibodies			Amino Acid	
Antiserum #246	KK45	KK46	Peptide	Number	Sequence
+	+	+	SP-1	327-342	(C)MSGLVFHSQPLTDRPK
+	+	+	SP-1V	327-346	MSGLVFHSQPINDRPKQAWC
-	-	-	DP-146	337-346	INDRPKQAWC
+	±	±	DP-147	327-339	MSGLVFHSQPIND
+	-	-	DP-154	329-339	GLVFHSQPIND
-	ND	ND	DP-153	331-339	VFHSQPIND
-	ND	ND	DP-152	332-339	FHSQPIND
-	ND	ND	DP-151	333-339	HSQPIND
-	ND	ND	DP-150	334-339	SQPIND
-	-	-	DP-171	329-337	GLVFHSQPI
-	-	-	DP-176	329-338	GLVFHSQPIN
ND	-	-	RG-1	329-340	GLVFHSQPINDR
ND	-	-	RG-2	329-341	GLVFHSQPINDRP

ND = not determined; - = <10% adsorption or binding relative to positive control peptide SP-1V; + = 90% adsorption or binding relative to positive control peptide SP-1V; ± = 25-50% binding relative to positive control peptide SP-1V. Mab KK45 and KK46 from Kent et al. (1991, 1992).

Figure Legends

Figure 1: Synthetic peptides used in the study. Antisera raised in goats, guinea pigs and mice to peptides SP-1, 1V, 2-18, V5 and DP-314 were tested in ELISA and neutralization assays (Table 1). The peptide panel DP-146 to DP-176, RG-1 and RG-2 were used either to adsorb neutralizing antisera or to map the binding sites of monoclonal antibodies raised to SIVmac251 or HIV-2 gp120. There is a premature stop codon in the env gene encoding the gp32 transmembrane glycoprotein (break in solid bar). Δ = proteolytic cleavage site of gp160.

Figure 2: Dose-dependent adsorption of neutralizing antibodies in goat #18 antiserum raised against peptide SP-1V (a.a. 327-346) containing a V3 sequence of SIVmac251 gp120. Neutralizing goat anti-SP-1V antiserum was pre-adsorbed with varying amounts of SIVmac251 synthetic peptides and then tested for the ability to inhibit SIVmac251-mediated syncytium formation. A: Adsorption of anti-SP-1V antibodies with peptides SP-1 (a.a. 327-346, Franchini et al., 1987) or SP-1V (a.a. 327-346, Myers et al., 1993) but not with SP-5 (a.a. 46-67). B: Adsorption of anti-SP-1V antibodies with peptides DP-147 (a.a. 327-339) but not with peptides DP-146 (a.a. 337-346) or SP-5 (a.a. 46-67). Results indicate the presence of a V3 neutralizing site between a.a. 327-339 of SIVmac251 gp120. Data are representative of 3-5 experiments with various V3 and non-V3 peptides.

Figure 3: Adsorption of neutralizing anti-SP-1V goat #18 antiserum with peptides containing sequences of SIVmac251 gp120. Pre-adsorbed serum was tested for the ability to inhibit SIVmac251-mediated syncytium formation. A: pre-immune #18 goat serum, 1/10 dilution; B: #18 anti-SP-1V antiserum, 1/160 dilution; #18 anti-SP-1V antiserum adsorbed with C: SP-1 (a.a. 327-346, Franchini et al., 1987), D: SP-1V (a.a. 327-346, Myers et al., 1993), E: SP-5 (a.a. 46-67), F: DP-146 (a.a. 337-346), G: DP-147 (a.a. 327-339). H: rhesus anti-SIVmac251 antiserum, diluted 1/10. Results are representative of 3 experiments.

Figure 4: Adsorption of goat #246 anti-SP-1V antiserum with peptides containing V3 sequences of SIVmac251 gp120 and with peptides A1-A11 in which amino acids GLVFHSQPIND (a.a. 329-339) were sequentially replaced with alanine. After adsorption, antiserum was tested for the ability to inhibit SIVmac251-mediated killing of CEMx174 human T-cells or for inhibition of syncytium formation (data for DP-171, 176, DP-151-154). Peptide DP-154 contained the minimal neutralizing sequence (a.a. 329-339) within the V3 region of SIVmac251 gp120 (summarized in Table 2).

Figure 5: Antibody reactivity in ELISA to V3 peptides of SIVmac251 gp120 in sera (N=31) from rhesus macaques infected with SIVmac251. Results are expressed as a ratio of mean optical density readings obtained from duplicate microtiter wells containing sera (1/50 dilution) from SIVmac251-infected and uninfected macaques.

FIGURE 1

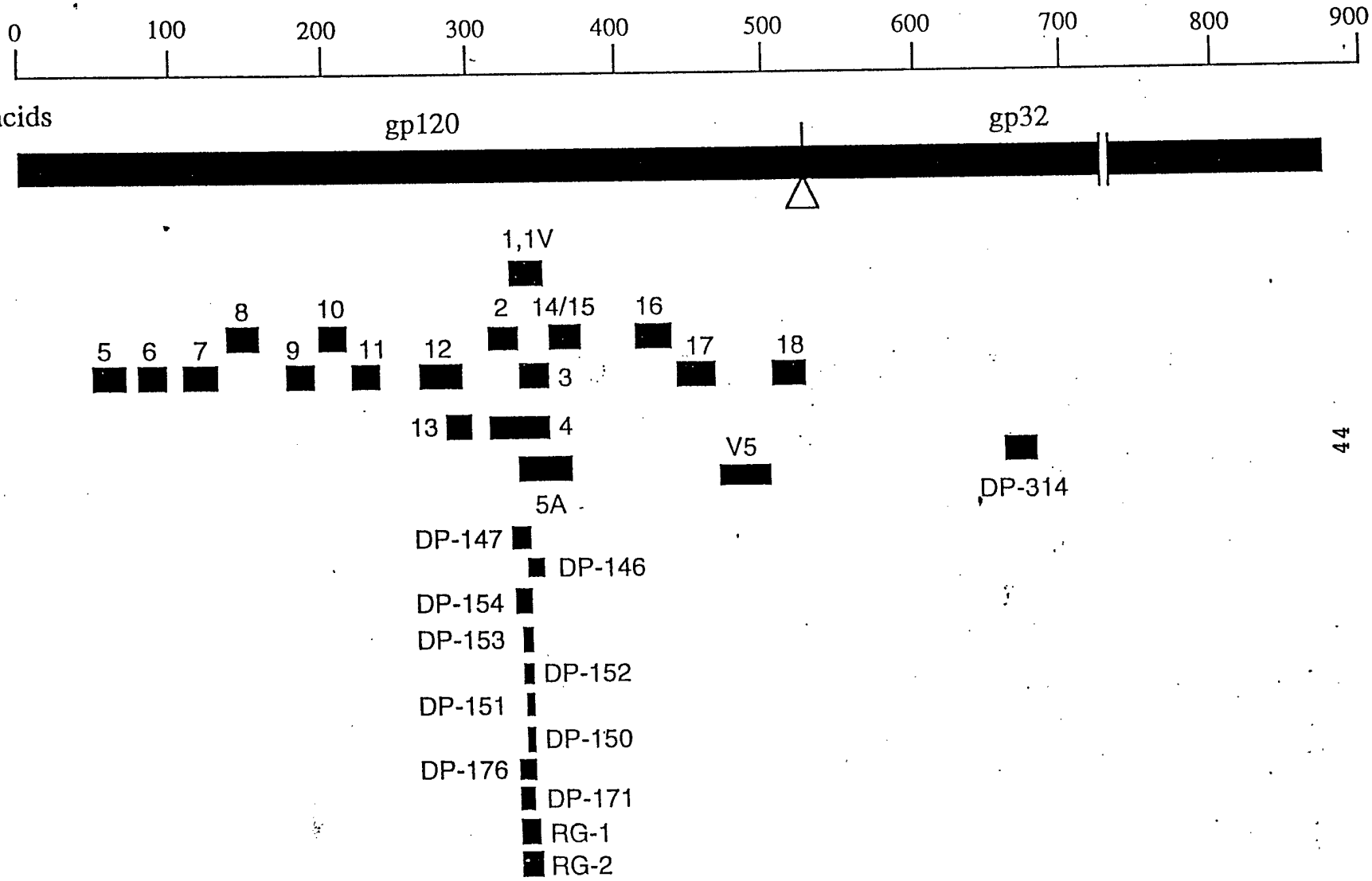
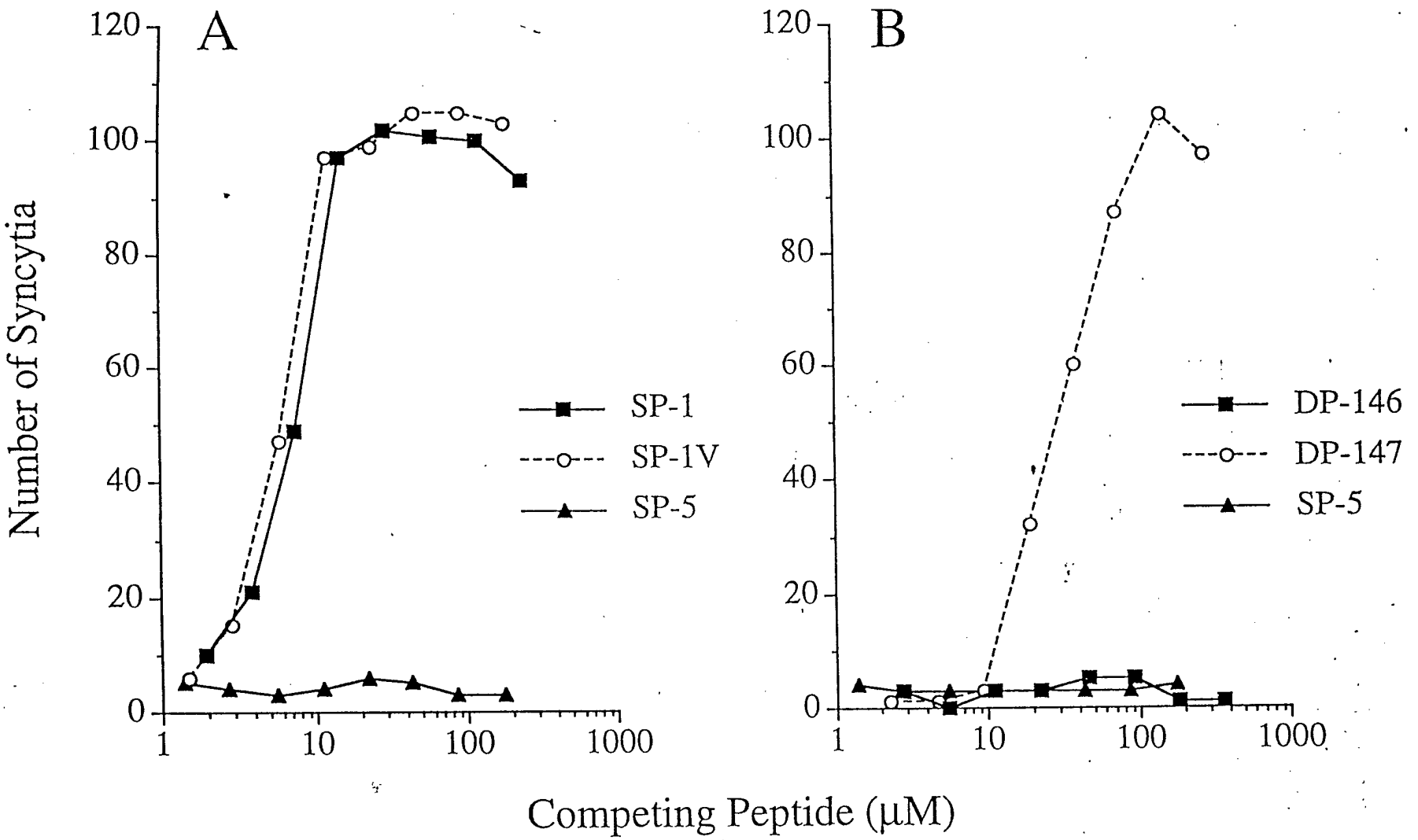

0
100
200
300
400
500
600
700
800
900
Amino acids
gp120
gp32
1,1V
8
10
2
14/15
16
5
6
7
9
11
12
17
18
3
13
4
V5
DP-314
5A
DP-147
DP-146
DP-154
DP-153
DP-152
DP-151
DP-150
DP-176
DP-171
RG-1
RG-2

FIGURE 2


A
B
120
100
80
60
40
20
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1
10
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1000
Number of Syncytia
Competing Peptide (μM)
SP-1
SP-1V
SP-5
DP-146
DP-147
SP-5

FIGURE 3

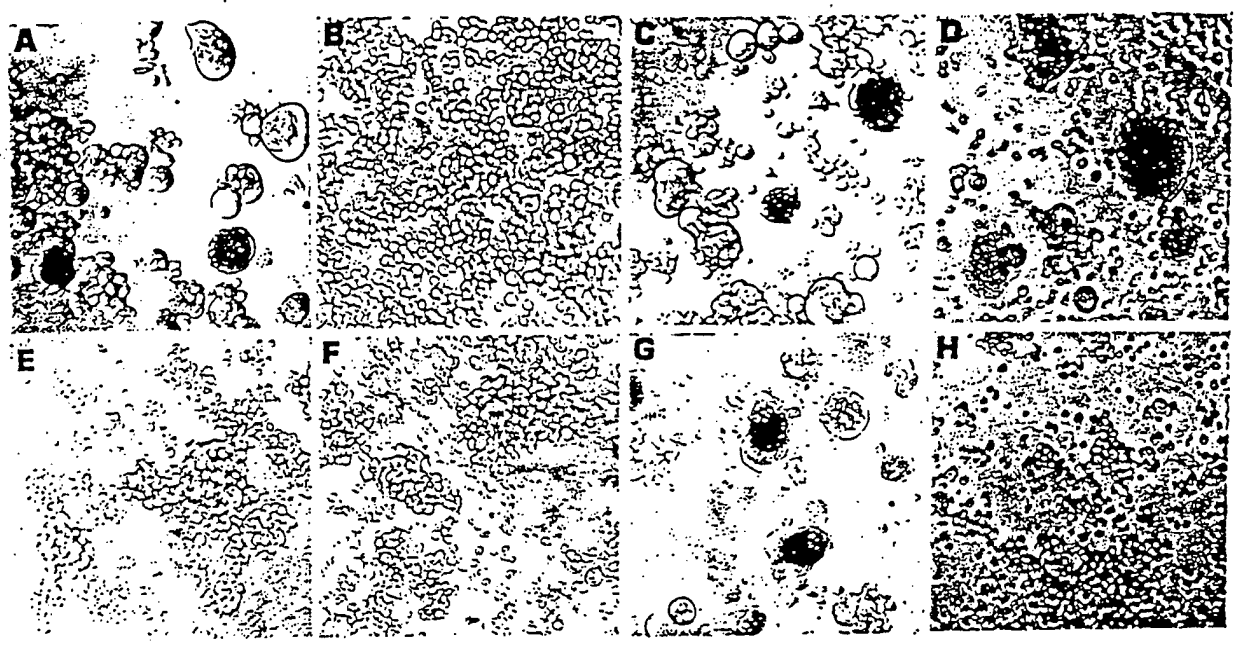


FIGURE 4

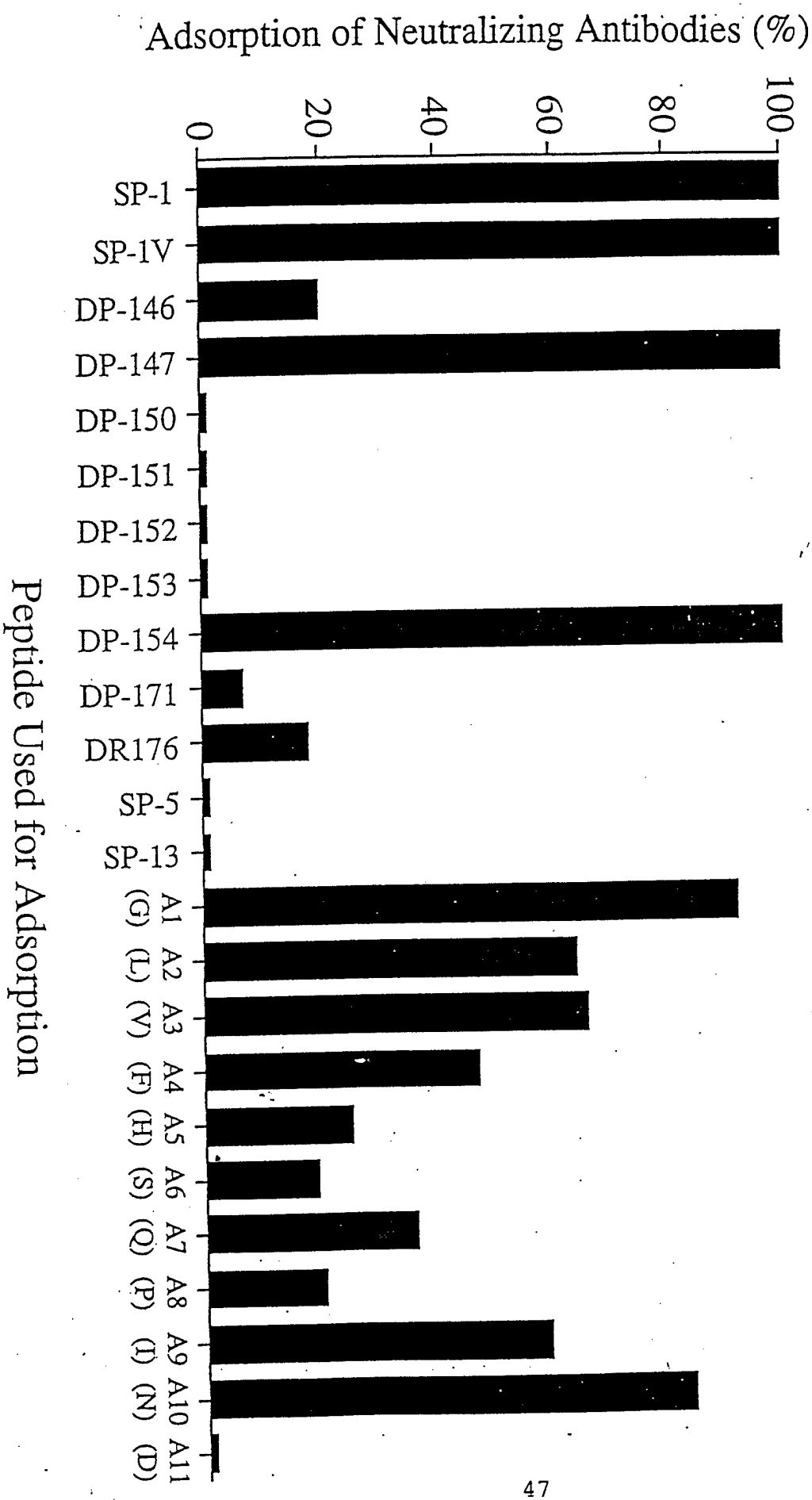
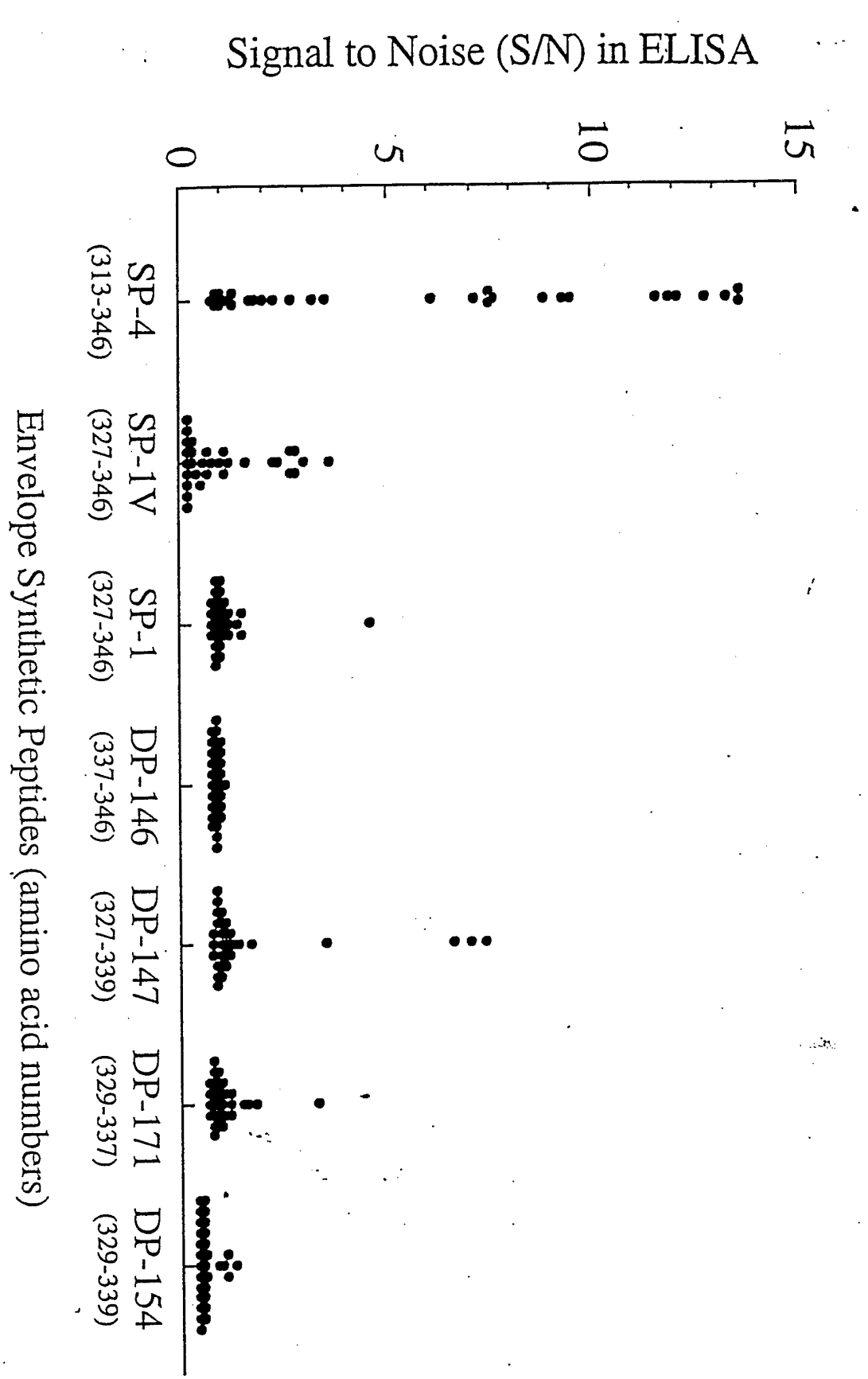

Adsorption of Neutralizing Antibodies (%)
0
20
40
60
80
100
SP-1
SP-1V
DP-146
DP-147
DP-150
DP-151
DP-152
DP-153
DP-154
DP-171
DR176
SP-5
SP-13
(G) A1
(L) A2
(V) A3
(F) A4
(H) A5
(S) A6
(Q) A7
(P) A8
(I) A9
(N) A10
(D) A11
Peptide Used for Adsorption

FIGURE 5


Signal to Noise (S/N) in ELISA
0
5
10
15
SP-4 (313-346)
SP-1V (327-346)
SP-1 (327-346)
DP-146 (337-346)
DP-147 (327-339)
DP-171 (329-337)
DP-154 (329-339)
Envelope Synthetic Peptides (amino acid numbers)