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GRANT NUMBER DAMD17-94-J-4467

TITLE: Structural and Functional Studies of Experimental HIV Synthetic Peptide Immunogens

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REPORT DATE: October 1997

DTIC QUALITY INSPECTED 2

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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19971230 145

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1997	Annual (30 Sep 96 - 29 Sep 97)

4. TITLE AND SUBTITLE
Structural and Functional Studies of Experimental HIV Synthetic Peptide Immunogens

5. FUNDING NUMBERS
DAMD17-94-J-4467

6. AUTHOR(S)
Barton F. Haynes, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27706

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Work performed in this grant continues to address 2 major problems in HIV synthetic peptide vaccine development: 1) the ability of synthetic peptides to mimic conformational antibody epitopes of HIV envelope proteins and induce immune responses capable of neutralizing primary HIV isolates , and 2) the design of optimally immunogenic multivalent peptide immunogens capable of being recognized by MHC Class I and II molecules in outbred populations. In technical aim #1, intranasal immunization with HIV synthetic peptide immunogens was found to be effective for the induction of serum anti-peptide IgG antibody responses. Studies are currently being performed to determine if the qualities of the antibodies induced by intranasal immunization are different from antibodies induced by systemic immunization. Amino acid substitutions that enhanced the immunogenicity of systemically administered peptides were associated with enhanced immune responses when the peptides were administered intranasally. In technical aim #2, mutant peptides are being designed and analyzed for structure and immunogenicity in association with Aim #1. In technical aim #3, HIV synthetic peptides are being tested for their ability to bind human HLA Class I molecules.

14. SUBJECT TERMS	15. NUMBER OF PAGES
HIV synthetic peptides; oil adjuvants; water adjuvants; intranasal immunization; NMR, peptide-MHC; neutralization sites.	62
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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DAMD 17-94-J-4467. Structural and functional studies of experimental HIV synthetic peptide immunogens.

Technical Aim #1. Use existing and new human and mouse monoclonal antibodies (mAbs) as templates for definition of native gp120 conformations and determine synthetic peptides that bind to these mAbs.

Investigators: Barton F. Haynes, M.D. and Herman F. Staats, Ph.D.

INTRODUCTION

The hypotheses to be tested in Technical Aim 1 are:

1. Regarding the B cell anti-HIV immune responses, synthetic peptides can be designed that mimic not only linear determinants of neutralizing epitopes of gp120 but also mimic more conserved, broadly neutralizing HIV conformational determinants.

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

3. 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

HIV synthetic peptide immunogens are immunogenic when intranasally administered to guinea pigs with the mucosal adjuvant cholera toxin.

The Duke DOD HIV vaccine group continues to make progress in the area of designing synthetic peptide immunogens that induce antibodies reactive with native gp120 and that neutralize HIV primary isolates. Our group first designed the "C4-V3" peptides nearly a decade ago. The C4-V3 peptide elicits B and T cell responses to various strains of human immunodeficiency virus type 1 (HIV-1) (1-4). These peptides consist of two segments derived from non-contiguous regions of the gp120 *env* protein. One segment contains 16 residues from the fourth constant (C4) domain of gp120, and includes a potent T-helper epitope, termed T1 (5). Linked to the carboxyl terminus of this gp120 C4 region-derived peptide is a 23 amino acid segment from the third variable (V3) domain of gp120, that includes a B cell neutralizing antibody epitope for cell line-adapted HIV strains (1, 6, 7), a T-helper epitope (1), and a cytotoxic T cell epitope (8). These C4-V3 peptides induce antibodies that bind to native gp120 and neutralize the particular cell line-adapted strain of HIV from which the V3 segment was derived.

We have previously reported that the C4-V3 CanO (A) HIV peptide was recognized by the human anti-HIV monoclonal antibody (mAb) 48d. This result suggested that the C4-V3 CanO(A) peptide mimicked the native epitope recognized by 48d. Therefore, if the C4-V3 CanO(A) peptide mimics a conformational epitopes in HIV gp120, immunization with the C4-V3 CanO(A) peptide should induce antibodies reactive with native, conformational epitopes on HIV gp120. One important point to consider is that the ELISA assays that determined C4-V3 CanO(A) was recognized by 48d were performed in a aqueous environment. In contrast, C4-V3 CanO(A) used as an immunogen would be formulated in

complete Freund's adjuvant (CFA), an adjuvant with mineral oil as its main constituent. Therefore, the structure of the peptide in an oil adjuvant (CFA) may be dramatically different from the structure of the peptide in a water environment (i.e. ELISA). This may result in the induction of antibody responses that do not recognize native, conformational epitopes. Recent work published by Van Cott et al. (9) supports the hypothesis that the same immunogen may induce qualitatively different immune responses dependent upon the adjuvant used.

We are currently testing the following hypothesis: "The environment that the HIV synthetic peptide is presented to the immune system in affects the qualities of the anti-HIV antibody response induced.". Using the C4-V3 CanO(A) peptide as well as HIV touchpoint peptides [synthesized with funds from our NIH National Cooperative Vaccine Development Group (NCVDG) grant] (Table 1), we have intranasally immunized guinea pigs with a number of HIV peptides formulated in water. This work is based on our previous observation that intranasal immunization of mice with C4-V3 peptides and the mucosal adjuvant cholera toxin in water was an effective route of immunization for the induction of systemic and mucosal immune responses (10-12).

The current experiment was performed to determine if immunization with the peptides prepared in an aqueous environment was superior to immunization with peptides formulated in an oil environment for the induction of antibodies that react with native gp120 and that neutralize primary HIV-1 isolates. Guinea pigs were intranasally immunized with 200 μg of peptide and 1 μg of cholera toxin at day 0, 14, 28, 42, 56, 70, 159 and 175 (a total of 8 immunizations). Intranasal immunization of outbred guinea pigs with HIV peptide immunogens and the mucosal adjuvant cholera toxin induced a wide range of anti-peptide antibody responses (Table 2). Also, a number of guinea pigs die due to anaphylactic reactions with repeated intranasal immunizations. However, we feel this is a species-specific phenomenon since we have never observed this reaction in any of our murine studies and a number of groups have published successful intranasal immunization Consistent with the use of outbred hosts, the magnitude of anti-peptide antibody titers varied between individual guinea pigs immunized with the same peptide. Intranasal immunization of outbred guinea pigs with HIV peptide immunogens did not induce serum antibody responses with the same magnitude that we observed using an inbred murine system (10, 12). We are currently evaluating peptides with specific amino acid substitutions for their ability to enhance the immunogenicity of intranasally administered peptide immunogens (see below). Serum obtained from guinea pigs intranasally immunized with C4-V3 CanO(A) and other HIV peptide immunogens is currently being tested for its ability to neutralize laboratory-adapted and primary HIV isolates. This initial experiment has provided evidence that intranasal immunization of guinea pigs with HIV peptide immunogens in an aqueous environment is a viable route of immunization for the induction of anti-HIV antibody responses. Additional studies will determine if intranasal immunization induces qualitatively different antibody responses as compared to parenteral immunization strategies that utilize oil-based adjuvants.

Table 1. Amino Acid Sequence of HIV Peptide Immunogens Used For Guinea Pig Intranasal Immunizations

Name of Peptide	Region	Amino Acid Sequence
C4-SP440-BAL	C1	KQIINMWQEVGRAMYAASDRKAYDTEVHNVWATHA
C4-SP450-BAL	C1	KQIINMWQEVGRAMYAKLTPLCVTLNCTDLRNAT
C4-V3 89.6	V3	KQIINMWQEVGRAMYATRPNNNTRRRLSIGPGRAFYARR
C4-V3 89.6 scrambled	V3	KQIINMWQEVGKAMYARGYFTRRNAPSNTAEGRPILRRN
C4-V3 BAL	V3	KQIINMWQEVGRAMYATRPNNNTRKSIHIGPGRAFYTTG
C4-V3 Can0(A)	V3	KQIINMWQEVGKAMYATRPHNNTRKSIHMGPGKAFYTTG
C4-V3 EV91	V3	KQIINMWQEVGKAMYATRPGNNTRKSIPIGPGRAFIATS
C4-V3 MN	V3	KQIINMWQEVGKAMYATRPNYNKRKRIHIGPGRAFYTTK
GTH1-SP61	gp41	YKRWIILGLNKIVRMYSQQEKNEQELLELDKWAS
GTH1-SP70	V2	YKRWIILGLNKIVRMYSSFNISTSIRGKVQKEYAF
GTH1-SP75	gp41	YKRWIILGLNKIVRMYSRILAVERY
GTH1-SP80 BAL	V1	YKRWIILGLNKIVRMYSTTSSSRGMVGGGE
SP200	gp41	CGGGELDKWASELDKWASEKDKWAS
SP201	gp41	CGGGELDKWAAELDKWAAEKDKWAA
SP202	gp41	CGGGELDKWA aib ELDKWA aib EKDKWA aib
SP204	gp41	YKRWIILGLNKIVRMYSQQEKNEQELL aib LDKWA aib LWN
SP400-BAL	gp41	RVLAVERYLRDQQLLGIWGCSGKLICTTAVPWNASWSNKSLNKI
SP410-BAL	C5	PGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR

Table 2.

Peptide	G. Pig #	Day 28 Titer (E/C)	Day 56 Titer (E/C)	Day 84 Titer (E/C)	Bleedout Titer (E/C)
C4-SP440-BAL	1	<32	512 (3.1)	2048 (3.5)	DEAD
C4-SP440-BAL	2	<32	<32	<64	<32
C4-SP450-BAL	3	<32	256 (3.3)	512 (3.5)	512 (3.5)
C4-SP450-BAL	4	<32	256 (3.9)	8192 (3.3)	2048 (4.1)
C4-V3 89.6	5	<32	<32	2048 (3.5)	2048 (3.2)
C4-V3 89.6	6	<32	DEAD	DEAD	DEAD
C4-V3 89.6 scrmbld	7	<32	<32	<64	<32
C4-V3 89.6 scrmbld	8	<32	<32	<64	<32
C4-V3 BAL	9	<32	256 (3.4)	2048 (4.1)	1024 (3.2)
C4-V3 BAL	10	<32	1024 (3.3)	8192 (3.1)	4096 (3.3)
C4-V3 CanO(A)	11	<32	16384 (4.2)	DEAD	DEAD
C4-V3 CanO(A)	12	<32	2048 (3.4)	32768 (3.2)	DEAD
C4-V3 EV91(A)	13	<32	2048 (3.6)	4096 (3.9)	DEAD
C4-V3 EV91(A)	14	<32	1024 (3.2)	2048 (4.9)	2048 (3.0)
C4-V3 MN(A)	15	<32	1024 (3.9)	2048 (3.5)	DEAD
C4-V3 MN(A)	16	<32	1024 (3.9)	8192 (4.2)	4096 (4.2)
GTH1-SP61	17	<32	256 (3.85)	8192 (3.1)	512 (3.6)
GTH1-SP61	18	<32	<32	<64	<32
GTH1-SP70	19	<32	<32	<64	<32
GTH1-SP70	20	<32	<32	<64	<32
GTH1-SP75	21	<32	<32	<64	<32
GTH1-SP75	22	<32	<32	<64	<32
GTH1-SP80-BAL	23	<32	256 (3.4)	256 (4.5)	N.T.
GTH1-SP80-BAL	24	<32	32768 (3.6)	32768 (3.2)	DEAD
SP-200	25	<32	<32	128 (3.1)	N.T.
SP-200	26	<32	<32	256 (3.2)	N.T.
SP-201	27	<32	<32	<64	N.T.
SP-201	28	<32	<32	<64	N.T.
SP-202	29	<32	<32	<64	<32
SP-202	30	<32	<32	<64	<32
SP-204	31	<32	512 (3.8)	8192 (3.6)	N.T.
SP-204	32	<32	256 (3.3)	1024 (3.5)	N.T.
SP400-BAL	33	32 (3.9)	8192 (3.6)	16384 (3.5)	4096 (3.25)
SP400-BAL	34	512 (4.06)	4096 (3.7)	4096 (3.8)	2048 (4.45)
SP410-BAL	35	<32	<32	<64	<32
SP410-BAL	36	<32	<32	512 (3.6)	512 (3.17)

N.T. = Not tested.

Amino acid substitutions enhance the immunogenicity of intranasally delivered C4-V3 HIV synthetic peptides.

We previously reported that specific amino acid substitutions in the C4 region of a C4-V3 RF(A) peptide immunogen (Table 3) greatly enhanced the immunogenicity of that peptide when subcutaneously administered in Freund's adjuvant (Progress Report for last year). Because the formulation of the peptides in oil adjuvants may adversely alter the conformation of the peptide immunogen (see Hypothesis above), we intranasally immunized guinea pigs with the parent C4-V3 RF(A) peptide and the three mutants, C4-V3 RF(A) E9V, C4-V3 RF(A) E9G, and C4-V3 RF(A) K12E (Table 3). Guinea pigs were intranasally immunized with 200 μg of peptide and 1 μg of cholera toxin at day 0, 14, 28, 42, 56, 70, 159 and 175 (a total of 8 immunizations). Serum samples were collected at various times and tested for the presence of anti-peptide antibodies with the ELISA. The E9V mutant was more immunogenic than all other peptides and induced a peak serum anti-peptide IgG titer of 1:32,768 - 1:65,536 (Table 4). The observation that antibodies induced by the parent and E9G or E9V mutant C4-V3 peptides recognized the K12E peptide in the ELISA assay suggests that the substitutions did not alter the ability of the peptides to be recognized by antibodies (Table 5). Exactly how the mutations affect the *in vivo* immunogenicity of the peptides is not clear since previous studies from our group determined that the increased or decreased antibody responses were not related to increased or decreased T cell responsiveness, respectively. The relative immunogenicity of the intranasally administered C4-V3 RF(A) and mutant peptides in guinea pigs was similar to our previous observations with subcutaneous administration of the peptides in the murine system; in decreasing order of immunogenicity: E9V, E9G, parent C4-V3, K12E.

Table 3. Amino Acid Sequence of C4-V3 RF(A) Peptides.

	Amino Acid Sequence	
Peptide Name	**C4 Region**	**V3 Region**
C4-V3 RF(A)	KQIINMWQEVGKAMYA	TRPNNNTRKSITKGPGRVIYATG
C4E9G-V3 RF(A)	KQIINMWQ*G*VGKAMYA	TRPNNNTRKSITKGPGRVIYATG
C4E9V-V3 RF(A)	KQIINMWQ*V*VGKAMYA	TRPNNNTRKSITKGPGRVIYATG
C4K12E-V3 RF(A)	KQIINMWQEVG*E*AMYA	TRPNNNTRKSITKGPGRVIYATG

Table 4. Enhanced immunogenicity of intranasally administered C4-V3E9V RF(A) Peptide.

		Day 28	**Day 56**	**Day 84**	**Bleedout**
Peptide	**G. Pig #**	**Titer (E/C)**	**Titer (E/C)**	**Titer (E/C)**	**Titer (E/C)**
C4-V3 RF	37	<32	<32	2048 (4.2)	4096 (4.5)
C4-V3 RF	38	<32	4096 (3.1)	8192 (3.8)	DEAD
C4-V3 RF E9G	39	<32	8192 (3.0)	DEAD	DEAD
C4-V3 RF E9G	40	256 (3.28)	512 (3.2)	256 (4.6)	512 (5.1)
C4-V3 RF E9V	41	32 (3.04)	8192 (3.8)	16384 (3.04)	DEAD
C4-V3 RF E9V	42	1024 (3.09)	32768 (4.6)	DEAD	DEAD
C4-V3 RF K12E	43	<32	<32	<64	<32
C4-V3 RF K12E	44	<32	<32	256 (3.4)	<32

Table 5. Parent and Mutant C4-V3 RF Peptides recognized by antibodies induced by each RF Peptide.

		Serum IgG End-Point ELISA Titer Against Indicated HIV-1 RF Peptide			
Immunizing Peptide	**Guinea Pig #**	**C4-V3**	**C4E9G-V3**	**C4E9V-V3**	**C4K12E-V3**
C4-V3	37	2,048	4,096	4,096	2,048
	38	8,192	8,192	8,192	8,192
C4E9G-V3	39	8,192	16,384	8,192	4,096
	40	256	256	512	256
C4E9V-V3	41	16,638	32,768	16,384	8,192
	42	32,768	65,536	65,536	32,768
C4K12E-V3	43	<64	<64	<64	<64
	44	256	512	512	256

Our group continues to investigate mechanisms to enhance the immunogenicity of synthetic HIV peptides. Literature reports suggest that a number of factors may affect the immunogenicity of synthetic peptide immunogens. The presence of residual trifluoroacetic acid (TFA) was reported to reduce the biological activity of an IL-1β peptide (13). Indeed, removal of residual TFA from the IL-1β peptide increased the activity of the peptide 10^4 - 10^6 fold (13). Others have reported that the presence of a C-terminal amide increases the immunogenicity of synthetic peptide immunogens by a factor up to 100 fold (14-16). The mechanisms responsible for the enhanced immunogenicity are not known.

As mentioned above, our group is now developing mucosal immunization protocols using HIV peptide immunogens. Because mucosal tissues contain a number of aminopeptidases and carboxypeptidases (17, 18), peptide immunogens may be rapidly degraded when delivered via mucosal routes. Although there are only several reports that address enhancing the stability of mucosally administered synthetic peptides, amino acid substitutions that result in acidic N-terminal amino acids may increase the half-life of synthetic peptides by decreasing the sensitivity of the peptides to aminopeptidase degradation (17). Likewise, the use of C-terminal amides may reduce peptide sensitivity to carboxypeptidase degradation (14-16). Systematic studies are planned to determine the effect the addition of N-terminal acidic amino acids and the use of C-terminal amides has on the immunogenicity of peptides delivered via parenteral as well as mucosal routes.

<u>Intranasal immunization with a mixture of HIV CTL epitope peptides induces MHC-restricted CTL specific for each peptide in the immunizing mixture.</u>

In addition to the induction of neutralizing antibody responses, prevention of HIV infection may depend upon the induction of anti-HIV cytotoxic T lymphocytes (CTL). Previous studies from our group have determined that subcutaneous immunization with C4-V3 peptides (3) or intranasal immunization with HIV CTL epitope peptides (11) induced HIV-specific CTL. The induction of CTL with peptide immunogens is dependent upon the host expressing an MHC Class I molecule that will bind and present the CTL epitope to the appropriate $CD8^+$ T cells. If the immunized host does not express an MHC Class I molecule that will bind the CTL epitope peptide, peptide-specific CTL responses will not be induced. Because humans represent an outbred population with variable expression of MHC Class I molecules, a mixture of CTL epitope peptides will be required to effectively immunize humans against HIV. Therefore,

we determined if immunization with a mixture of CTL epitope peptides would induce CTL specific for each peptide. BALB/c, C57BL/6, or (BALB/c x C57BL/6)F1 mice were intranasally immunized with equimolar amounts of the H-2^d-restricted HIV gp120 CTL epitope peptide R15K, the H-2^b-restricted ovalbumin CTL epitope peptide OVA, a combination of the two peptides, or a hybrid peptide that contained both the R15K and the OVA CTL epitopes (R15K-OVA) and 1 μg of cholera toxin on day 0, 5, 10, and 15. Because BALB/c mice are of the H-2^d haplotype, we predicted that BALB/c mice would only be able to respond to immunization with R15K. Because C57BL/6 mice are of the H-2^b haplotype, we expected C57BL/6 mice to respond only to the OVA CTL epitope peptide. (BALB/c x C57BL/6)F1 mice that express both H-2^b and H-2^d molecules should respond to both the R15K HIV CTL epitope peptide as well as the OVA CTL epitope peptide. As indicated in Table 6, intranasal immunization of mice with CTL epitope peptides induced peptide-specific, MHC-restricted CTL. Intranasal immunization of outbred [(BALB/c x C57BL/6)F1] mice with a mixture of CTL epitope peptides or the hybrid CTL epitope peptide induced peptide-specific, MHC-restricted CTL for each peptide contained in the immunizing mixture. These results suggest that immunization of humans with a mixture of peptides, each corresponding to a CTL epitope restricted by a unique HLA molecule, will be a viable means of inducing HIV-specific, MHC-restricted CTL in the human population.

Table 6. Intranasal immunization with a mixture of CTL epitope peptides induces MHC-restricted, peptide-specific CTL for each peptide in the mixture.

		% lysis of H-2^b restricted target cells pulsed with the indicated peptide (E:T 80:1)		**% lysis of H-2^d restricted target cells pulsed with the indicated peptide (E:T 80:1)**	
Mouse Strain	**Peptide(s) Used for Immunization**	**R15K**	**OVA**	**R15K**	**OVA**
BALB/c	R15K	2	N.T.	40	N.T.
	OVA	N.T.	2	N.T.	10
	R15K & OVA	N.T.	N.T.	20	2
	R15K-OVA	N.T.	N.T.	10	N.T.
C57BL/6	R15K	2	N.T.	2	N.T.
	OVA	4	57	N.T.	5
	R15K & OVA	4	55	5	N.T.
	R15K-OVA	5	35	4	3
(BALB/c x C57BL/6)F1	R15K	2	5	35	7
	OVA	2	60	10	10
	R15K & OVA	2	30	22	7
	R15K-OVA	2	27	20	2

N.T. = not tested.

Additional studies are underway to identify the most immunogenic length of CTL epitope peptide immunogens. Previous studies in our laboratory compared the induction of CTL by intranasal immunization with 3 different peptides that all contained the same CTL epitope core. One peptide, R10I, is a 10 amino acid peptide that contains a CTL epitope from the V3 loop of HIV-1 gp120. R15K contained the same 10 amino acid sequence as R10I with additional flanking residues from gp120 to increase the length of the peptide to 15 residues. The last peptide, N24G, contained the same sequences as

R10I and R15K with additional flanking residues to produce a peptide of 24 amino acids in length. Intranasal immunization of BALB/c mice with equimolar doses of R10I, R15K, or N24G and 1 μg of cholera toxin on day 0, 5, 10, and 15 induced peptide-specific CTL activity in all groups. Even though animals were immunized with equimolar doses of the different peptides, the R15K and N24G immunized mice consistently had higher peptide-specific CTL activity at 1, 3, or 6 weeks after the last immunization. There are at least two potential explanations for this observation. First, amino- and carboxypeptidases in the nasal cavity degrade the peptides after intranasal immunization. The longer peptides have "extra" amino acids that protect the CTL epitope from protease degradation. Mice immunized with the longer peptides were effectively immunized with higher doses of peptides (due to protease degradation of the 10 amino acid peptide) and therefore had higher peptide-specific CTL responses. Second, the longer peptides contained T-helper epitopes in addition to the CTL epitope and the induction of T cell help enhanced the induction of higher peptide-specific CTL responses.

Additional studies are planned to identify characteristics that enhance the immunogenicity of CTL epitope peptides. A CTL epitope from HIV-1 89.6 from the same V3 region as the R10I peptide has been identified and will be used for additional studies (Table 7). For these studies 9, 15, and 24 amino acid peptides all containing the same CTL epitope will be compared for their ability to induce peptide-specific CTL. Two additional peptides will also be tested. One peptide will be a hybrid peptide composed of the HIV-1 89.6 V3 loop CTL epitope synthesized at the C terminus of the known HIV-1 gp120 helper determinant T1. The other peptide will be a control peptide containing the 89.6 V3 CTL epitope synthesized at the C terminus of a scrambled version of the T1 T helper epitope. Mice will be intranasally immunized with equimolar amounts of the peptide and 1 μg of cholera toxin on day 0, 5, 10, and 15 and monitored for the induction of peptide-specific CTL at 1, 3 and 6 weeks after the last immunization. Once the optimal design of CTL epitope peptides is determined, CTL epitope peptides for use in rhesus macaques will be designed and tested.

Table 7. Determination of the optimal CTL epitope peptide immunogen.

Peptide Name	Amino Acid Sequence	Characteristics
89.6P-9	*IGPGRAFYA*	9 amino acid HIV-1 89.6 CTL peptide
89.6P-15	RLS*IGPGRAFYA*RRN	15 amino acid HIV-1 89.6 CTL peptide
89.6P-24	PNNNTRERLS*IGPGRAFYA*RRNII	24 amino acid HIV-1 89.6 CTL peptide
89.6P Th-CTL	KQIINMWQEVGKAMYA*IGPGRAFYA*	Th-CTL epitope chimera
89.6P ThScr-CTL	AVWQAMQKYKIEMING*IGPGRAFYA*	Scrambled Th epitope - CTL epitope chimera

CONCLUSIONS:

1. Intranasal immunization with HIV peptide immunogens and the mucosal adjuvant cholera toxin is a viable route of immunization for the induction of serum anti-HIV peptide antibodies. Additional studies are required to determine if intranasal immunization with the peptides formulated in water (vs. subcutaneous immunization with peptides formulated in oil) affects the qualities of the antibodies induced (i.e. neutralization activity, surface binding of HIV infected cells, etc.). Those studies are currently being performed.

2. Single amino acid substitutions may drastically enhance or reduce the immunogenicity of HIV peptide immunogens. Our goal is to enhance the immunogenicity of HIV peptide immunogens so that antibody responses capable neutralizing primary HIV isolates may be achieved.

3. Intranasal immunization with HIV CTL epitope peptides and cholera toxin induces peptide-specific CTL. Intranasal immunization with a combination of CTL epitope peptides, each restricted by a different MHC Class I molecule, induced MHC-restricted, peptide-specific CTL for each peptide in the mixture. Effective immunization of humans will most likely require immunization with a mixture to peptides to ensure that the entire population is effectively immunized. Therefore, additional studies are being performed to optimize the immunogenicity of CTL epitope peptides.

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DAMD 17-94-J-4467. Structural and functional studies of experimental HIV synthetic peptide immunogens.

Technical Aim #2. Structural Studies Using NMR

Investigators: L. D. Spicer, R. de Lorimier, H. M. Vu

INTRODUCTION

For Technical Aim 2 the immunogenic peptide subjects of this grant are being studied using solution NMR spectroscopy. The object is to determine conformational requirements needed to design peptide immunogens capable of inducing broadly cross-reactive anti-HIV neutralizing antibodies and anti-HIV T cell responses targeting HIV isolates grown either in T-cell lines or PBMC. Included in these studies are peptides already being characterized for immunogenic properties as well as new peptide constructs based on iterative evaluation of structure/function and design. We have completed characterization of the initial four peptides, C4-V3 RF, C4-V3 Can0, C4-V3 MN and C4-V3 EV91, 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 B and T cell 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 C4-V3 peptides Can0, EV91, MN, and RF 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 and Can0 studies have been published (de Lorimier et al., 1994; Vu et al., 1996), and a manuscript describing EV91, MN 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 C4 segment (also termed T1), 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 V3 (also called SP10) segments, which differ in sequence, show different preferred conformations in each peptide. The GPGX sequence at the tip of the V3 loop tends to form a Type II b-turn in peptides Can0 and MN, but a Type I b-turn in RF and EV91. The RPXX sequence toward the C-terminal end of the V3 sequences tends to form a Type I b-turn in RF and MN, a Type II b-turn in EV91, and is in an extended conformation in Can0. Residues just preceding the GPGX turn in RF and Can0 are extended, but in EV91 form another turn. And residues immediately following the GPGX turn form nascent helices in EV91 and MN, but are extended in RF and Can0.

To further evaluate the organization of the peptides, particularly with respect to potentially important side chain orientations, the above-listed conformational elements suggested by NMR data were used to create models of the 13-residue region encompassing the GPGX motif at the tip of the V3 loop. Using molecular modeling software, 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 structure study and shown to interact extensively with residues in the antigen-binding pocket. This hydrophobic patch was observed in our modeling of Can0 and MN, but not in EV91 or RF. The RF model showed a disruption in the middle of this apolar surface due to protrusion of a charged side chain from a lysine residue. The corresponding hydrophobic region in the EV91 model was seen to be twisted and appeared more compact than its extended counterparts in Can0 and MN. The structural similarity of Can0 and MN, and their differences with EV91 and RF, parallels their immunogenicities in that Can0 and MN 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 and in our Can0 model may act as a key conformational motif in inducing antibodies that are cross-reactive. Peptides EV91 and RF 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 C4-V3 RF peptide, is complete (de Lorimier et al., in preparation). As noted above, the C4 sequence, which contains T cell epitope called T1, 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 C4 portion of C4-V3 peptides, even though the C4 portion of native gp120 is antigenic. Thus we proposed to alter the sequence of C4 by single amino-acid substitutions that might reduce its helical tendency in solution and thus render it immunogenic for antibodies which recognize the C4 segment of gp120. Three variants of C4-V3 RF 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 originally reported T1SP10RF(A) peptide (de Lorimier et al., 1994) at the junction between C4 and V3. A control peptide, C4-V3 RF, has the native C4 sequence but no cysteine residue. All four peptides (control and three variants) were studied for immunogenicity and conformation.

In terms of B-cell immunogenicity, none of the C4 sequence variants elicited detectable antibodies in mice which bind to C4 of intact gp120. One possible explanation for this finding is that the sequence alterations removed determinants important for recognition of native C4. Another is that more stringent constraints are required for C4, as a peptide, to be immunogenic for a B-cell response. In terms of T-cell immunogenicity, measured by antibody titer and splenocyte proliferation, the C4 variants induced very different responses. Variant E9V was a much more potent inducer of T-helper responses than the original C4 sequence, while K12E was significantly less immunogenic. Since C4 contains T1, 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 C4-V3 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 control peptide C4-V3 RF showed that its conformation was nearly identical to cysteine-containing T1SP10RF(A), including a helical propensity in C4. Variant E9V retained this helical character, while variant E9G lacked any NOEs indicative of a helical tendency. Variant K12E showed helical conformations in C4, 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.

Several immunogenic hybrid peptides have been designed based on a T cell epitope in gag p24 termed GTH1. In light of results outlined above on C4 sequence variants of the T1 epitope in C4-V3 peptides, we considered altering the sequence of GTH1 to enhance immunogenicity. Sequence alignment of T1 and GTH1 reveals potential homology. We propose that insertion of an alanine residue in GTH1 will increase homology to T1 and improve the antigenicity of GTH1. Sequence alterations at positions in GTH1 analogous to positions 9 and 12 in T1 (see above) were also proposed which might lead to increased immunogenicity.

CONCLUSIONS

The objectives of Technical Aim 2 regarding solution conformations of peptides have been achieved. The original four C4-V3 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 proposed from these studies, a set of peptides based on C4 sequence variants of C4-V3 RF 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: STRUCTURAL AND FUNCTIONAL STUDIES OF EXPERIMENTAL HIV SYNTHETIC PEPTIDE IMMUNOGENS

Technical Aim #3: HLA-peptide interactions

Investigator: Donna D. Kostyu, Ph.D.
Co-investigator: Jane Treweek, M.D.

INTRODUCTION

Our ability to elicit T cell responses to synthetic peptide immunogens depends, at the most basic level, on whether the peptide or sequences derived from it are presented effectively on an HLA class I or class II molecule. There have been two developments that affect the way in which we view the potential immunogenicity of peptide vaccines in eliciting a CTL response. First, the number of alleles has grown to extraordinary numbers, with over 300 class I alleles now defined and still rising (1). For example, there are currently 22 subtypes of HLA-A2 , 19 subtypes of HLA-B35 and 36 subtypes of HLA-B15. Population distributions, where known, can be dramatic. HLA-A*0201 is found in all populations, but A*0202, A*0205 and A*0214 are found in Blacks and A*0203, A*0207 and A*0210 are found in Asians. HLA-A*0202 and A*0205 may bind similar sets of peptides, but A*0207 does not (2). Second, Sette and coworkers have raised the issue of whether widely divergent alleles such as B7, B35 and B51 may nevertheless present similar peptides, as the amino acid sequences around the peptide binding pockets is identical (3). This suggests that peptides or peptide analogs may be designed for presentation on multiple HLA class I alleles. These two issues represent contrasting views on the ease with which peptide vaccines will be immunogenic. We consequently are extending our peptide binding studies to test the influence of HLA class I subtypes, have carefully analyzed the location and extent of class I polymorphism, and have applied an algorithm to identify allele targets in 15 populations in order to establish basic rules for design and testing of peptide vaccines.

BODY

A. HLA-A AND HLA-B RESTRICTED RECOGNITION OF V3 PEPTIDES

We have characterized the interactions of the SP50 and AP10 peptides with the following alleles: A*0101, A*0201, A*0202, A*0301, A*2402, A*6801, A*3101, A*11, B*0702, B*0801, B*4402, B*2705, B*35, B*5101, B*5701. In almost all instances testing involved replicate experiments with at least two different cell lines carrying the appropriate allele. The HLA-B7 restricted CTL site identified by Safrit (4) is readily found in all of the SP50 peptides. There is a second HLA-B7 restricted site in AP10EV91. There are numerous instances of very weak binding with some HLA-A or B alleles, but as we continue to test cell lines, binding appears to be of low affinity, not always reproducible, and is probably indicative of unstable interactions. There appears to be an HLA-A24 restricted site in the AP10MN and AP10CANOA peptides, but only when we test one A*2402 cell line. We have been unable to find any recognition of this peptide with other A24 positive cells, suggesting that our A24 reactive monoclonal antibody picks up another allele on one individual, or that we are dealing with several A24 subtypes. This is currently under study.

B. NON-MHC BINDING OF THE T1 PEPTIDE

In the previous progress report, we were part way through the analysis of the 16mer C4-derived T1 peptide. Binding was found to be anomalous in several ways. The binding that was observed was unaccountably strong, and beyond that which could be accounted for by expression of HLA class I molecules on the cell surface. Although the T1 sequence contained internal anchor residues that should enable the peptide to bind to multiple HLA-A and HLA-B alleles, studies with protease inhibitors provided no evidence of processing the 16mer to a shorter, more bindable form. Moreover, T1 bound to some cell lines in ways that could not be accounted for by known motifs. Binding of several preparations of T1 was not always reproducible. Flow cytometry analysis indicated agglutination and sometimes cell death of cell lines exposed to the T1 peptide. All activities were observed with T1 dissolved in water, but not when dissolved in DMSO.

These results suggested that the T1 peptide forms extensive complexes in water. Direct binding of the peptide to cells could be monitored by incubating the T1 peptide with B or T cell lines for as little as 5 minutes, then exposing the cells to an avidin-FITC reagent. We believe that the peptide adopts a conformation similar to an amphipathic alpha helix, stacking extensively, and able to bind directly to cell surfaces and avidin-FITC. The ability to monitor binding allowed further characterization. We synthesized a series of truncated versions of T1 and localized a critical site to the amino terminal end. The T1.1 peptide (KQIINMWQEV) and T1.2 peptide (IINMWQEVGKA) were both capable of binding and agglutinating cells, albeit less efficiently that the T1 peptide itself, whereas the removal of the four amino-terminal residues (T1.3-T1.6 peptides) eliminated binding in this assay (Table 1). The T1, T1.1 and T1.2 peptides agglutinated many, but not all cell lines.

We synthesized two T1 variant peptides with amino acid substitutions at positions 9 and 12 to mimic the RF E9V and K12E peptides, and tested them for their ability to bind to cells. T1.8 (E9V) bound to cells quite well, whereas T1.7 (K12E) showed minimal binding, approximately 20% of that observed with T1.8 (Table 1).

There are two possible explanations for these observations. The T1 peptide could spontaneously adapt an extended, aggregated conformation in water, capable of binding to and agglutinating cells in a charge-related, but nonspecific manner. The alternate explanation is that the sequence KQIINMWQEVGKA contains or mimics a heparin-sulfate binding site. In this regard, the binding of T1 to the cell surface is inhibited by heparin and chrondroitin sulfate. It is possible that the different degrees with which T1 binds and agglutinates cells is dependent on the level of heparin-like molecules on the cell surface.

The unusual behavior of the T1 peptide probably explains the anomalous and inexplicable binding of the 39mer T1SP10MN(A) peptide to some HLA-A and B alleles in our usual binding assay. A 39 mer is too long to bind to a class I molecule, and we found no evidence of short cleavage products in the T1SP10MN(A) preparation. The other three 39mers T1SP10RF(A), T1SP10EV91(A) and T1SP10CANOA(A) failed to show such effects. We tested whether T1SP10MN(A), T1SP10RF(A) E9G, and T1SP10RF(A) K12E could bind directly to cells, using the secondary avidin-FITC to detect binding as was done for T1. We found extremely small, but discernible shifts in fluorescence with the T1SP10MN(A) and T1SP10RF(A) E9G peptides (Table 2), indicating that some aggregation and binding to the cell surface was occurring in an MHC-independent manner. It was not observed with the T1SP10RF(A) K12E peptide. We believe that the sequence of the V3 peptide affects the conformation of T1 or it's ability to spontaneously aggregate. The T1SP10MN(A) and T1SP10RF(A) E9G peptides maintain some of the

conformational characteristics of the T1 peptide itself, whereas the T1SP10RF(A) K12E peptide does not. This suggests that the immunogenicity of the 39mer peptides may in part be due to the ability of T1 to spontaneously form aggregates or specific complexes, bind cells, and enter a class I or class II pathway.

C. HIGH RESOLUTION HLA-A AND HLA-B TYPING

The analysis of peptide interactions with HLA-A or HLA-B alleles requires specific knowledge of the allele subtype being tested. Thirty primers have been tested for generic and family-specific amplification of exons 2 and 3 of HLA-A and HLA-B and we currently have 135 probes for high resolution typing. Specificity and reactivity of the exon 2 primers and probes have been evaluated on reference cells and on a set of approximately 250 members of multiplex rheumatoid arthritis families.

D. EFFECT OF HLA POLYMORPHISM ON PEPTIDE RESPONSES

We have analyzed a database of 275 HLA-A, HLA-B and HLA-C alleles in order to identify the exact sites of polymorphic residues and probable impact on peptide binding or interaction with other molecules. Modeling was done in collaboration with Dr. Linda Hannick in the Laboratory of Molecular Structure, NIAID. Despite the often quoted conclusion that polymorphism is concentrated around the peptide binding groove, there is significant polymorphism outside the peptide binding groove in the a1a2 domains and even in the a3, transmembrane and cytoplasmic regions. Some of this polymorphism will have no effect on peptide binding and may be ignored. In other instances, substitutions could affect interactions with β2microglobulin (leading to weaker interactions and less stable peptide binding), or with NK receptors, CD8, or chaperones in the endoplasmic reticulum. Two alleles (HLA-A68 and HLA-B48) have amino acid substitutions near the CD8 binding site (5,6) and it is known that peptide presentation on HLA-A68 can lead to CD8-independent CTLs (7). There is also evidence that some polymorphism is locus-specific. Thus, design of peptide vaccines to elicit class I restricted CTLs must test whether a particular molecule will not only bind and present a peptide, but also whether all subtypes of that allele will bind and present in a similar manner and if all give rise to efficient production of CTLs.

The T1SP10 peptides tested here bind to the commonly found HLA-B*0702 allele. However, there have been five other HLA-B7 alleles identified (B*0703-B*0707), with from 1 to 3 amino acid substitutions at residues 69, 70, 71, 97, 114 or 156. All are highly polymorphic residues and likely to affect peptide binding. The population distribution of these new HLA-B7 subtypes is not known, so it is possible that some HLA-B7 individuals will fail to produce CTLs to the vaccine because they carry a B7 subtype other than *0702. Sette and coworkers have identified an HLA-B7 supertype, where HLA-B7, B35 and B51 alleles may bind similar peptides. However, in our testing to date, the HLA-B7 presented SP50 and AP10 peptides have failed to bind HLA-B35 and HLA-B51 molecules.

As immunogens become shorter and more defined, the likelihood of nonresponders on the basis of HLA alleles increases. In collaboration with Dr. Deborah Dawson in the Department of Epidemiology and Biostatistics at Case Western Reserve University, we have applied an algorithm to identify the predominant alleles 5 Caucasian populations (French, Spanish, US, Canadian and Brazilian), 5 Black (Capetown, South African, US, Brazilian, and San Bushmen), and 5 Asian (Japanese, Korean, Northern Han Chinese, Southern Han Chinese, and Thai) populations. Tables 3 and 4 show the cumulative frequencies of HLA-A and HLA-B alleles in these groups, with those alleles providing 80% coverage in boxes. In Caucasian populations, three alleles (HLA-A1, A2, and A3) are present in all five populations and at high frequency. In Black populations, the predominant alleles are HLA-A2 and A30, with A28 and A23 found occasionally and A3, A34, A1 and A26 sporadically. The Asian populations are remarkable in the prevalence of HLA-

A2, A24 and A11 such that 80% coverage can be achieved with only 2 alleles in Japanese, Northern Han, Southern Han, and Thai individuals. It should be noted that the A11 in Asian populations and the A33 in Korean and Thai populations are rarely found in Caucasians or Blacks.

There are more HLA-B alleles and at lower allele frequencies, leading to extreme heterogeneity in the 15 populations. Three alleles are found in approximately 80% of individuals in the South African and San Bushmen populations, and four alleles are observed in the Southern Han Chinese population. For all the remaining groups, the number of alleles required for 80% coverage grows rapidly to six or eight B alleles and to ten HLA-B alleles in Brazilian Blacks. Whereas HLA-A2 is shared among all populations, no commonly shared, high frequency HLA-B allele can be identified. Further, even dividing the populations into three major ethnic groups (Caucasians, Blacks, Asians), there is extreme heterogeneity in HLA-B alleles.

This algorithm identifies the predominant alleles in any population of interest, allows for calculation of coverage given knowledge of HLA allele frequencies in that populations, and can be used to predict efficiency of immune responses based on HLA class I alleles. Our data, currently based on the 1991 International Workshop, is of general utility for vaccine trials but is also illustrative of trends and problems. A peptide vaccine presented on a common HLA-A molecule may have broader coverage than vaccines targeting HLA-B alleles. Mixed ethnic groups are especially problematic. Inbred populations, on the other hand, require special attention because the alleles they carry may be unusual. However, higher coverage may be possible. For the peptides studied here, the inclusion of an HLA-B7 restricted CTL site should make the vaccine effective in approximately 10% of US, Canadian and French Caucasians and in US and Brazilian Black populations. However, it would have minimal effects in Asians where HLA-B7 is rarely observed (Table 4).

CONCLUSIONS

Our ability to design a peptide vaccine that elicits CTLs is constrained by the striking polymorphism at HLA-A and HLA-B, the occurrence of multiple subtypes of alleles (not all of which will bind the same set of peptides), and ethnic distribution. How much of a problem these will be, and whether it is possible to circumvent them, is still not clear. In this next year, we intend to obtain and test cell lines that express HLA-B*0703, 0704, 0705, 0706, and 0707 for their ability to bind and present V3 peptides. As some of these cells are rare, we may use site-directed mutagenesis to create transfectants for clear and unambiguous answers. Our ability to type all HLA-A and HLA-B alleles at high resolution is nearly complete.

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Table 1

Binding of the T1 peptides to the cell surface

Peptide	Sequence	Mean fluorescent intensity
experiment 1		
none		6
T1	K Q I I N M W Q E V G K A M Y A	2406
T1.1	K Q I I N M W Q E V	445
T1.2	I I N M W Q E V G K A	663
T1.3	N M W Q E V G K A M Y A	7
T1.4	N M W Q E V G K A	5
T1.5	W Q E V G K A M Y A	7
T1.6	E V G K A M Y A	6
experiment 2		
none		13
T1.8 (E9V)	K Q I I N M W Q V V G K A M Y A	591
T1.7 (K12E)	K Q I I N M W Q E V G E A M Y A	197

Table 2

Direct binding of two peptides
to the cell surface

Cells	Peptide	Avidin-FITC	GMFI *	SD
HSB	none	+	3.14	.20
HSB	T1	+	215.58	
HSB	T1SP10MN(A)	+	6.00	.18
HSB	T1SP10RF(A) E9G	+	6.32	.81
HSB	T1SP10RF(A) K12E	+	2.80	.11
HSB	SP50 MAL	+	2.47	.18
HSB	Mage3	+	2.80	.28

* Geometric mean fluorescence intensity +/- standard deviation of triplicates

Table 3
Cumulative frequencies of HLA-A alleles in Caucasian, African American and Asian populations

Allele	Freq	CP
French		
A2	0.213	0.38
A1	0.137	0.58
A3	0.133	0.73
A24	0.089	0.82
A29	0.064	0.87
A11	0.062	0.91
A28	0.056	0.94
A26	0.044	0.96

Allele	Freq	CP
Spanish		
A2	0.276	0.48
A1	0.113	0.63
A29	0.093	0.73
A3	0.090	0.82
A11	0.070	0.87
A24	0.068	0.92
A30	0.051	0.94
A26	0.045	0.96

Allele	Freq	CP
Brazilian		
A2	0.260	0.45
A1	0.099	0.59
A24	0.096	0.70
A3	0.069	0.77
A28	0.064	0.83
A30	0.060	0.88
A26	0.053	0.91
A31	0.048	0.94
A11	0.047	0.96

Allele	Freq	CP
USA		
A2	0.283	0.49
A1	0.169	0.70
A3	0.122	0.82
A24	0.096	0.89
A11	0.055	0.92
A32	0.051	0.95

Allele	Freq	CP
Canadian		
A2	0.267	0.46
A1	0.186	0.70
A3	0.113	0.81
A24	0.113	0.90
A32	0.058	0.93
A26	0.053	0.96

Allele	Freq	CP
South African		
A2	0.149	0.28
A30	0.136	0.49
A28	0.111	0.64
A23	0.097	0.74
A34	0.069	0.81
A3	0.062	0.86
A29	0.059	0.90
A29	0.050	0.93
A24	0.041	0.95

Allele	Freq	CP
San Bushmen		
A30	0.204	0.37
A2	0.184	0.63
A3	0.155	0.79
A23	0.126	0.89
A43	0.117	0.95

Allele	Freq	CP
Capetown		
A2	0.165	0.30
A30	0.113	0.48
A1	0.099	0.61
A28	0.092	0.72
A26	0.070	0.79
A24	0.062	0.84
A33	0.060	0.89
A3	0.057	0.92
A11	0.053	0.95

Allele	Freq	CP
Brazilian		
A23	0.144	0.27
A2	0.128	0.47
A30	0.109	0.62
A28	0.090	0.72
A3	0.080	0.80
A33	0.060	0.85
A1	0.056	0.89
A31	0.054	0.92
A24	0.035	0.94
A11	0.034	0.96

Allele	Freq	CP
USA		
A2	0.167	0.31
A28	0.109	0.48
A30	0.095	0.60
A3	0.089	0.71
A23	0.081	0.79
A33	0.081	0.86
A1	0.053	0.89
A34	0.051	0.92
A24	0.047	0.95

Allele	Freq	CP
Japanese		
A24	0.351	0.58
A2	0.244	0.84
A26	0.109	0.91
A11	0.104	0.96

Allele	Freq	CP
Korean		
A2	0.293	0.50
A24	0.228	0.77
A33	0.149	0.89
A11	0.094	0.94
A26	0.081	0.98

Allele	Freq	CP
Northern Han (Chinese)		
A2	0.367	0.60
A11	0.203	0.82
A24	0.126	0.91
A1	0.047	0.93
A30	0.044	0.10

Allele	Freq	CP
Southern Han (Chinese)		
A2	0.337	0.56
A11	0.319	0.88
A24	0.199	0.98

Allele	Freq	CP
Thai		
A11	0.325	0.54
A2	0.255	0.82
A24	0.146	0.92
A33	0.136	0.98

Table 4
Cumulative frequencies of HLA-B alleles in Caucasian, African American and Asian populations

Allele	Freq	CP
French		
B44	0.108	0.20
B35	0.084	0.35
B7	0.075	0.46
B14	0.072	0.56
B51	0.069	0.65
B8	0.068	0.73
B62	0.053	0.78
B18	0.048	0.82
B55	0.037	0.85
B27	0.036	0.88
B13	0.030	0.90
B38	0.030	0.92
B49	0.030	0.93
B60	0.030	0.95

Allele	Freq	CP
Spanish		
B44	0.170	0.31
B35	0.084	0.44
B51	0.078	0.55
B7	0.076	0.65
B14	0.069	0.73
B8	0.064	0.79
B18	0.055	0.84
B49	0.037	0.87
B62	0.037	0.89
B38	0.030	0.91
B27	0.028	0.93
B50	0.027	0.94
B13	0.025	0.95

Allele	Freq	CP
Brazilian		
B35	0.137	0.26
B44	0.106	0.43
B51	0.071	0.53
B18	0.053	0.60
B7	0.051	0.66
B14	0.050	0.72
B8	0.049	0.77
B39	0.049	0.81
B62	0.042	0.85
B38	0.036	0.87
B27	0.031	0.89
B49	0.030	0.91
B60	0.024	0.93
B57	0.022	0.94
B45	0.021	0.95

Allele	Freq	CP
USA		
B44	0.104	0.20
B7	0.100	0.37
B8	0.100	0.52
B35	0.085	0.63
B62	0.055	0.69
B18	0.049	0.74
B60	0.045	0.79
B14	0.041	0.82
B27	0.041	0.86
B38	0.041	0.89
B57	0.039	0.91
B51	0.037	0.93
B61	0.033	0.95
B13	0.030	0.96

Allele	Freq	CP
Canadian		
B44	0.133	0.25
B7	0.111	0.43
B8	0.111	0.58
B35	0.076	0.68
B62	0.070	0.75
B14	0.065	0.81
B18	0.058	0.86
B60	0.056	0.90
B27	0.043	0.92
B51	0.040	0.94
B57	0.036	0.96

Allele	Freq	CP
South African		
B70	0.226	0.40
B58	0.172	0.64
B42	0.128	0.78
B8	0.098	0.86
B7	0.081	0.91
B44	0.071	0.95

Allele	Freq	CP
San Bushmen		
B58	0.356	0.59
B8	0.133	0.74
B7	0.128	0.85
B70	0.104	0.92
B18	0.071	0.96

Allele	Freq	CP
Capetown		
B58	0.110	0.21
B44	0.086	0.35
B70	0.086	0.48
B51	0.062	0.57
B8	0.057	0.64
B18	0.057	0.71
B35	0.048	0.76
B57	0.048	0.80
B7	0.043	0.84
B14	0.043	0.87
B42	0.038	0.90
B27	0.029	0.91
B60	0.029	0.93
B13	0.024	0.94
B37	0.024	0.95

Allele	Freq	CP
Brazilian		
B35	0.100	0.19
B7	0.096	0.35
B44	0.070	0.46
B42	0.060	0.55
B53	0.044	0.61
B51*	0.041	0.66
B52	0.036	0.70
B18	0.034	0.74
B49	0.031	0.77
B55	0.031	0.80
B57	0.031	0.82
B14	0.026	0.85
B45	0.026	0.86
B8	0.022	0.88
B70	0.022	0.90
B13	0.018	0.91
B50	0.017	0.92
B51	0.017	0.93
B58	0.017	0.93
B62	0.017	0.94
B39	0.013	0.95

Allele	Freq	CP
USA		
B53	0.128	0.24
B7	0.083	0.38
B70	0.082	0.50
B35	0.077	0.60
B58	0.069	0.69
B44	0.062	0.75
B42	0.047	0.80
B57	0.042	0.83
B45	0.038	0.86
B18	0.033	0.89
B8	0.032	0.91
B51	0.032	0.92
B14	0.030	0.94
B63	0.028	0.95

Allele	Freq	CP
Japanese		
B52	0.107	0.20
B61	0.107	0.38
B51	0.093	0.52
B62	0.083	0.63
B35	0.081	0.72
B44	0.074	0.79
B54	0.063	0.85
B60	0.056	0.89
B7	0.050	0.92
B39	0.045	0.94
B46	0.044	0.96

Allele	Freq	CP
Korean		
B62	0.105	0.20
B44	0.099	0.37
B61	0.092	0.50
B51	0.078	0.61
B35	0.070	0.69
B54	0.065	0.76
B13	0.063	0.82
B58	0.052	0.86
B60	0.042	0.89
B7	0.041	0.91
B46	0.040	0.94
B48	0.040	0.95

Allele	Freq	CP
Northern Han (Chinese)		
B13	0.164	0.30
B51	0.118	0.48
B62	0.118	0.64
B60	0.060	0.71
B61	0.042	0.75
B44	0.041	0.79
B35	0.036	0.82
B55	0.032	0.85
B58	0.032	0.87
B7	0.031	0.89
B46	0.028	0.91
B48	0.028	0.93
B57	0.028	0.94
B63	0.026	0.95

Allele	Freq	CP
Southern Han (Chinese)		
B60	0.171	0.31
B46	0.154	0.54
B62	0.147	0.72
B13	0.085	0.80
B61	0.054	0.85
B54	0.049	0.88
B58	0.049	0.92
B51	0.043	0.94
B55	0.040	0.96

Allele	Freq	CP
Thai		
B46	0.140	0.26
B13	0.093	0.41
B60	0.083	0.53
B75	0.083	0.64
B51	0.064	0.71
B27	0.060	0.77
B44	0.054	0.82
B57	0.052	0.86
B62	0.050	0.90
B61	0.043	0.92
B38	0.035	0.94
B52	0.031	0.96

COOPERATION - EDITOR

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HLA Class I Polymorphism: Structure and Function and Still Questions

Donna D. Kostyu, Linda I. Hannick, Jane L. Traweek, Manar Ghanayem, Drew Heilpern and Deborah V. Dawson

ABSTRACT: The HLA-A, HLA-B and HLA-C molecules have turned out to be highly polymorphic and functionally complex. They not only serve as peptide receptors, but also interact with β_2-microglobulin, an $\alpha\beta$ T cell receptor, CD8 and NK inhibitory molecules, all at different sites. The fact that more than 300 class I alleles have now been defined prompted us to ask the question of where polymorphism really occurs in a class I molecule. We have used a database of 272 HLA-A, HLA-B and HLA-C alleles to illustrate how extensive the polymorphism is. The data is presented here for comparison of alleles and allele families and to facilitate studies of class I structure and function. *Human Immunology 56, 00–00 (1997).* © American Society for Histocompatibility and Immunogenetics, 1997. Published by Elsevier Science Inc.

ABBREVIATIONS

β_2m	β_2-microglobulin	BIP	Ig binding protein
CDR1, 2, 3	complementarity determining regions	TAP	transporter associated with antigen processing
NK	natural killer		
TCR	T cell receptor		

INTRODUCTION

The HLA class I molecules have turned out to be surprisingly complex. The $\alpha1\alpha2$ domains bind more than peptides. Some residues are in direct contact with the $\alpha\beta$ TCR, establishing the fundamental basis of MHC restriction [1]. Residues at one end of the $\alpha1$ domain α helix interact with NK receptors to generate an inhibitory signal and prevent cell lysis [2]. Nearby is residue 86, a conserved *N*-glycosylation site that facilitates interaction with calnexin in the endoplasmic reticulum [3]. β_2-microglobulin (β_2m), required for stable peptide binding, extends diagonally beneath the $\alpha1\alpha2$ domains [4]. The fact that β_2m can engage and dis-engage allows for peptide loading in the endoplasmic reticulum and at the cell surface. The $\alpha3$ domain is more than a structural support. Residues at the top of the $\alpha3$ domain connect to the $\alpha1\alpha2$ domains while residues along one strand interface with β_2m. An extended loop contains the binding site for CD8 [5]. Overall, the generic class I molecule has evolved into a multi-site receptor, interacting with β_2m, a peptide, an $\alpha\beta$ T cell receptor, and CD8 in order to stimulate a cytotoxic T cell response, as well as with calnexin, BIP, TAP and other molecules in the endoplasmic reticulum to facilitate construction, and with NK inhibitory receptors at the cell surface for quality control.

In addition to this is the remarkable degree of polymorphism. There were fewer than 50 class I alleles recognized in 1975. Today the number is over 300 and still growing. Such polymorphism was foreshadowed early. Some will remember the twenty-nine "Z" specificities identified in the 1972 Histocompatibility Work-

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Key words: HLA-A, HLA-B, HLA-C MHC class I, polymorphism

Received August 1997.

Human Immunology 56, 00–00 (1997)

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Published by Elsevier Science Inc.

0198-8859/97/$17.00

PII S0198-8859(97)00175-4

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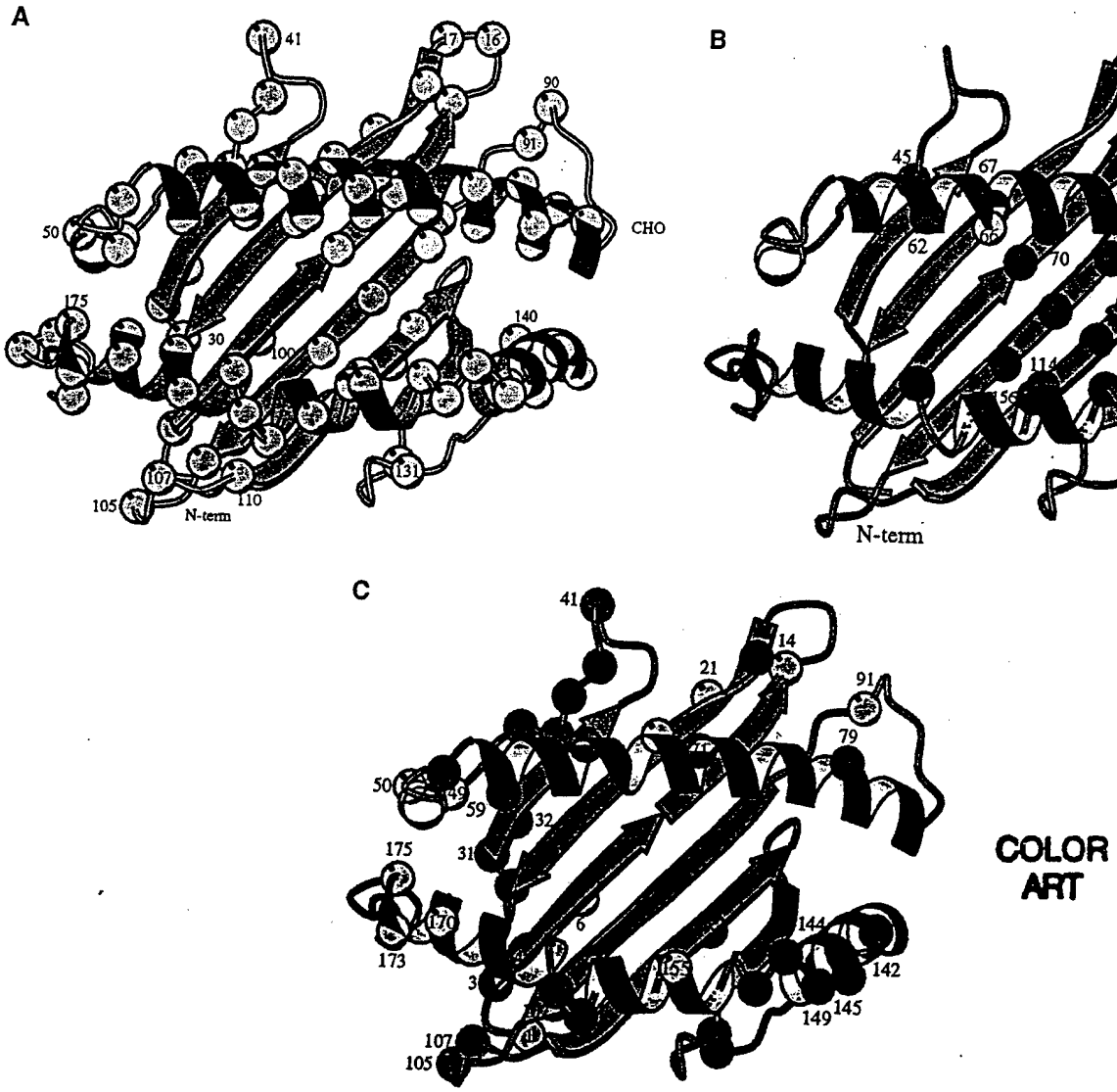

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FIGURE 1 Polymorphism in the α1 and α2 domains. (a) The yellow balls denote the location of all of the polymorphic residues of the α1 and α2 domains of HLA-A, HLA-B, and HLA-C. (b) The locations of the most polymorphic residues are indicated by red balls (those residues with four or more amino substitutions in any one class I molecule) and yellow balls (residues that show fewer amino acid substitutions but that are polymorphic in all three molecules). A listing of these residues is given in Table 2. The figure is turned slightly from part (a) in order to show all of the residues. (c) Locus-specific polymorphism is indicated by residues that are polymorphic only at HLA-A (red balls), HLA-B (green balls), or HLA-C (yellow balls). A listing of these residues is given in Table 3. Residues 178 and 180 are in the connecting strand to the α3 domain and are not visible here.

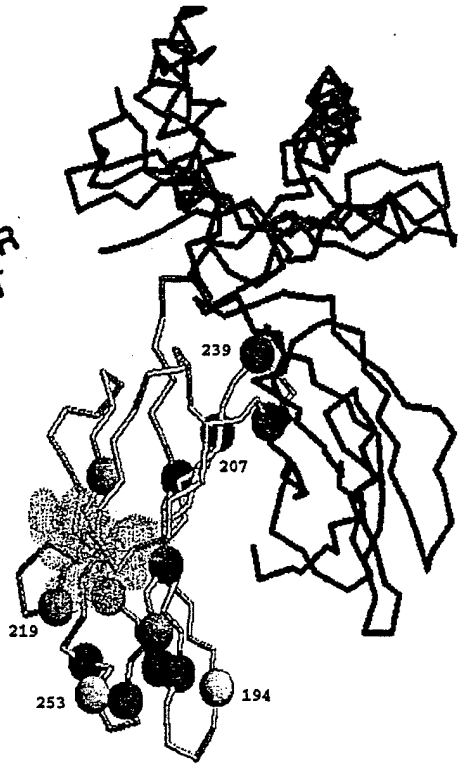

FIGURE 2 Polymorphic residues in the α3 domain, showing residues that are polymorphic at all three loci (white balls), residues that are polymorphic only at HLA-A (red balls), HLA-B (green balls) or HLA-C (yellow balls). For clarity, seven residues that are polymorphic in two of the three class I molecules are not shown here, but are listed in Table 4. The magenta ball represents the transmembrane residue included in the crystal structure of HLA-B27 and the silver stippled area indicates the CD8 binding site.

shop [6], the multiple subtypes of B5 in non-Caucasian populations [7] and the fourteen variants of B62 in the 1984 Workshop [8]. Most unsettling were the observations that sera concordant in Caucasians were notoriously heterogeneous in other ethnic groups [9]. The identification of the dialleleic Bw4/Bw6 on the HLA-B molecule [10, 11] and cross-reactivity between HLA-A and HLA-B [12, 13] were indications of complex, serologically defined epitopes. Cytolytic T cells were equally discriminating [14]. This complexity has led to interesting twists and turns in nomenclature. For example, the original allele LND was formally named HLA-B15, split into B62 and B63, and now with DNA typing reclassified back to B15 [15]. There are currently 37 HLA-B15 alleles. The Native American allele BN21 is serologically a B50 but by DNA sequencing is a B40 (B*4005) [16]. The BU and SV alleles first identified in 1977 [17] have been variably considered as 8w59 [18], the supertypic allele B70, the subtypic alleles B71 and B72 [19] and by DNA sequencing are B*1503 and B*1510 [15].

Class I polymorphism has important biologic implications for allotransplantation, the design of vaccines for tumor immunotherapy or infectious disease and for the design of peptides for modification of T cell responses. However, it is difficult to get a clear grasp of the amino acid substitutions that define the extraordinary number of alleles identified to date, and to determine the effect some might have on peptide binding or perhaps on interactions with β_2m, CD8, NK cells or a TCR. Even management of the database has become cumbersome. To simplify class I polymorphism, we have taken the amino acid sequence of 272 HLA class I alleles and asked where exactly the polymorphism is. The data is presented here for comparison of alleles and allele families and to facilitate studies of structure and function.

MATERIALS AND METHODS

We have used the October 1996 data base of 272 HLA-A, HLA-B, and HLA-C alleles compiled by Kelly Arnett and Peter Parham for the American Society of Histocompatibility and Immunogenetics (http://www.smed.edu/home_pages/ASHI/~~sequences/A_P.htm~~), excluding three alleles (A*3005, A*3302 and Cw*0201) that were withdrawn in the latest nomenclature report [20]. ~~Our analysis is therefore based on the sequences of 272 class I alleles.~~ Thirty-three new alleles (A*0218, 0221, 0222, 2411, 2413, 2608, 2903, 6803, 7403, B*0708, 0804, 1304, 1536, 1804, 1805, 2711, 3514, 3520, 3521, 3911, 3912, 4010, 4409, 4702, 5002, 5109, 5302, 5505, 5603, Cw*0707, 1204, 1604 and 1802) are not included in this analysis as these sequences were not available.

All of the modeling has been done by L.I.H. using the program MOLSCRIPT [21] for Fig. 1 and the program RasMol [22] for Figs. 2–4. Three-dimensional coordinates are based on the crystal structure of HLA-B27 [23].

RESULTS

The full length HLA class I concensus sequence and all possible amino acid substitutions at each residue and for each class I molecule are provided in Table 1, beginning with the leader sequence and extending through the cytoplasmic region. Polymorphism is spread throughout the molecule, i.e. 15/24 residues (63%) are polymorphic in the leader sequence, 47/90 residues (52%) in the α1 domain, 41/92 residues (45%) in the α2 domain, 21/92 residues (23%) in the α3 domain, 20/40 residues (50%) in the transmembrane region, and 7/28 residues (25%) in the cytoplasmic region. We have not considered as polymorphic those single amino acid substitutions that have occurred between loci but that are conserved within a

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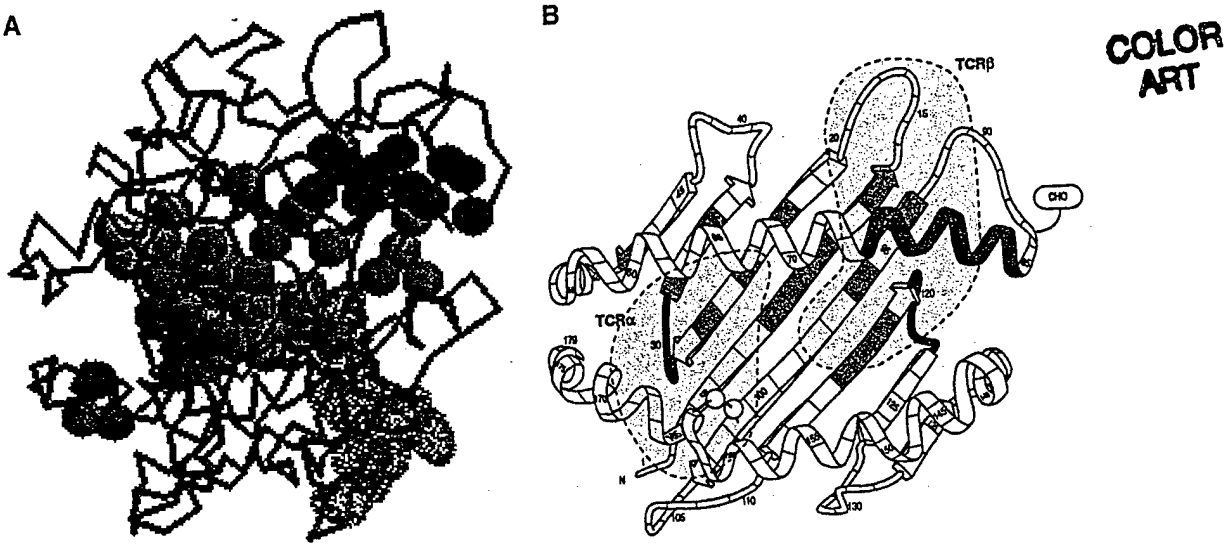

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FIGURE 3 Schematic diagrams of how the α1α2 domains interact with NK molecules, the α3 domain, β_2m and TCR. In (a), the site that is recognized on HLA-B and HLA-C molecules by NK inhibitory receptors is represented by purple balls. The α3 domain contacts in the downward loop (residues #29-31) and in the connecting strand between the α2 and α3 domains (#181-183) are green balls. The extended loop of the α3 domain (residues #223-229) which forms part of the CD8 binding site is shown by the silver stippling. The residues of the α1α2 domain that interface with β_2m are pink balls. In (b), the downward loops of the α1α2 domains are shown by the green and black loops; the NK recognition site is purple. The TCR α and β chains overlay the HLA α1α2 domains as shown in yellow, although direct contact only occurs at the peptide-binding groove and along the α helices. The gray shaded residues are those in contact with β_2m. The figure on the right was modified from ref. 55; the TCR contact area is from ref. 31; the NK site is from ref. 32–34; the β_2m contact area is from ref. 4.

locus, e.g. residue 182 which is an Ala (A) in all HLA-B and HLA-C alleles and a Thr (T) in all HLA-A alleles.

Residues that are involved in interdomain interactions (i.e. the α1α2 domains with the α3 domain) or that interact with β_2m or CD8 are also indicated in Table 1. We indicate which positions contribute to six peptide-binding pockets (A-F). These interactions should be considered in general terms only. These contact residues are based on the crystal structure of HLA-A2 [4] and the pockets and the residues that contribute to them may differ in other alleles. An alternative and broader view of peptide-MHC contacts has recently been proposed [24].

The location of all the polymorphic residues in the α1α2 domains of HLA-A, HLA-B and HLA-C is shown in Fig. 1A. Much of the polymorphism occurs at the bottom of the peptide binding cleft and especially along the alpha helices, serving as touch points for peptide and/or TCR. Interestingly, many of the positions on the loops outside the peptide-binding site, (e.g. # 16, 17, 41, 90, 91, 105, 107 and 131) are also polymorphic.

T2 Which positions are the most variable? Table 2 lists the highly polymorphic residues (defined arbitrarily as positions with at least four observed amino acids in any one of the class I molecules or positions that are polymorphic in all three class I molecules) in the α1 and α2 domains of HLA-A, HLA-B and HLA-C. Fourteen residues (#9, 45, 62, 67, 70, 77, 95, 97, 99, 114, 116, 152, 156, 163) have at least four observed amino acid substitutions. Another three residues (#66, 80, 90) show fewer amino acid substitutions but are polymorphic in all three class I molecules. The location of these is shown in Fig. 1B. Only one (#90) of the seventeen residues lies outside of the peptide binding groove.

Locus-specific polymorphism is observed in both the α1 and α2 domains. Nineteen residues are polymorphic only at HLA-A (red balls in Fig. 1C), 13 residues only at HLA-B (green balls in Fig. 1C), and 12 residues only at HLA-C (yellow balls in Fig. 1C). The amino acid substitutions at these positions are listed in Table 3. The T3 variability between loci does not appear to be randomly dispersed but instead occurs in discrete areas. In HLA-B molecules for instance, five amino acid substitutions are found at position 45, a position that is not polymorphic at HLA-A and HLA-C. HLA-B specific amino acid substitutions are also observed at residues 30 and 32 which make interdomain contacts and probably stabilize the three dimensional structure, residue 46 which has a side chain that points outward, residue 59 which is a tyrosine that points down into the peptide binding groove and residue 64, which might stabilize the alpha helix on which it sits relative to the α2 domain area. In HLA-A molecules, a cluster of substitutions occurs at the *N*-terminal end of the α2 domain α helix (Fig. 1C), at residues 127, 142, 144, 145, 149, 150 and 151. The side

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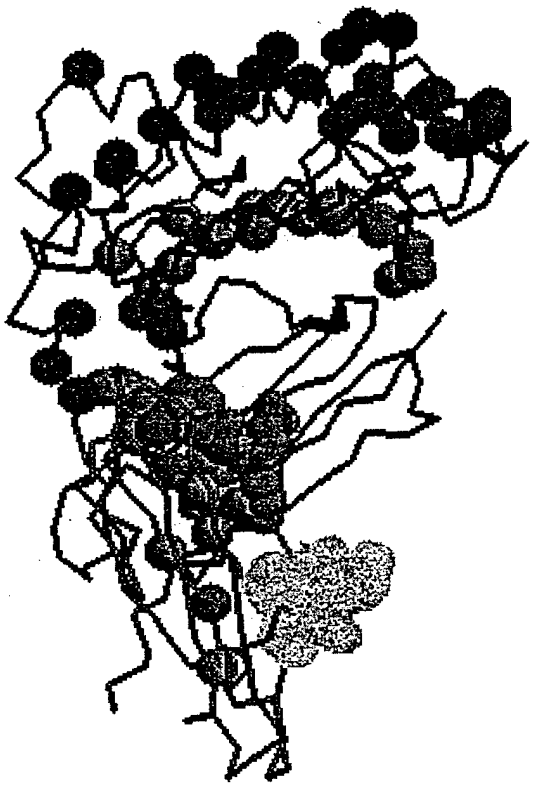

FIGURE 4 A lateral view of a class I molecule. The HLA residues which contact the TCR α chain (red balls, residues #58, 65, 66, 68, 69, 155, 158, 159, 163, 166, 167, 170) and the TCR β chain (black balls, residues #72, 149, 150, 151, 155) are based on the HLA-A2-HTLV1 Tax peptide crystal structure. Also shown are the NK site (purple balls, residues #77-83), the contact residues between the α1α2α3 domains and β_2m (pink balls), the CD8 binding site (the silver stippled area, residues #223-229), and the α1α2 residues that contact the α3 domain (green balls, residues #29-31 and #181-183). The TCR contact sites are from ref. 31; the NK site from ref. 32–34, the CD8 binding site from ref. 5; the β_2m contacts from ref. 4.

chains of these residues point away from the peptide binding groove.

The α3 domain is also polymorphic, as summarized in Table 4. Two residues (#194, 253) are polymorphic in all three class I molecules; these are located in the membrane-proximal region of the α3 domain. Seven residues are polymorphic in two of the three loci. Twelve residues show locus-specific polymorphism, five unique to HLA-A, three to HLA-B and four to HLA-C. The location of these is shown in Fig. 2.

The transmembrane region shows a small number of amino acid substitutions. These are primarily conservative substitutions of one hydrophobic amino acid to another hydrophobic amino acid. The cytoplasmic region includes three polymorphic residues in HLA-A molecules, one in HLA-B molecules and four in HLA-C molecules. Those at HLA-B and HLA-C involve transitions to cysteines.

The analysis above focuses on the overall three-dimensional conformation of a class I molecule and on the variability of each residue, but does not permit comparison of alleles. In order to visualize the polymorphism that exists within allele families, we collapsed the data set of sequences and removed the invariant positions, leaving only those residues that were polymorphic. Tables 5–7 summarize the polymorphism that occurs at HLA-A, HLA-B, and HLA-C, respectively, in the α1, α2, α3, transmembrane and cytoplasmic regions. The leader sequence was not included as it does not contribute to the protein structure at the cell surface. The differences within an allele family can be very small. For example HLA-A*0101 and A*0102 differ by two amino acids in the α1 domain. The two subtypes of A29 differ by one amino acid in the α2 domain ~~and one in the cytoplasmic region~~. In contrast is the heterogeneity of the A2 and B15 allele families. Correlations between serology and sequence have been well explored and widely published and are not discussed here. We might note however the uniqueness of the A*8001 allele [26]. The HLA-A69 allele carries the α1 domain of HLA-A28 (68) and the α2 domain of HLA-A2 [27]. HLA-B*4201 is a hybrid of B*0702 and B*0801 [28]. The unusual pattern of substitutions in the α3 domain and transmembrane regions in the HLA-B*7301 allele was shown to be due to duplication and deletion [29].

The polymorphism in the α3 domain in HLA-A is interesting. There are two basic α3 regions for HLA-A, governed by substitutions at residues 184 (P or A), 193 (P or A), 194 (I or V), 207 (G or S), 253 (E or Q) and sometimes 246 (A or S) (Table 5). The AAVSQ pattern occurs in the A1, A3, A11, A9 and A30 allele families. The remaining HLA-A alleles carry a sequence which is similar to that of HLA-B. These five residues occur on one face of the α3 domain. Residues 207 and 184 are at the top of the α3 domain near the interface with the α1α2 domains. Residues 193, 194 and 253 sit at the base of the α3 domain. Residue 246 might be predicted to have an effect on CD8 binding. The α3 domain in HLA-B molecules shows little polymorphism and in HLA-C, there are scattered amino acid substitutions.

DISCUSSION

One hypothesis for the generation and maintenance of the extensive class I polymorphism is that broader peptide binding leads to more adaptable immune responses [1, 30]. Polymorphism is indeed found at the base of the peptide binding cleft and especially on the α helices surrounding the peptide binding groove. However, a considerable amount of polymorphism in the α1α2 do-

TABLE 1 Polymorphic and invariant residues at HLA-A, HLA-B and HLA-C

Consensus	HLA-A	HLA-B	HLA-C	Interaction with*	Consensus	HLA-A	HLA-B	HLA-C	Interaction with*
Leader sequence					32 Q		QL		β2m
					33 F				
1 M					34 V				pocket B
2 R	AT	LR			35 R	RQ		RQ	β2m
3 V	VI				36 F				
4 M		MT			37 D				
5 A	PA		AE		38 S				
6 P					39 D				
7 R			QR		40 A				
8 T			AT		41 A		AT		
9 L		LV			42 S				
10 L	LV		IL		43 P	RQ			
11 L					44 R	RK			
12 L					45 M		METKG	G	pocket B
13 L					46 E		EA		
14 S	LS	WS			47 P				
15 G		AG			48 R				β2m
16 A			GA		49 A			AES	
17 L		LV			50 P			PR	
18 A					51 W				
19 L					52 I		IV	V	
20 T			IT		53 E				
21 E	QH				54 Q				
22 T					55 E				
23 W	WR				56 G	GRE			
24 A					57 P				
Alpha 1 domain					58 E				
					59 Y		YH		pocket A
1 G			GC	α3	60 W				
2 S					61 D				
3 H	HQ				62 R	RQGEL	RG		
4 S		SF			63 E	ENQ	EN		pockets A, B
5 M				pocket A	64 T		TI		
6 R			RK	β2m	65 Q	RG	QR		
7 Y				pockets A, B	66 I	NK	INK	KN	pockets A, B
8 F				β2m	67 V	MV	YFSCM	Y	pocket B
				β2m, pockets B, C	68 K			KN	
9 Y	YFST	YDH	YFSD		69 T	A	TAR	R	
10 T				β2m	70 N	HQ	NQSK	Q	pockets B, C, D
11 A	S	AS	AS		71 T	S	TA	A	
12 V	MV	VM		β2m	72 Q				
13 S					73 T	TI		TA	pockets C, D
14 R			RW		74 D	DHN	DY		pocket C
15 P					75 R				
16 G		GV	GS		76 E	AVE	EV	V	
17 R	RS		RA		77 S	NDS	SNDG	SN	pocket F
18 G					78 L				
19 E	EK				79 R	GR			
20 P					80 N	TI	NTI	NK	pocket F
21 R			RH		81 L	LA	LA		pocket F
22 F					82 R	RL	RL		
23 I				β2m	83 G	GR	GR		
24 A		AST	AS	pocket B	84 Y				pocket F
25 V				β2m, pocket B	85 Y				
26 G					86 N				carbohydrate
27 Y				β2m	87 Q				
28 V					88 S				
29 D				α3	89 E				
30 D		DG		α3	90 A	DA	DA	DA	
31 T	TS			α3					

TABLE 1 Polymorphic and invariant residues at HLA-A, HLA-B and HLA-C (*Continued*)

Consensus	HLA-A	HLA-B	HLA-C	Interaction with*
Alpha 2 domain				
91 G			GR	
92 S				
93 H				
94 T		TI	TI	β2m
95 L	LIVR	LIW	LIF	
96 Q				β2m
97 R	RIM	RSTWNV	RWN	pockets C, D, E
98 M				β2m
99 Y	YCF	YFS	YCFS	pockets A, B, D
100 G				
101 C				
102 D	DH			
103 V		VLM	VL	
104 G				
105 P	PS			
106 D				
107 G	GW			
108 R				
109 L	LF	LF		
110 L				
111 R				
112 G				
113 Y		YH	YH	pocket D
114 D	RHQE	DNH	DN	pockets D, E
115 Q	QR			β2m
116 Y	YDNH	YLFSDH	YFSL	β2m, pocket F
117 A				β2m
118 Y				
119 D				β2m
120 G				β2m
121 K				β2m
122 D				
123 Y				pocket F
124 I				
125 A				
126 L				
127 N	NK			
128 E				
129 D				
130 L				
131 R		RS		
132 S				
133 W				pocket E
134 T				
135 A				
136 A				
137 D				
138 T	M	TK	TK	
139 A				
140 A				
141 Q				
142 I	IT			
143 T		TS	TS	pocket F
144 Q	QK			
145 R	RL			
146 K				pocket F
147 W		WL	WL	pocket E, F
148 E				
149 A	AT			
150 A	AV			
151 R	RH			
152 V	VAEWR	VE	EAT	pocket E
153 A				
154 E				
155 Q			QE	pocket D
156 L	LRWQ	LRDW	LRWDQ	pocket D, E
157 R				
158 A	AV	AT		
159 Y				pocket A, D
160 L				pocket D
161 E	ED			
162 G		GD		
163 T	TREL	TLE	TLE	pocket A
164 C				
165 V				
166 E	ED	ED		
167 W	WG	WGS		pocket A
168 L				
169 R				
170 R			RG	
171 Y	YH	YH		pocket A
172 L				
173 E			EK	
174 N				
175 G			GR	
176 K				
177 E		ED	EK	
178 T		TK		
179 L				α3
180 Q		QE		
181 R				α3
182 A	T			α3
Alpha 3 domain				
183 D			E	α1α2
184 P	PA		PHR	
185 P				
186 K	KR			
187 T				
188 H				β2m
189 V	M			
190 T				
191 H				
192 H				β2m
193 P	PA		PL	
194 I	IV	IV	LV	
195 S				
196 D				
197 H				
198 E				
199 A		AV		
200 T				
201 L				β2m
202 R				
203 C				β2m
204 W				
205 A				β2m
206 L				

TABLE 1 (*Continued*)

Consensus	HLA-A	HLA-B	HLA-C	Interaction with*
207 G	GS			
208 F				
209 Y				α1α2
210 P				α1α2
211 A		AG	AT	α1α2
212 E				
213 I				
214 T				
215 L				
216 T				
217 W				
218 Q				
219 R			RW	
220 D				
221 G				
222 E				
223 D				CD8
224 Q				CD8
225 T				CD8
226 Q				CD8
227 D				CD8
228 T				CD8
229 E				CD8
230 L				
231 V				β2m
232 E				β2m
233 T				β2m
234 R				β2m
235 P				β2m
236 A	AE			β2m
237 G				β2m
238 D				β2m
239 R	G	RG	G	
240 T				
241 F				α1α2
242 Q				β2m
243 K				
244 W				β2m
245 A	AV	AT		CD8
246 A	AS			
247 V				
248 V			VM	
249 V				
250 P		PL		
251 S				
252 G				
253 E	EQK	EQ	EQ	
254 E				
255 Q	QK			
256 R				
257 Y				
258 T				
259 C				
260 H				
261 V			VM	
262 Q				
263 H				
264 E				α1α2
265 G				
266 L				
267 P		PQ	PQ	
268 K	KE	KE	E	
269 P				
270 L		LC	LC	
271 T				
272 L				
273 R			RS	
274 W				
Transmembrane region				
275 E		EK	EKG	
276 P	PL			
277 S				
278 S				
279 Q				
280 S	P		P	
281 T				
282 I	IV	IV		
283 P	PH			
284 I				
285 V			VM	
286 G				
287 I				
288 V	IL			
289 A				
290 G				
291 L				
292 A	V			
293 V	L			
294 L	LF			
295 A	G	AV	AV	
296 V	A			
297 L		**		
298 V	VM		A	
299 V	IF	VA		
300 I	TA	IV	L	
301 G		GV		
302 A				
303 V				
304 V				
305 A				
306 A		AT	AV	
307 V				
308 M	MR	MV		
309 C	W			
310 R				
311 R	RK			
312 K	KN			
313 S				
314 S				
Cytoplasmic region				
315 G	DV			
316 G	R			
317 K				
318 G				
319 G				
320 S				
321 Y	—			

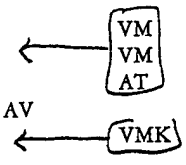

TABLE 1 Polymorphic and invariant residues at HLA-A, HLA-B and HLA-C (*Continued*)

Consensus	HLA-A	HLA-B	HLA-C	Interaction with *
322 S	STF			
323 Q				
324 A			AV	
325 A				
326 S		SC	SC	
327 S				
328 D				N
329 S				
330 A				
331 Q				
332 G				
333 S				
334 D				
335 V	VM		E	
336 S				
337 L				
338 T				I
339 A				AT
340 C				CS
341 K				
342 V				A

*Interaction with β2m (beta-2-microglobulin), the α1α2 domains or the α3 domain and residues that contribute to pockets A-F are based on HLA-A2 (2). Residues contributing to the CD8 binding site are from ref. 41.

**Residue 297 (T) is only found in the HLA-B73 allele.

mains also occurs in the loops outside of the peptide-binding region. Why is there polymorphism there? These are unlikely to be T cell contact residues. Garboczi and colleagues [31] solved the structure of an αβ TCR-

TABLE 2 Highly polymorphic residues in the α1 and α2 domains of HLA-A, HLA-B and HLA-C

Residue	HLA-A	HLA-B	HLA-C
9	YFST	YDH	YFSD
45	← —	METKG	—
62	RQGEL	RG	—
67	MV	YFSCM	—
70	MQ	NQSK	—
77	NDS	NDSG	SN
95	LIV	LIW	LIF
97	RIM	RSTWNV	RWN
99	YCF	YFS	YCFS
114	RHQE	DNH	DN
116	YDNH	YLFSDH	YFSL
152	VAEWR	VE	EAT
156	LRWQ	LRDW	LRWDQ
163	TREL	TLE	TLE
66	NK	INK	NK
80	TI	NTI	NK
90	DA	DA	DA

TABLE 3 Locus-specific polymorphism in the α1 and α2 domains

Residue	HLA-A	HLA-B	HLA-C
3	HQ	—	—
19	EK	—	—
31	TS	—	—
43	RQ	—	—
44	RK	—	—
56	GRE	—	—
79	GR	—	—
102	DH	—	—
105	PS	—	—
107	GW	—	—
115	QR	—	—
127	NK	—	—
142	IT	—	—
144	QK	—	—
145	RL	—	—
149	AT	—	—
150	AV	—	—
151	RH	—	—
161	ED	—	—
4	—	SF	—
30	—	DG	—
32	—	QL	—
41	—	AT	—
45	—	METKG	—
46	—	EA	—
59	—	YH	—
64	—	TI	—
71	S	TA	A
131	—	RS	—
162	—	GD	—
178	—	TK	—
180	—	QE	—
1	—	—	GC
6	—	—	RK
14	—	—	RW
21	—	—	RH
49	—	—	AES
50	—	—	PR
68	—	—	KN
91	—	—	GR
155	—	—	QE
170	—	—	RG
173	—	—	EK
175	—	—	GR

Tax peptide-HLA-A2 complex and identified the TCR-HLA contacts as residues on the α1 and α2 helices. The TCR extends diagonally across the class I molecule (Figure 3B). CDR1, CDR2, and CDR3 of the TCRα chain and CDR3 of the TCRβ chain contact peptide and the α helices [31]. CDR1 and CDR2 of the TCR β chain are displaced over the outlying loops but are not in direct contact with the class I molecule. Perhaps TCR/HLA contacts are more extensive under natural cell-cell inter-

TABLE 4 Polymorphic residues in the α3 domain

Residue	HLA-A	HLA-B	HLA-C
194	IV	IV	LV
253	EQK	EQ	EQ
184	PA	—	PHR
193	PA	—	PL
211	—	AG	AT
245	AV	AT	—
267	—	PQ	PQ
268	KE	KE	—
270	—	LC	LC
186	KR	—	—
207	GS	—	—
236	AE	—	—
246	AS	—	—
255	QK	—	—
199	—	AV	—
239	—	RG	—
250	—	PL	—
219	—	—	RW
248	—	—	VM
261	—	—	VM
273	—	—	RS

actions and in the presence of CD8. Perhaps some of the polymorphic residues on the outer loops come in contact with CD8 or an NK molecule, or amino acid substitutions at these sites influence pocket formation and consequently peptide binding in more subtle ways. These all imply some significance to the observed polymorphism. The alternate explanation is that the polymorphism outside the binding groove is of no selective advantage, but is generated because the mechanism for generating polymorphism operates randomly in the α1α2 domains (and perhaps also in the leader sequence). It is preserved because of the selective forces acting on other residues that do contribute to peptide binding.

Schematic views of the interactive sites of the α1α2 domains are shown in Figs. 3 and 4. One of the most curious findings has been that the HLA class I molecules which function as peptide receptors are themselves ligands for natural killer cells. NK cells recognize polymorphic sites on class I molecules and the interaction generates a negative signal which prevents a cell from being lysed by the NK cell. For example, NK1 and NK2 clones represent a diallelic system of monitoring HLA-C expression. NK1 clones recognize HLA-Cw2, Cw4, Cw5, Cw6 (alleles sharing the sequence NK at residues 77 and 80) and NK2 clones recognize HLA-Cw1, Cw3, Cw7 and Cw8 (alleles sharing the sequence SN at residues 77 and 80) [32] (see also Table 7). Other NK receptors recognize the Bw4 site (residues 77 to 83) on HLA-B [33, 34]. Recognition does not extend to those HLA-A alleles like A24 which also carry a Bw4 sequence, suggesting that NK recognition is complex [33]. The NK receptor sites on the carboxy terminal end of the α1 domain α helix are separate from the TCR binding site (Fig. 4) Still other NK receptors recognize polymorphic sites on HLA-A, B and C molecules, but the actual sequences that are recognized are not yet defined and may be separate from residues 77 to 83 [35–37]. Are there multiple NK recognition sites on class I molecules? How do class I molecules evolve so that interaction with peptides is encouraged while interaction with NK receptors is preserved?

β_2m extends beneath the α1α2 domains. In the crystal structure of HLA-A2, fifteen residues of β_2m interact with seventeen residues (#8, 10, 12, 23, 25, 27, 32, 35, 48, 94, 96, 115, 116, 117, 119, 120, 121) of the α1α2 domains (pink balls in Figs. 3A and 4; grey residues in Fig. 3B). Two other α1α2 domain residues (#6, 98) lay within the interface region but are not in direct contact with β_2m [4]. Some of these β_2m-contact residues are polymorphic. Five (#12, 32, 35, 94, 115) show what we consider to be low degrees of polymorphism, i.e. only two amino acids are observed. One residue (#116) is highly polymorphic, with as many as six observed substitutions in HLA-B. In the α3 domain of HLA-A2, fifteen residues interact with β_2m; one of these (#236) is polymorphic at HLA-A. The β_2m-α1α2 domain interactions are necessary for stable peptide binding and conformation. Mutations in β_2m can affect HLA conformation [38] and the binding affinity of β_2m may vary between alleles [39]. The interaction of β_2m and a class I heavy chain is also reported to lead to conformational changes in the cytoplasmic tail [40]. These observations suggest that class I polymorphism in the α1, α2 and α3 domains may affect peptide binding indirectly through β_2m interactions.

Interaction of the α1α2 domains with the α3 domain occurs in the downward loop at residues 29–31, in the connecting segment between the α2 and α3 domains (residues 179–183), and at the top of the loops in the α3 domain (residues 209, 210, 211, 241, 264). Three of these residues (#30, 31, 211) are polymorphic.

The α3 domain contains the CD8 binding site, primarily involving the extended loop between residues 223–229 [41]. These are not polymorphic residues. However, position 245 contributes to CD8 binding, as an A (Ala) to V (Val) substitution that occurs in A*68 alleles causes a decreased affinity for CD8 [5] and allows the generation of CD8-independent CTLs [42]. An A (Ala) to T (Thr) substitution at position 245 is seen in HLA-B*4801 and B*8101 alleles. Could this, or the unusual AAVSQ pattern of substitutions that occurs in

TABLE 5 Polymorphic Residues at HLA-A

		Alpha 1	Alpha 2	Alpha 3	TM	C
		1113344566666777777888889	1111111111111111111111	11112222222	22222223333	333
		39279153462356703467901230	99900001112444455555666667	88990344556	78889990011	123
		HYVRETRPRGREQIVNTDESRNLRGA	LRYDPGLDQYNIQRAARVLAETEWY	PKPIGAAAEQK	PIPVLVVIMRK	GSV
A1	A*0101	-F-----QK-Q-RNMH--ANGT---D	II----FR-D--K--VHARV-RDG-	----------	L--I--IT---	DT-
	A*0102	-S-S---QK-Q-RNMH--ANGT---D	II----FR-D--K--VHARV-RDG-	----------	L--I--IT---	DT-
A2	A*0201	-F-----Q--G-RK-H-HVDGT----	V---SWFH--KTKH--H--------	A-AVS---Q--	---IF-IT---	D--
	A*0202	-F-----R--G-RK-H-HVDGT----	----SWFH--KTKH--H-W------	A-AVS---Q--		
	A*0203	-F-----Q--G-RK-H-HVDGT----	V---SWFH--KTKHT-HEW------	A-AVS---Q--	---IF-IT---	D--
	A*0204	-F-----Q--G-RK-H-HVDGT----	VM--SWFH--KTKH--H--------	A-AVS---Q--	---IF-IT---	D--
	A*0205	-------R--G-RK-H-HVDGT----	----SWFH--KTKH--H-W------	A-AVS---Q--	---IF-IT---	D--
	A*0206	-------Q--G-RK-H-HVDGT----	V---SWFH--KTKH--H--------	A-AVS---Q--	---IF-IT---	D--
	A*0207	-F-----Q--G-RK-H-HVDGT----	V-C-SWFH--KTKH--H--------	A-AVS---Q--	---IF-IT---	D--
	A*0208	-------R--G-RN-H-HVDGT----	----SWFH--KTKH--H-W------	A-AVS---Q--		
	A*0209	-F-----Q--G-RK-H-HVDGT----	V---SWFH--KTKH--H--------	A-AVSE--Q--		
	A*0210	-------Q--G-RK-H-HVDGT----	V-F-S-FH--KTKH--H--------	A-AVS---Q--	---IF-IT---	D--
	A*0211	-F-----Q--G-RK-HI-VDGT----	V---SWFH--KTKH--H--------	A-AVS---Q--	---IF-IT---	D--
	A*0212	-F-----Q--G-RK-H-HVDGT----	V---SWFH--KTKH--H-Q------	A-AVS---Q--	---IF-IT---	D--
	A*0213	-F-----Q--G-RK-H-HVDGT----	V---SWFH--KTKH--HEQ------	A-AVS---Q--	---IF-IT---	D--
	A*0214	-------R--G-RK-H-HVDGT----	----SWFH--KTKH--H--------			
	A*0215N	-F-----Q--G-RK-H-HVDGT----	V-C-SWFH--KTKH--H--------	A-AVS---Q--	---IF-IT---	D--
	A*0216	-F-----Q--G-RK-H-HVDGT----	V---SWFH--KTKH--H----E---	A-AVS---Q--	---IF-IT---	D--
	A*0217	-F-----Q--G-RK-H-HVDGT----	-MF-SWFH--KTKH--H--------	A-AVS---Q--	---IF-IT---	D--
	A*0219	-F-----Q--G-RK-H-HVDGT----	V---SWFH--K-K---H-Q---DG-			
	A*0220	-F-----Q--G-RN-H-HVDGT----	V---SWFH--KTKH--H--------			
A3	A*0301	-F-----Q--Q-RN-Q--VDGT----	II--S-FR-D--K---HE--D----	----------	L--I--IT---	DT-
	A*0302	-F-----Q--Q-RN-Q--VDGT----	II--S-FR-D--K---H-Q-D----	----------		
	A*0303	-F-----Q--Q-RN-Q--VDGT----	II-.S-FR-D--K---HE--D----	----------	L--I--IT---	DT-
A11	A*1101	-------Q--Q-RN-Q--VDGT---D	II----FR-D--K---HAQ--R---	----------	L--I--IT---	DT-
	A*1102	----K--Q--Q-RN-Q--VDGT---D	II----FR-D--K---HAQ--R---	----------		
	A*1103	-------Q--Q-RN-Q-VDGT---D	II----FR-D--K----EQ--R---			
	A*1104	-------Q--Q-RN-Q--VDGT---D	II----FR-D--K---HAQ------	----------	L--I--IT---	DT-
A9	A*2301	-S-----Q--E-GK-H---N-IALR-	-MF-S-FH--K----------DG-	----------	-VHI--IT--N	D--
	A*2402	-S-----Q--E-GK-H---N-IALR-	-MF-S-FH--K-K---H-Q---DG-	----------	-V-I--IT--N	D--
	A*2403	-S-----Q--E-GK-H---N-IALR-	-MF-S-FH--K-K---H-Q------	----------	-V-I--IT--N	D--
	A*2404	-S-----Q--E-GK-H--ANGT----	-MF-S-FH--K-K---H-Q---DG-	----------	-V-I--IT--N	D--
	A*2405	-S-----Q--E-GK-H---N-IALR-	-MF-S-FH--K-----H-Q---DG-			
	A*2406	-S-----Q--E-GK-H---N-IALR-	-MF-S-FH--K-K---H-W---DG-			
	A*2407	-S-----Q--E-GK-Q---N-IALR-	-MF-S-FH--K-K---H-Q---DG-	----------	-V-I--IT--N	D--
	A*2408	QS-----Q--G-RK-H---N-IALR-	-MF-S-FH--K-K---H-Q---DG-	----------	-V-I--IT--N	D--
	A*2409	-S-----Q--E-GK-H---N-IALR-	-MF-S-FH--K-K---H-Q---DG-	----------	-V-I--IT--N	D--
	A*2410	-S-----Q--E-GK-H---N-IALR-	-MF-S-FH--K-K---H-Q--R---			
	A*2414	-S-----Q--E-GK-H---N-IALR-	V---SWFH--K-K---H-Q---DG-			
A10	A*2501	-------Q---NRN-H-----IALRD	I-----FQ-D----T-HEW--R---	A-AVS--SQ--	---IF-IA---	D-M
	A*2502	-------Q---NRN-Q-----IALRD	I-----FQ-D----T-HEW--R---	A-AVS--SQ--	---IF-IA---	D-M
	A*2601	-------Q---NRN-H--ANGT---D	I-----FQ-D----T-HEW--R---	A-AVS--SQ--	---IF-IA---	D-M
	A*2602	-------Q---NRN-H--ANGT---D	I-----FQ-N----T-HEW--R---	A-AVS--SQ--	---IF-IA---	D-M
	A*2603	-------Q---NRN-H-HVDGT---D	I-----FQ-D----T-HEW--R---	A-AVS--SQ--	---IF-IA---	D-M
	A*2604	-------Q---NRN-H--ANGT---D	I-----FQ-D----T-HEW--L---	A-AVS--SQ--	---IF-IA---	D-M
	A*2605	-------Q---NRN-H---NGT---D	I-----FQ-D----T-HEW--R---	A-AVS--SQ--	---IF-IA---	D-M
	A*2606	-------Q---NRN-H-HVDGT---D	I-----FQRD----T-HEW--R---			
	A*2607	-------Q--G-RK-H--ANGT---D	I-----FQ-D----T-HEW--R---	A-AVS--SQ--	---IF-IA---	D-M
A29	A*2901	-T-----Q--LQRN-Q--ANGT----	IM-HS-FR-D--------------	A-AVS--SQ--	---IF-FAR--	D-M
	A*2902	-T-----Q--LQRN-Q--ANGT----	IM--S-FR-D--------------	A-AVS--SQ--	---IF-FAR--	D-M

TABLE 5 Polymorphic Residues at HLA-A (*Continued*)

		Alpha 1	Alpha 2	Alpha 3	TM	C
A30	A*3001	-S-S---Q-RQ-RN-Q--VDGT----	II--S-FE-H-------W-------	----------	L--I--IT---	DT-
	A*3002	-S-S---Q-RQ-RN-H---NGT----	II--S-FE-H-------R-------	----------	L--I--IT---	DT-
	A*3003	-S-S---Q--Q-RN-H---NGT----	II--S-FE-H-------R-------	----------		
	A*3004	-S-S---Q-RQ-RN-H---NGT----	II--S-FE-H------H-W------			
A31	A*31012	-T-----Q-RQ-RN-HI-VDGT---	IM--S-FQ-D--------------	--AVS--SQ--	---IF-FAR--	D-M
A32	A*3201	-F-----Q--Q-RN-H-----IALR-	IM-----Q-D--------------	A-AVS--SQ--	---IFMFAR--	D-M
	A*3202	-F-----Q--Q-RN-H-----IALR-	IM-----Q-D------H-Q------	A-AVS--SQ--	---IFMFAR--	D-M
A33	A*3301	-T-----Q---NRN-HI-VDGT----	IM--S-FQ-D--------------H	-RAVS--SQ--	---IF-FAR--	D-M
	A*3303	-T-----Q---NRN-HI-VDGT----	IM-S-FQ-D--------------	--AVS--SQ--	---IF-FAR--	D-M
A34	A*3401	-------Q---NRK-Q--VDGT---D	I-----FQ-D----T-HEW------	A-AVS--SQ--	---LF-IA---	D-M
	A*3402	-------Q---NRN-Q--VDGT---D	II--S-FR-D----T-HE-------	A-AVS--SQ--	---IF-IA---	D-M
A36	A*3601	-F-----QK-Q-RNMH--ANGT----D	II----FR-D--K--VHARV-----	----------	L--I--IT---	DT-
A43	A*4301	-------Q--LQRN-H--ANGT---D	I-----FQ-D----T-HEW--R---	A-AVS--SQ--	---IF-IA---	D-M
A66	A*6601	-------Q---NRN-Q--VDGT---D	I-----FQ-D----T-HEW--R---	A-AVS--SQ--	---IF-IA---	D-M
	A*6602	-------Q---NRN-Q--VDGT----	I-----FQ-D----T-HEW--E---	A-AVS--SQ--	---IF-IA---	D-M
	A*6603	-------Q---NRN-H--VDGT----	I-----FQ-D----T-HEW--E---			
A28	A*68011	-------Q---NRN-Q--VDGT----	IM--S-FR-DKTKH--H-W------	A-AVS-V-Q--	---IF-IT---	D--
	A*68012	-------Q---NRN-Q--VDGT----	IM--S-FR-DKTKH--H-W------	A-AVS-V-Q--	---IF-IT---	D--
	A*6802	--M----Q---NRN-Q--VDGT----	I-----FH--KTKH--H-W------	A-AVS-V-Q--	---IF-IT---	D--
	A*6901	-------Q---NRN-Q--VDGT----	V---SWFH--KTKH--H--------	A-AVS---Q--	---IF-IT---	D--
A74	A*7401	-F-----Q--Q-RN-H--VDGT----	IM-----Q-D--------------	A-AVS--SQ--	---IFMFAR--	D-M
	A*7402	-F-----Q--Q-RN-H--VDGT----	IM-----Q-D--------------			
A80	A*8001	-F---SQQ-EE-RN-H-NANGT---D	II--S-FR-D--K----R---EDG-	----S---KKE	---I--IA-K-	V--

the α3 domain of HLA-A but not HLA-B, have any effect on CD8 binding or function?

The effect of polymorphism in the transmembrane and cytoplasmic regions in unexplored. Residues in the cytoplasmic domain can be phosphorylated [43] and class I molecules are reported to be physically associated with insulin receptors in cross-linking studies [44]. HLA molecules may be internalized in clathrin coated pits [45] or involved in signalling [46]. Class I heavy chains are known to cluster in the absence of β_2m [47]. The molecules also appear to associate with calnexin, calreticulin, BiP, gp96, tapasin and TAP in the endoplasmic reticulum (reviewed in [48]), suggesting still other interaction sites.

In terms of biologic impact, the contribution of class I polymorphism to allograft rejection is well known. Perhaps polymorphism at some residues is more important than others. Class I polymorphism also raises pivotal questions on the effectiveness of peptide vaccines. In tumor immunotherapy, HLA-A restricted peptides may be used to stimulate CTLs in melanoma patients [49, 50] and a synthetic HIV hybrid peptide containing B cell, Th and Tc determinants from gp120 is a vaccine candidate for HIV [51]. Advantages of such synthetic immunogens are their safety and defined character. A disadvantage is that as an immunogen becomes shorter and smaller, the number of individuals who have class I alleles capable of binding and presenting the peptide to T cells also decreases. It has been suggested that this is surmountable. Some serologically unique alleles such as HLA-B*0702, B*3501, B51, B*5301 and HLA-B*5401 share common sequences around peptide binding pockets and have overlapping peptide binding patterns [52].

One must appreciate the coordinate evolution and regulation of a molecule so polymorphic and with multiple active sites. Thirty-five years after the first descriptions of HLA alleles [10, 53] and 10 years after the first crystallographic analysis of HLA-A2 [54], there are still questions remaining. These are unlikely to be solved by sequencing more alleles, but will require functional studies and understanding of complex cell-cell interactions. ~~Rose Payne and coworkers stated in 1964 that "the leukocyte antigens provide a rich new source of material for the study of generic polymorphism in human populations" [53]. How prophetic.~~

TABLE 6 Polymorphic Residues at HLA-B

		Alpha 1	Alpha 2	Alpha 3	TM	C
		111233444556666666777778888 9	1111111111111111111111 9999001113344455566667778	1122222222 9913455667	222223333 789990000	3 2
		491264021562923456790146701230	457939346183572682367178 0	4919503780	525790168	6
		SYAVGADQAMEIYRETQIVTNTDESNLRGA	TLRYVLYDYRTTRWVLAGTEWYETQ	IAARAPEPKL	EIALVIGAM	S
B7	B* 702	--S--S---E----N---YAQA--------	--S---H-------ER--E---DKE	----------	-V-.-----	C
	B* 703	--S--S---E----N---Y----------	--S---H-------ER--E---DKE	----------	-V-.-----	C
	B* 704	--S--S---E----N---YAQA--------	--S---H-------ED--E---DKE	----------	-V-.-----	C
	B* 705	--S--S---E----N---YAQA--------	--S---HN------ER--E---DKE	----------	---.-----	C
	B* 706	--S--S---E----N---YAQA--------	--S---HN------ER--E---DKE	----------	-V-.-----	C
	B* 707	--S--S---E----N---YAQA--------	------H-------ER--E---DKE			
B8	B* 801	-D-M-S---E----N---F----------	--S---HN------L--D------D-E	----------	-V-.-----	C
	B* 802	-D-M-S---E----N---F-----NTALR-	--S---HN------E--D------D-E	----------	-V-.-----	C
	B* 803	.D-M-S---E----N---F---Y-NIALR-	--S---HN-------D------D-E			
B13	B* 1301	---M-T--T-A-------S---Y-NTALR-	II--L-HNLS--L-----E------	----------	-V-.-----	C
	B* 1302	---M-T--T-A-------S---Y-NTALR-	-WT-L-HNLS--L-----E-----	----------	-V-.-----	C
	B* 1303	---M-T--T-A-------S---Y-NTALR-	-WT-L-HNLS--------L-----	----------	-V-.-----	C
B14	B* 1401	--S--S---E----N---C----------	--W----NFS----E------H---	----------	-V-.-----	-
	B* 1402	-----S---E----N---C------- ---	--W----NFS----E------H---	----------	-V-.-----	-
B15	B* 1501	---M------A-------S---Y-------	------H-SS----EW--L------	----------	---.---T-	-
	B* 1502	---M------A---N---S---Y-------	II------SS----E---L------	----------	---.---T-	-
	B* 1503	---M-S---E--------S---Y---- --	------H-SS----E---L------	----------	---.---T-	-
	B* 1504	---M------A-------S---Y-------	-WT---H-SS----EW--L------	----------	---.---T-	-
	B* 1505	---M------A-------S---Y-------	------H-SS--------L------	----------	---.---T-	-
	B* 1506	---M------A-------S---Y-------	---F----SS----E---L------	----------	---.---T-	-
	B* 1507	---M------A-------S---Y-------	--S---H-SS----EW--L------	----------	---.---T-	-
	B* 1508	---M------A---N---F---Y-------	------H-SS----EW--L------	----------	---.---T-	-
	B* 1509	---M-S---E----N---C---Y-------	------HN-S----E---L------	----------	---.---T-	-
	B* 1510	---M-S---E----N---C-^-Y-------	------H--S----E---L------	----------	---.---T-	-
	B* 1511	---M------A---N---Y---Y-------	------H-SS----EW--L------	----------	---.---T-	-
	B* 1512	---M------A-------S---Y-------	------H-SS----EW--LDG----	----------	---.---T-	-
	B* 1513	---M------A---N---S---Y-NIALR-	II------SS----E---L------	----------	---.---T-	-
	B* 1514	---M------A-------S---Y-------	------H-SS----EW--L-S----	----------	---.---T-	-
	B* 1515	---M------A---N---S---Y-------	------H-SS----EW--L------	----------	---.---T-	-
	B* 1516	F--M------A-----RNMASAY-NIALR-	-W--L-H-SS----E---L------	----------	---.---T-	-
	B* 1517	---M------A-----RNMASAY-NIALR-	-------HDS----E---L------	----------	---.---T-	-
	B* 1518	---M-S---E----N---C---Y-------	------H-SS----E---L------	----------	---.---T-	-
	B* 1519	---M------A-------S---Y-------	------H-SS----EW--LDG----	-----L----	---.---T-	-
	B* 1520	---M------A-------S---Y-------	II--L-H-SS--------L------	V---------	---.---T-	-
	B* 1521	---M------A---N---C---Y-------	II------SS----E---L------	----------	---.---T-	-
	B* 1522	---M-----T----N---F---Y-------	------H-SS----EW--L------			
	B* 1523	---M-S---E----N---C---Y-NIALR-	------H-SS----E---L------	----------	---.---T-	-
	B* 1524	---M------A-------S---Y-NIALR-	------H-SS----EW--L------			
	B* 1525	---M------A-------S---Y-------	II------SS----E---L------	----------	---.---T-	-
	B* 1526N	---M------A-------S---Y-------	---*--H-SS----EW--L------	----------	---.---T-	-
	B* 1527	--M------A-------S---Y------	---F--H-SS----EW--L------			
	B* 1528	---M------A----I--S---Y-------	------H-SS----EW--L------	----------		
	B* 1529	---M-S---E----N---F---Y-------	------H-SS----E---L------	----------		
	B* 1530	--M------A-------S---Y-------	------HN-S----EW--L------			
	B* 1531	--M------A---N---S---Y-------	--------SS-----			
	B* 1532	---M------A-------S---Y-------	---S--H-SS----EW--L------			
	B* 1533	---M------A-------S---Y-------	------H-S-K---EW--L------			
	B* 1534	---M------A-------S---Y-------	----L-H-SS----EW--L------			
	B* 1535	---M------A-------S---Y-------	--T---H-SS----EW--L------			
	B* 1537	---M-S---E----N---C---Y-------	------H--S----E---L--H---			
B18	B* 1801	-HS--SG--T----N---S---Y-------	------H-SS----------H---	----------	---.---T-	-
	B* 1802	-HS--SG--T----N---S---Y-------	--N---H-SS----------H---	----------	---.---T-	-
	B* 1803	-HS--SG--T----N---S----------	------H-SS----------H---			

TABLE 6 Polymorphic Residues at HLA-B (*Continued*)

		Alpha 1	Alpha 2	Alpha 3	TM	C
B27	B* 2701	-HS--T-L-E--------CAKAY-NTALR-	--N----HDS--------E------			
	B* 2702	-HS--T-L-E--------CAKA--NIALR-	--N----HDS--------E------	----------	-V-.-----	C
	B* 2703	-HS--T-L-E--H-----CAKA--DT-LR-	--N----HDS--------E-----	----------	-V-.-----	C
	B* 2704	-HS--T-L-E--------CAKA---T-LR-	--N----HDS----E---E------	--G-------	-V-.-----	C
	B* 27052	-HS--T-L-E--------CAKA--DT-LR-	--N----HDS--------	----------	-V-.-----	C
	B* 27053	-L-E--------CAKA--DT-LR-	--N----HDS--------E--			
	B* 2706	-HS--T-L-E--------CAKA---T-LR-	--N------S----E---E------	----------	-V-.-----	C
	B* 2707	-HS--T-L-E--------CAKA--DT-LR-	--S---HN----------E------	----------	-V-.-----	C
	B* 2708	-HS--T-L-E--------CAKA--------	--N----HDS--------E------	----------	-V-.-----	C
	B* 2709	-HS--T-L-E--------CAKA--DT-LR-	--N----HHS--------E------	----------	-V-.-----	C
	B* 2710	-HS--T-L-E--------CAKA--DT-LR-	--N----HDS----E---E------			
B35	B* 3501	---M-----T----N---F---Y-------	II--L-H-SS--------L------	V---------	---.---T-	-
	B* 3502	---M-----T----N---F---Y-------	II--LFHN-S--------L------	V---------	---.---T-	-
	B* 3503	---M-----T----N---F---Y-------	II--L-H-FS--------L------	V---------	---.---T-	-
	B* 3504	---M-----T----N---F---Y-------	II--L-HN-S--------L------	V---------	---.---T-	-
	B* 3505	---M-----T----N---F---Y-------	--S-L-H-SS--------L------	V---------	---.---T-	-
	B* 3506	---M-----T----N---F---Y-------	II--L-HNFS--------L------	V---------	---.---T-	-
	B* 3507	---MV----T----N---F---Y-------	II--L-H-SS--------L------	V---------	---.---T-	-
	B* 3508	---M-----T----N---F---Y-------	II--L-H-SS-----R--L------	V---------	---.---T-	-
	B* 35091	---M-----T----N---F---Y-------	II--L-HN----------L------	V---------	---.---T-	-
	B* 35092	---M-----T----N---F---Y-------	II--L-HN----------L------			
	B* 3510	---M-----T-------F---Y-------	II--L-H-SS--------L---			
	B* 3511	---M-----T----N---F---Y-------	II--L-H-SS----E---L------	V---------	---.---T-	-
	B* 3512	---M-----T----N---F---Y-------	II----HN-S--------L------	V---------	---.---T-	-
	B* 3513	---M-----T--------F---Y-------	II--L-H-FS--------L------			
	B* 3515	---M-----T----N---F---Y-------	II--L-H-SS--------E------	V---------	---.---T-	-
	B* 3516	---M-----T--------F---Y-------	IIS---H-SS--------L------	V---------	---.---T-	-
	B* 3517	---M-----T----N---F---Y-------	IIS---H-SS--------L------			
	B* 3518	---M-----T----N---F---Y-------	II--L-HN-------R--L------			
	B* 3519	---M-----K----N---F---Y-------	II--L-H-SS--------L------			
B37	B* 3701	-HS--S---T--------S---Y-DT-LR-	-I-S---NFS-----D---------	----------	---.---T-	-
	B* 3702	-HS--S---T--------S---Y-DT-LR-	--N----HDS--------E------	----------	-V-.-----	C
B16	B* 3801	--S--S---E----N---C---Y-NIALR-	------HNFS------T--------	----------	-V-.-----	-
	B* 3802	--S--S---E----N---C---Y-NTALR-	------HNFS------T--------	----------	-V-.-----	-
	B* 39011	--S--S---E----N---C----------	------HNFS------T--------	----------	-V-.-----	-
	B* 39013	--S--S---E----N---C----------	------HNFS------T--------	----------	-V-.-----	-
	B* 39021	--S--S---E--------S----------	------HNFS------T--------	----------	-V-.-----	-
	B* 39022	--S--S---E--------S----------	------HNFS------T--------	----------	-V-.-----	-
	B* 3903	--S--S---E----N---C----------	------HNFS------T--------	----------	-V-.-----	-
	B* 3904	---M-S---E----N---C----------	------HNFS------T--------	----------	-V-.-----	-
	B* 3905	--S--S---E----N---C---Y-------	------HNFS------T--------	----------	-V-.-----	-
	B* 39061	--S--S---E----N---C----------	-WT---HNFS------T--------	----------	-V-.-----	-
	B* 39062	--S--S---E----N---C----------	-WT---HNFS------T--------	----------	-V-.-----	-
	B* 3907	-E----N---C---Y-------	------H-SS------T-----			
	B* 3908	--S--S---E--------S---Y-------	------HNFS-----RT--------	----------	-V-.-----	-
	B* 3909	--S--S---E----N---C----------	---S--HNFS------T--------	----------	-V-.-----	-
	B* 3910	--S--S---E----N---Y----------	------HNFS------T--------	----------	-V-.-----	-
B40	B* 40011	-H-M-T-LTK--------S-----Y-------	------HN---S-L----E---DKE	----------	-V-.-----	C
	B* 40012	-H-M-T-LTK--------S-----Y-------	------HN---S-L----E---DKE	----------	-V-.-----	C
	B* 4002	-HS--T-LTK--------S-----Y-------	--S---HN----------E------	----------	-V-.-----	C
	B* 4003	-HS--T-LTK--------S-----Y-------	--S---H-S---------E---DKE	----------	-V-.-----	C
	B* 4004	-HS--T-LTK--------S-----Y-------	II--L-HN----------E------	----------	-V-.-----	C
	B* 4005	-HS--T-LTK--------S-----Y-------	--S---HN------E---L------	----------	-V-.-----	C
	B* 4006	-HS--T-LTK--------S-----Y-------	-WT---HN----------E------	----------	-V-.-----	C
	B* 4007	-H-M-T-LTK--------F-----Y-------	------HN---S-L----E---DKE	----------	-V-.-----	C
	B* 4008	-HS--T-LTK----N---F-----Y-------	--S---HN----------E---DKE	----------	-V-.-----	C
	B* 4009	-HS--T-LTK--------S-----Y-------	--S---------------E------			
B41	B* 4101	-H-M-T-LTK--------S-----Y-------	-W----HN-------D------D-E	----------	-V-.-----	C
	B* 4102	-H-M-T-LTK--------S-----Y-------	--S---HN-------D------D-E	----------	-V-.-----	C

TABLE 6 (*Continued*)

		Alpha 1	Alpha 2	Alpha 3	TM	C
B42	B* 4201	--S--S---E----N---YAQA--------	--S---HN-------D------D-E	----------	-V-.-----	C
B12	B* 4402	---M-T-LTK--------S---Y-NTALR-	II------DS-----D--L-S----	-V--------	-V-.-----	C
	B* 44031	---M-T-LTK--------S---Y-NTALR-	II------DS--------L-S----	-V--------	-V-.-----	C
	B* 44032	---M-T-LTK--------S---Y-NTALR-	II------DS--------L-S----	-V--------	-V-.-----	C
	B* 4404	---M-T-LTK--------S---Y-NTALR-	II------DS-----R----S----	-V--------	-V-.-----	C
	B* 4405	^-------S---Y-NTALR-	II-------S-----D--L-S----			
	B* 4406	---M-----T----N---F---Y-NIALR-	II------DS-----D--L-S----			
	B* 4407	---M-T-L-K--------S---Y-NTALR-	II------DS--------L-S----	-V--------	-V-.-----	C
	B* 4408	---M-T-L--A-------S---Y-NTALR-	II------DS-----D--L-S----	-V--------	-V-.-----	C
	B* 4410	---M-T-LTK--------S---Y-NTALR-	II-FL-HNLS--------L-S----			
	B* 4501	-H-M-T-LTK--------S---Y-------	-W--L--NLS-----D--L-S----	----------	---.---T-	-
B46	B* 4601	---M------A------KYRQA-V------	------H-SS----EW--L------	----------	---.---T-	-
B47	B* 4701	---M-T-LTK--------S---Y-DT-LR-	---F---HDS--------E------	----------	-V-.----V	C
B48	B* 4801	--S--S---E--------S---Y-------	--S---HN---S-L----E---DKE	----T-----	-V-.-----	C
	B* 4802	--S--S---E--------S---Y-------	II--L-H-SS--------L------	V---------	---.---T-	-
	B* 4803	--S--S---E--------S---Y-------	------HN---S-L----E---DKE			
B21	B* 4901	-H-M-T-LTK--------S---Y-NIALR-	-W--L--NLS----E---L------	----------	---.---T-	-
	B* 5001	-H-M-T-LTK--------S---Y-------	-W--L--NLS----E---L------	----------	---.---T-	-
B5	B* 51011	---M-----T----N---F---Y-NIALR-	-WT---HN-S----E---L--H---	V---------	---.---T-	-
	B* 51012	---M-----T----N---F---Y-NIALR-	-WT---HN-S----E---L--H---			
	B* 51021	---M-----T----N---F---Y-NIALR-	-WT---HN-S----E---L------	V---------	---.---T-	-
	B* 51022	---M-----T----N---F---Y-NIALR-	-WT---HN-S----E---L------	V---------	---.---T-	-
	B* 5103	---M-----T----N---F---Y-NIALR-	-WT---HN-S----E---L-GH---	V---------	---.---T-	-
	B* 5104	---M-----T----N---F---Y-NIALR-	II----HN-S----E---L--H---	V---------	---.---T-	-
	B* 5105	---M-----T----N---F---Y-NIALR-	-WT---HN-S-----R--L------	V---------	---.---T-	-
	B* 5106	---M-----T----N---F---Y-NIALR-	------HN-S----E---L--H---			
	B* 5107	---M-----T----N---S---Y-NIALR-	-WT---HN-S----E---L--H---			
	B* 5108	..-M-----T----N---F---Y-NIALR-	-WT---HN-S-----D--L--H---			
	B* 52011	---M-----T--------S---Y-NIALR-	-WT---HN-S----E---L--H---	V---------	---.---T-	-
	B* 52012	---M-----T--------S---Y-NIALR-	-WT---HN-S----E---L--H---	V---------	---.---T-	-
	B* 5301	---M-----T----N---F---Y-NIALR-	II--L-H-SS--------L------	V---------	---.---T-	-
B22	B* 5401	---M-----G-V--N---YAQA--------	-WT-L-HNLS----------	----------	---.---T-	-
	B* 5501	---M-----E----N---YAQA--------	-WT-L-HNLS----E-----	----------	---.---T-	-
	B* 5502	---M-----E----N---YAQA--------	-WT-L-HNLS----------	----------	---.---T-	-
	B* 5503	---M-----E----N---YAQA-V------	-WT-L-HNLS----E-----			
	B* 5504	---M-----E----N---YAQA--------	--S---HN----------			
	B* 5601	---M-----E----N---YAQA--------	-WT-L-HNLS--------L------	----------	---.---T-	-
	B* 5602	---M-----E----N---YAQA--------	----L-HNLS--------L------	----------	---.---T-	-
B17	B* 5701	---M------A--G--RNMASAY-NIALR-	IIV---H-SS--------L------	----------	-V-.-----	C
	B* 5702	---M------A--G--RNMASAY-NIALR-	IIV---HN-S-----R--L------	----------	-V-.-----	C
	B* 5703	---M------A--G--RNMASAY-NIALR-	IIV---HN-S--------L------			
	B* 5704	---M------A--G--RNMASAY-NIALR-	IIV-----DS-----R--L------			
	B* 5801	---M-----T---G--RNMASAY-NIALR-	II--L-H-SS--------L------	V---------	---.---T-	-
	B* 5802	---M-----T---G--RNMASAY-NIALR-	--W-L-H-SS--------L------	V---------	---.---T-	-
B59	B* 5901	---M-----E----N---F---Y-NIALR-	-WT-L-HNLS----------	----------	---.---T-	-
B67	B* 67011	--S--S---E----N---YAQA--------	------HNFS------T--------	----------	-V-.-----	-
	B* 67012	--S--S---E----N---YAQA--------	------HNFS------T--------			
B73	B* 7301	-HS--T---E----N---CAKA-VG----D	-WT-M--NF---------E--H---	---G--QQEC	K-VTAVV--	-
B78	B* 7801	---M-----T----N---F----------	-WT---HN-S----E---L--H---	V---------	---.---T-	-
	B* 78021	---M-----T--^N---F---Y-------	-WT---HN-S----E---L--H---	V---------	---.---T-	-
	B* 78022	---M-----T----N---F---Y-------	-WT---HN-S----E---L--H---			
B81	B* 8101	--S--S---E----N---YAQA--------	--S---HN---S-L----E---DKE	----T-----	-V-.-----	C
B82	B* 8201	---M-S---E----N---YAQA--------	---FL-HNLS-----D-DL-S----			

TABLE 7 Polymorphic Residues at HLA-C

		Alpha 1	Alpha 2	Alpha 3	TM	C
		1 2345 6 78 1111222867889 16914671459068370 0 GRYARGRRARAPIKTSNA	111111111111111 999990111344555677 77 145793346837256303 57 GTLRYVYDYTTWVQLTREGE	11122222222 89911456677 43419831703 PPIARVEVPLR	22233333 78900000 55534567 EVAVVAAV	3333 2234 4690 ASAC
Cw1	Cw*0102	CKFS----S---K-----	---WCL------E-R-----	H-V-WM-----	------V-	----
	Cw*0103	CKFS----S---K-----	---WCL-NF---E-R-----	H-V-WM-----	------V-	----
Cw2	Cw*0202	C----S-H----K--NK-	-----L--S---E-WE----	H-VT-------	------V-	----
	Cw*0203	----S-H----K--NK-	-----L--S---E-WE----			
Cw3	Cw*0302	-------H----K-----	-I------S---E--L-K--	H-V-W------	------V-	----
	Cw*0303	-------H----K-----	RII---------E--L-K--	H-V-W------	------V-	----
	Cw*0304	-------H----K-----	-II---------E--L-K--	H-V-W------	------V-	----
Cw4	Cw*0401	--SSW-----E-K-ANKD	----FL-NF---E-R-----	H-V-W------	K--M--V-	----
	Cw*0402	--SSW-----SRKNANKD	----FL-NF---EER-----	H-V-W------	K--M--V-	---S
	Cw*0403	-----S-H----K-ANKD	----FL-NF---E-R-----	H-V-W------	K-----V-	----
Cw5	Cw*0501	C--------Q--K--NK-	-----L-NFK--E-R----K	H-V--------	G-----V-	----
Cw6	Cw*0602	C-D-----S---K-ANKD	---W-L--S---E-W-----	H-V--------	----M-V-	----
Cw7	Cw*0701	C-D-----S---N-A--D	-----L--S--LA-------	--L---QMQ-S	--V--T-M	-CT-
	Cw*0702	C-D-----S---K-A--D	----SL--S--LA-------	--L---QMQ-S	-MV--T-M	-CT-
	Cw*0703	C-D---A-S---K-A--D	----SL--S---A--L----	--L---QMQ-S	-MV--T-M	-CT-
	Cw*0704	C-D-----S---K-A--D	--F--L--F--LA-D----K	--L---QMQ-S	-MV--T-M	-CT-
	Cw*0705	-D-----S---K-A--D	---N-L--S--LA-------			
	Cw*0706	C-D-----S---N-A--D	-----L--S--LA-------	--L---QMQ-S	-MV--T-K	VCT-
Cw8	Cw*0801	C--------Q--K-----	-----L-NF---T------K	H-V--------	G---M-V-	----
	Cw*0802	C--------Q--K-----	-----L-NFK--E-R----K	H-V--------	G---M-V-	----
	Cw*0803	C--------Q--K-----	-----L-NF---T-----RK	H-V--------	G---M-V-	----
Cw12	Cw*12021	C----------K-A---	-----L--S---E-W-----	H-V--------	----M-V-	----
	Cw*12022	C----------K-A---	-----L--S---E-W-----	H-V--------	---M-V-	----
	Cw*1203	C----------K-A---	---W-L--S---E-W-----	H-V--------	----M-V-	----
Cw13	Cw*1301	C------H----K-A---	-----L--S---E-W-----	H-V--------	------V-	----
Cw14	Cw*1402	C-SS--------K-----	---WFL--S---E-R-----	H-V-W------	------V-	----
	Cw*1403	C-SS---H----K-----	---WFL--S---E-R-----	H-V-W------	------V-	----
Cw15	Cw*1502	C------H----N--NK-	-II--LH-L---E-------	H-V--------	----M-V-	----
	Cw*1503	C------H----N-ANK-	-II--LH-L---E-------	H-V--------	----M-V-	----
	Cw*1504	C------H----N--NK-	-II--L--S---E-------	H-V--------	----M-V-	----
	Cw*15051	C------H----N--NK-	-II--LH-F---E-------	H-V--------	----M-V-	----
	Cw*15052	C------H----N--NK-	-II--LH-F---E-------	H-V--------	----M-V-	----
Cw16	Cw*1601	C----------K-----	---W-L--S---A-Q-----	HLV--------	------V-	----
	Cw*1602	C----------K--NK-	---W-L--S---A-Q-----	HLV--------	------V-	----
Cw17	Cw*1701	----------K-ANK-	--I--L-NF-SLE--EG---	R-V---Q-QC-		
	Cw*1702	----------K-ANK-	--I--L-NF-SLE--EG---	R-V---Q-QC-		
Cw18	Cw*1801	C-D-----S---K-ANKD	----FL-NF---E-R-----	H-V-W------	K-V---V-	----

ACKNOWLEDGEMENTS

We thank Virginia B. Pett for invaluable assistance and David Garboczi for helpful comments and discussion. We also gratefully acknowledge the insight and enthusiasm of Bernard Amos who was always curious about details that didn't fit. This work was supported by grants DAMD17-94-J-4467 and NIH NCRR-RR03655.

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Conformational Preferences of a Chimeric Peptide HIV-1 Immunogen from the C4−V3 Domains of gp120 Envelope Protein of HIV-1 CAN0A Based on Solution NMR: Comparison to a Related Immunogenic Peptide from HIV-1 RF[†]

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Received November 8, 1995; Revised Manuscript Received February 13, 1996[⊗]

ABSTRACT: A critical problem to overcome in HIV vaccine design is the variability among HIV strains. One strategy to solve this problem is the construction of multicomponent immunogens reflective of common HIV motifs. Currently, it is not known if these motifs should be based primarily on amino acid sequence or higher-order structure of the viral proteins or a combination of the two. In this paper, we report NMR-derived solution conformations for a synthetic peptide taken from the C4 and V3 domains of HIV-1 CAN0A gp120 envelope protein. This peptide, designated T1-SP10CAN0(A), is compared to a recently reported C4−V3 peptide, T1-SP10RF(A) from the HIV-1 RF strain [de Lorimier *et al.* (1994) *Biochemistry 33*, 2055−2062], in terms of conformational features and immune responses in mice [Haynes *et al.* (1995) *AIDS Res. Hum. Retroviruses 11*, 211−221]. The T1 segment of 16 amino acids from the gp120 C4 domain is identical in both peptides and exhibits nascent helical character. The SP10 region, taken from the gp120 V3 loop, differs from that of T1-SP10RF(A) in both sequence and conformations. A reverse turn is observed at the conserved GPGX sequence. The rest of the SP10 domain is extended with the exception of the last three residues which show evidence for a helical arrangement. Modeling of the turn region of the T1-SP10CAN0(A) peptide shows exposure of a continuous apolar stretch of side chains similar to that reported in the crystal structure of a V3 peptide from HIV-1 MN complexed with a monoclonal antibody [Rini *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6325−6329]. This hydrophobic patch is interrupted by a charged Lys residue in the T1-SP10RF(A) peptide. This observation suggests that the HIV-1 CAN0A and HIV-1 RF C4−V3 peptides can induce widely different anti-HIV antibodies, consistent with immunogenic results.

Recent work defining appropriate targets of anti-HIV[1] antibodies which inactivate or neutralize the virus has centered on the third variable (V3) and fourth constant (C4) domains of HIV-1 envelope glycoprotein gp120 (Palker *et al.*, 1988, 1989; Harouse *et al.*, 1991; Girard & Sheare, 1993; Letvin, 1993; Moore *et al.*, 1993; Thali *et al.*, 1993; Haynes *et al.*, 1995a) and specific regions of HIV-1 envelope glycoprotein gp41 (Purtscher *et al.*, 1994). Since peptide segments corresponding to certain epitopes of pathogens can induce antibodies that recognize original intact antigens and neutralize the virus (Lerner, 1984), Haynes *et al.* (1995a) have synthesized four chimeric peptides, each of which includes segments taken from the gp120 C4 domain and from the gp120 V3 loops of four HIV-1 variants. The two peptides under consideration here are C4−V3 CAN0A, whose primary sequence is KQIINMWQEVGKAMYATR-PHNNTRKSIHMGPGKAFYTTG, and C4−V3 RF, whose primary sequence is KQIINMWQEVGKAMYATRPNNN-TRKSITKGPGRVIYATG.

These peptides are of interest in that epitopes of the gp120 V3 region of the C4−V3 peptides induce potent anti-HIV neutralizing antibodies that inhibit the growth of HIV in T cell lines *in vitro* (Palker *et al.*, 1989; Haynes *et al.*, 1995a) and the C4 T helper region of the peptides activates peptide-primed T cells that recognize native HIV gp120 (Palker *et al.*, 1989; Hart *et al.*, 1990). The gp120 V3 segment of C4−V3 peptides also contains an HLA class I restricted cytotoxic T lymphocyte (CTL) epitope restricted by HLA-B7 (Safrit *et al.*, 1994). Moreover, these four C4−V3 peptides are currently being used as a multicomponent (polyvalent) immunogen to boost anti-HIV immune responses in HLA-B7-typed, HIV-infected patients (Haynes, 1995b). Recent studies of this polyvalent HIV envelope C4−V3 peptide mixture in mice demonstrated that the V3 sequences of each C4−V3 peptide had separate and distinct immunogenic properties in that HIV RF and HIV CAN0A V3 peptides

[†] Supported in part by National Institutes of Health Grants GM 41829 (L.D.S.) and AI35351 (B.F.H.) and the Department of Defense, Army (DAMD 17-94-J-4467). H.M.V. and R.d.L. were supported by NIH Training Fellowships AI07392-06 and AI07217, respectively. B.F.H. is a Carter Wallace Fellow in Retroviral Research. The Duke NMR Center was established with partial support from the NIH, NSF, and North Carolina Biotechnology Center.

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[⊗] Abstract published in *Advance ACS Abstracts*, April 1, 1996.

[1] Abbreviations: HIV, human immunodeficiency virus; gp120, 120 kDa surface glycoprotein; gp41, 41 kDa transmembrane glycoprotein; C4, fourth constant domain of gp120; V3, third variable domain of gp120; C4−V3 peptide, synthetic 39-mer that begins with 16 residues from the C4 domain and ends with 23 residues from the V3 domain of HIV gp120; CTL, cytotoxic T lymphocytes; HLA, human leukocyte-associated antigen; CAN0A, T1-SP10CAN0(A); RF, T1-SP10RF(A); NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; DQF-COSY, double-quantum-filtered correlation spectroscopy.

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induce different anti-HIV antibody responses (Haynes *et al.*, 1995a). These data prompted us to undertake the analysis of the solution conformations of these C4−V3 peptides, using high-field NMR spectroscopy, in order to explore the contribution of structural features to their differential antigenicities.

Nuclear magnetic resonance is an effective method of studying conformational propensity of small immunogenic peptides in solution (Dyson & Wright, 1991, 1995; de Lorimier & Spicer, 1994). Thus, a nonapeptide derived from influenza virus haemagglutinin was shown to adopt a type II β turn in water (Dyson *et al.*, 1985), whereas preference for helical conformations in an aqueous solution was demonstrated for a 22-amino acid T cell-stimulating peptide of sperm whale myoglobin (Waltho *et al.*, 1989). In recent years, HIV-1 immunogenic peptides have become a subject of interest in this field. A stable α helix was reported for a segment of 25 residues from HIV-1 Tat that constituted the RNA-binding domain (Mujeeb *et al.*, 1994). Dyson *et al.* (1992) observed helical turns in a 23-mer derived from a transmembrane protein of simian immunodeficiency virus of rhesus macaque origin.

Solution conformations of synthetic peptides corresponding to the major neutralizing determinants of various HIV-1 strains have also been reported. In one case, a sequence of 24 amino acids taken from the V3 region of the HIV-1 IIIB isolate was shown by two-dimensional NMR to exist in a nascent helical conformation in aqueous solution and as a more stable helix in trifluoroethanol (Zvi *et al.*, 1992). Others have seen evidence for reverse β turns at the tips of the V3 loop peptides from HIV-1 MN gp120 (Chandrasekhar *et al.*, 1991) and HIV-1 RF gp120 (de Lorimier *et al.*, 1994). These turns were also observed by X-ray crystallography in HIV-1 MN V3 peptides bound to their antibodies (Rini *et al.*, 1993; Ghiara *et al.*, 1994). In addition, initial efforts to map a specific antibody binding epitope from an HIV-1 IIIB V3 peptide complex using NMR have recently been reported (Zvi *et al.*, 1995). In order to identify conformational motifs that may confer immunogenic specificity, we describe here detailed studies of solution conformational preferences for a C4−V3 peptide, T1-SP10CAN0(A) (C4−V3 CAN0A), for comparison with a previously reported C4−V3 peptide, T1-SP10RF(A) (C4−V3 RF) (de Lorimier *et al.*, 1994).

MATERIALS AND METHODS

Peptide Synthesis and Purification. The T1 peptide is a T helper epitope from the C4 HIV gp120 region, and the SP10 segment contains CTL, T helper, and B cell neutralizing antibody determinants from the gp120 V3 loop region (Cease *et al.*, 1987; Palker *et al.*, 1989; Takahashi *et al.*, 1992). The C4−V3 CAN0A peptide was synthesized by the FMOC method on an ABI 431A peptide synthesizer, consistent with earlier reported methods (de Lorimier *et al.*, 1994; Haynes *et al.*, 1995a). The end product was N-terminal amine and C-terminal acid. High-performance reversed phase liquid chromatography was used to purify the product by running a gradient of 0.1% trifluoroacetic acid (TFA) in H_2O and 0.08% TFA in CH_3CN through a Vydac C18 column. The molecular weight of the purified peptide was determined by electrospray mass spectroscopy to be 4463.51 (calculated MW is 4464.19).

NMR Experiments. Peptide solutions of 4 mM concentration were prepared in 90% H_2O and 10% D_2O (pH 3.95). All spectra were obtained on a Varian Unity 500 MHz spectrometer at 5 °C with a 5 mm inverse probe optimized for proton detection. Two-dimensional proton double-quantum-filtered correlation spectra, DQF-COSY (Piantini *et al.*, 1982; Rance *et al.*, 1983), total correlation spectra, TOCSY (Bax & Davis, 1985), and nuclear Overhauser effect spectra, NOESY (Jeener *et al.*, 1979), were acquired with mixing times of 60 and 85 ms for the TOCSY and 300 ms for the NOESY experiments. The water peak was suppressed by presaturation during the relaxation delay and the mixing period. For the TOCSY experiment, 256 free induction decays (64 scans each) of 1024 complex points per increment were recorded (512 FIDs for NOESY) in a spectral width of 10 ppm in each dimension. Temperature-shift coefficients of 35 amide protons were obtained from seven TOCSY spectra (60 ms mixing time) over the range of 278−296 K using a linear least squares method to fit the temperature dependence of the measured chemical shifts.

Data Analysis. Felix 2.1 (Biosym Technologies Inc.) was used to process data on an SGI Indigo workstation. Time domain data in the directly acquired dimension were zero-filled to 2048 points, phase-shifted by 90° (75° for NOESY), and multiplied by a sinebell-squared window function before Fourier transformation. Zero- and first-order phase adjustments and a polynomial baseline correction were applied to the F2 dimension before the second transformation. Spectra were referenced against the methyl protons of the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid which were set at 0 ppm.

Resonance assignments were analyzed using standard methods (Wüthrich, 1986). Proton resonances were assigned to their spin systems using scalar-coupling data from the DQF-COSY and TOCSY spectra. The sequential arrangements of the amino acids were in turn sorted out by NOESY spectra. The patterns of short- and medium-range NOE connectivities were used to determine conformations (Wüthrich, 1986; Dyson & Wright, 1991).

Molecular Modeling. Insight II (Biosym Technologies Inc.) was used to create CPK models for segments from the tip of the V3 loop: RKSIHMGPGKAFY for C4−V3 CAN0A and RKSITKGPGRVIY for C4−V3 RF. The conformational features and secondary structural elements represented by the NOE correlation patterns for short- and medium-range NOESY peaks were used to construct the backbone configurations. For C4−V3 CAN0A, residues RKSIHM and AFY were assigned with an extended β conformation, whereas a type II β turn was defined for the sequence GPGK. Similarly, RKSITK and VIY in C4−V3 RF were assigned with an extended β conformation, while GPGR was given a type I β turn. The coordinates of the initial conformers were exported to Discover 3.1 (Biosym Technologies Inc.), and parameters from a consistent valence force field were assigned to all atoms. Potential energies were calculated by a quadratic function and minimized first by the steepest descent and then by the Newton−Raphson algorithm. After the first 100 iterations, cross terms and Morse potentials were included in the calculation. Nonbond energy cutoffs for Coulombic and van der Waals interactions were 9.50 Å, and the dielectric constant was set to 1. The energy-minimized structure was used as a starting point to sample other conformational space by molecular dynamics (Discover 3.1). The system was equilibrated at a target temperature of 298 K in the initialization phase. An NVT

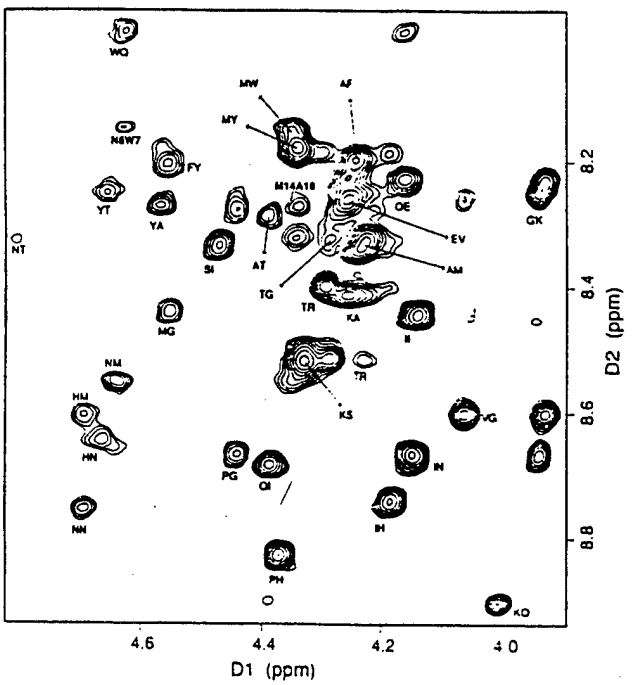


FIGURE 1: NOESY spectrum (300 ms mixing time) of the fingerprint region of T1-SP10 C4−V3 peptide from HIV CAN0A gp120. Interresidue correlations between the α protons of residues i and the amide protons of residues $i-1$ are labeled. Two dαN (i, $i+2$) NOEs, Asn^5−Trp^7 and Met^{14}−Ala^{16}, were also identified as additional evidence for a nascent helical conformation.

ensemble (constant volume, constant temperature) with a direct velocity scaling method was used to control the temperature. Dynamic trajectories were calculated by the velocity−Verlet algorithm every 1 fs for 300 ps, during which period energy minimization was performed at 5 ps intervals. The reported models were those of the molecular dynamics lowest-energy conformers after they were subjected to a final energy minimization.

RESULTS

Peptide Resonance Assignment and Overall Conformation. The assignments of the proton chemical shifts for C4−V3 CAN0A are available as supporting information. C4−V3 RF assignments have previously been reported (de Lorimier *et al.*, 1994). Cross-peaks between the main chain amide and α-carbon protons in C4−V3 CAN0A are well-dispersed in the fingerprint region of the COSY and TOCSY spectra (not shown) and the NOESY spectrum as shown in Figure 1. Sequential assignments were made on the basis of NOEs between the α-carbon protons of residue i and the amide protons of residue $i+1$ [dαN (i, $i-1$)] and were confirmed by dβN (i, $i+1$) and dNN (i, $i+1$) NOEs.

The N-terminal C4 gp120 region, termed T1, of C4−V3 CAN0A is identical in sequence to the C4 (T1) sequence in the C4−V3 peptide from HIV RF (de Lorimier *et al.*, 1994), and the corresponding proton chemical shifts for this 16-amino acid segment are nearly the same in the two C4−V3 peptides except for Ala^{16}. This residue is located at the junction of the T1 region with the gp120 V3 domain, termed SP10, which starts with Thr^{17} in CAN0A. In C4−V3 RF, the first residue in the SP10 domain is Cys^{17} which has been deleted from the T1-SP10 junction of the C4−V3 CAN0A peptide to avoid the potential for intermolecular cross-linking. This cysteine deletion did not affect the immunogenicities of the peptides (Palker *et al.*, 1989; Haynes *et al.*, 1995). Chemical shift values for main chain protons of residues 17−39 (17−40 for C4−V3 RF) in the SP10 domains of the two peptides vary considerably as expected due to differences in the amino acid sequences, and as illustrated below, NOE patterns show corresponding differences in preferred conformers in these regions. Analysis of the individual chemical shifts according to Wishart *et al.* (1991, 1992, 1994) suggests that the C4−V3 CAN0A peptide does not satisfy the criteria for stable secondary structures. As shown in Figure 2, C4−V3 CAN0A exhibits alternating and generally small shift differences from the residue specific average values determined from a large number of proteins. This implies that the peptide assumes an ensemble of conformations that are mostly random, a result similar to that reported earlier for the C4−V3 RF peptide (de Lorimier *et al.*, 1994).

Conformation of the HIV CAN0A gp120 C4 Region, T1. The NOE connectivities for the C4−V3 CAN0A peptide in aqueous solution at 5 °C and pH 4 are summarized in Figure 3. Although long-range proton−proton NOEs are not detected, short- and medium-range interactions are observed which can be used to identify peptide conformational features of the two domains. The presence of strong dαN (i, $i+1$) NOEs and the absence of dNN (i, $i+1$) as well as other medium-range NOEs between the same residues are indicative of backbone dihedral angles in the β region of ϕ,ψ space (Dyson *et al.*, 1988a) and are diagnostic of an extended conformation (Dyson & Wright, 1991). The first three residues of the T1 region in the C4−V3 CAN0A peptide, Lys^1−Ile^3, display this pattern as shown in Figure 3. The next 13 residues from Ile^4 to Ala^{16} show NOEs that are characteristic of a nascent helix (Dyson *et al.*, 1988a, 1991). These include two uninterrupted stretches of medium-strength dNN (i, $i+1$) NOEs illustrated by the data in Figure 4 and the dαN (i, $i+1$) NOEs shown in Figure 1. Two other types of medium-range NOEs that are signatures of a nascent helix, dNN (i, $i+2$) and dαN (i, $i+2$) (Dyson *et al.*, 1988b), are also identified in this region of T1. Thus, dNN (i, $i+2$) NOEs are observed for Ile^4−Met^6, Asn^5−Trp^7, Met^6−Gln^8, Gln^8−Val^{10}, and Gly^{11}−Ala^{13} as shown for the Ile^4−Met^6 and Gly^{11}−Ala^{13} pairs in Figure 5A. In addition, dαN (i, $i+2$) NOEs are detected for Ile^4−Met^6, Asn^5−Trp^7, Glu^9−Gly^{11}, and Met^{14}−Ala^{16}, illustrated by the Asn^5−Trp^7 and Met^{14}−Ala^{16} NOEs in Figure 1. Longer-range dαN (i, $i+3$) and dαβ (i, $i+3$) NOEs which are indicative of full helical turns may also be present but could not be unambiguously assigned in this region. Also in this region, protection of the Gln^8 backbone amide proton is suggested by an unusually low temperature-shift coefficient of 2.74×10^{-3} ppm/K. A similar low temperature-shift coefficient was observed at Gln^8 in C4−V3 RF (de Lorimier *et al.*, 1994) and is suggestive of hydrogen bond formation (Wright *et al.*, 1988).

Evidence for some cis conformations is also detected, mainly in the carboxyl end of the T1 region, as indicated by interresidual correlations between sequential α protons of Val^{10} and Gly^{11}, Gly^{11} and Lys^{12}, and Ala^{13} and Met^{14}. The NOEs indicating cis peptide bonds are weak, suggesting that the trans conformation dominates at these peptide bonds. Similar evidence for cis bonds between Ile^4 and Asn^5, Val^{10} and Gly^{11}, and Gly^{11} and Lys^{12} was previously reported in the T1 region of the C4−V3 RF peptide (de Lorimier *et al.*, 1994). A kink in the T1 region of C4−V3 CAN0A which is not detected in C4−V3 RF is suggested by a dβN (i, $i+2$)

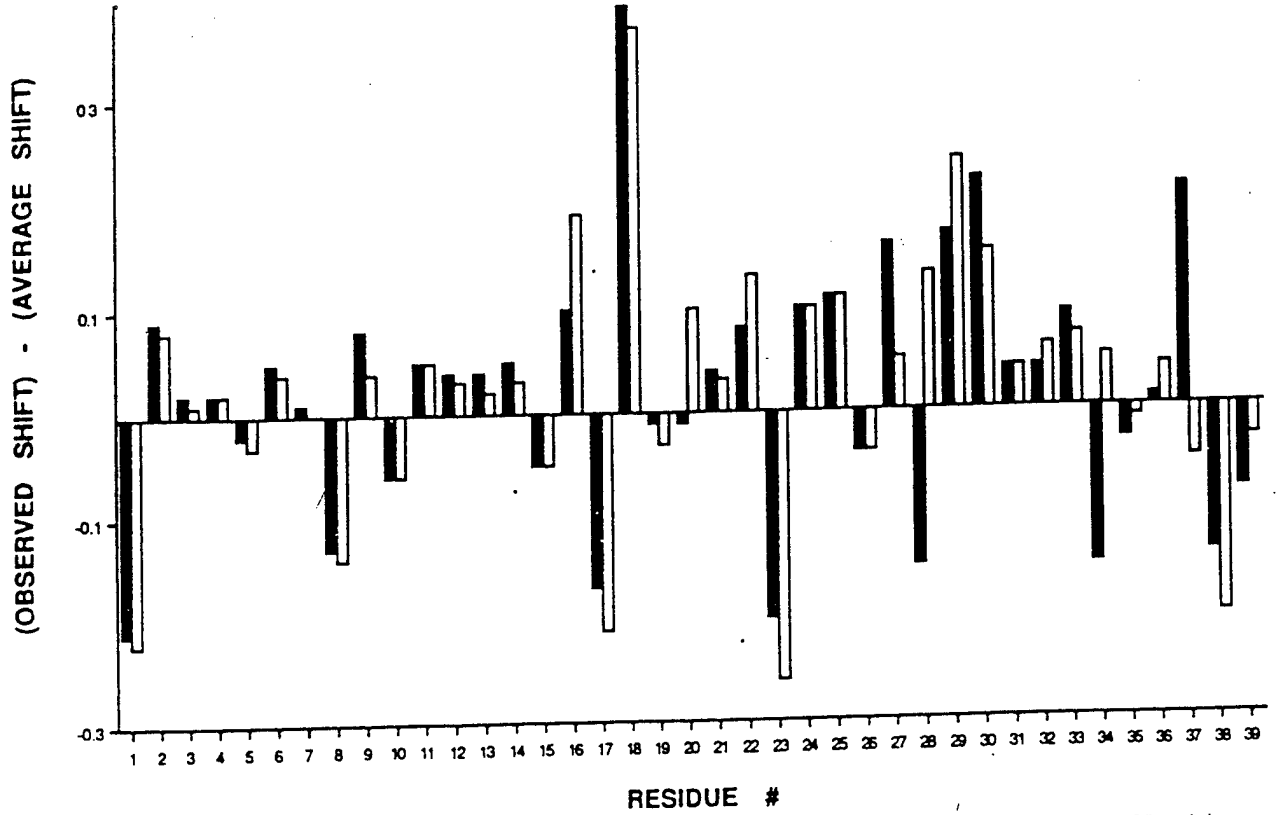


FIGURE 2: Deviation between the observed α proton chemical shifts of T1-SP10 C4−V3 peptide from HIV CAN0A gp120 and the average chemical shifts of the corresponding amino acids whose values were taken from Wishart *et al.* (1991). The values reported for the RF peptide are also included for comparison, but the chemical shift of Cys^{17} was omitted so that corresponding residues in the two SP10 regions can be aligned. Solid bars denote T1-SP10 C4−V3 peptide from HIV RF gp120, and open bars are for T1-SP10 C4−V3 peptide from HIV CAN0A gp120.

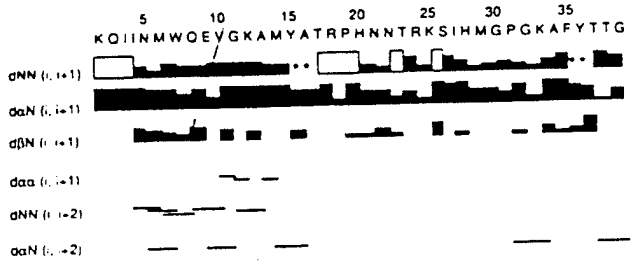


FIGURE 3: NOE connectivity patterns of T1-SP10 C4−V3 peptide from HIV CAN0A gp120. Open boxes denote unambiguously absent NOEs; black boxes and their heights show the presence of NOEs and their relative intensities. An asterisk signifies ambiguity of NOE assignment due to peak overlap.

NOE (Osterhout *et al.*, 1989) between a β proton of Trp^7 and the amide proton of Glu^9.

Conformation of the HIV CAN0A gp120 V3 Loop Region, SP10. The N-terminal sequence of the SP10 region of C4−V3 CAN0A tends to exhibit an extended β conformation as shown by the absence of dNN (*i*, *i*+1) and presence of dαN (*i*, *i*+1) connectivities for residues Thr^{17}−Pro^{19}, Asn^{22}, and Lys^{25} (Figure 3). In addition, all measurable vicinal spin−spin coupling constants between the amide and α proton in this region range from 9 to 10 Hz which further imply a β conformation (Wagner *et al.*, 1986). However, the most prominent conformational feature over the full SP10 domain is a reverse β turn comprised of residues Gly^{30}−Lys^{33}. This sequence is part of the V3 neutralizing determinant in gp120 of HIV-1. Previous reports have both predicted reverse turns in this region (La Rosa *et al.*, 1990) and presented evidence for turn structures within fragments of corresponding V3

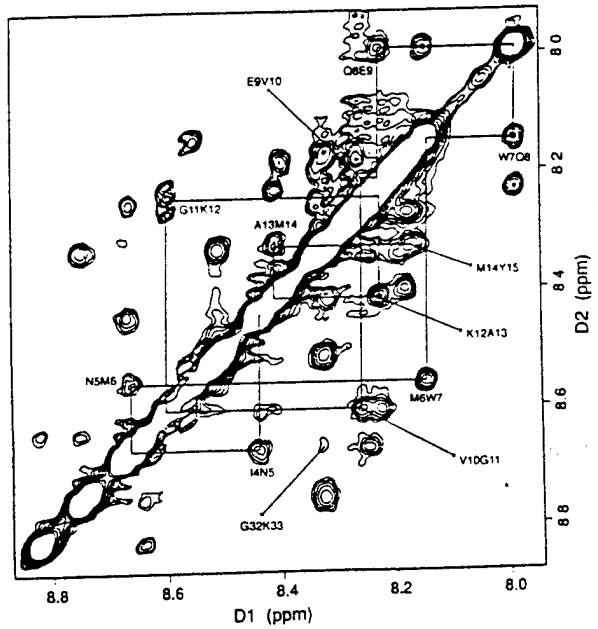
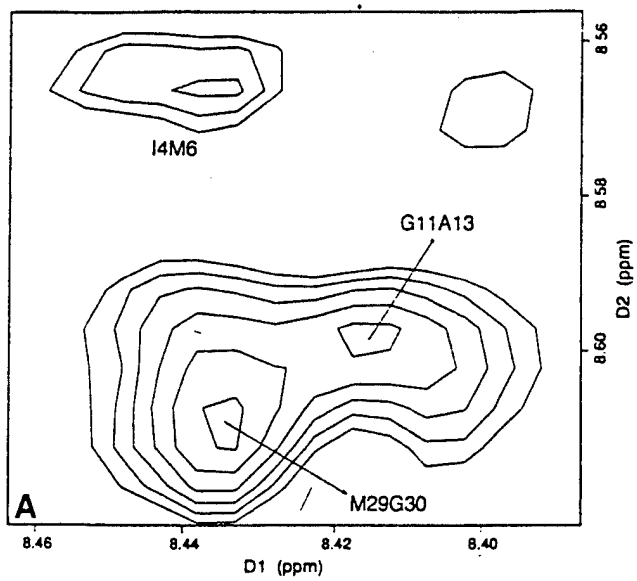


FIGURE 4: dNN (*i*, *i*+1) NOEs in the T1 region of T1-SP10 C4−V3 peptide from HIV CAN0A gp120. The sequential connectivity trace begins with Ile^4 and ends at Tyr^{15}. Also shown is a dNN (*i*, *i*+1) peak between Gly^{32} and Lys^{33} in the SP10 portion of the peptide. The spectrum was obtained with a 300 ms mixing time.

loop sequences derived from the HIV MN (Chandrasekhar *et al.*, 1991; Rini *et al.*, 1993; Ghiara *et al.*, 1994), HIV IIIB/LAI (Zvi *et al.*, 1992), and HIV RF (de Lorimier *et al.*, 1994) HIV-1 strains. Within this sequence of the CAN0A peptide, the following connectivities are detected: a weak dNN (*i*,



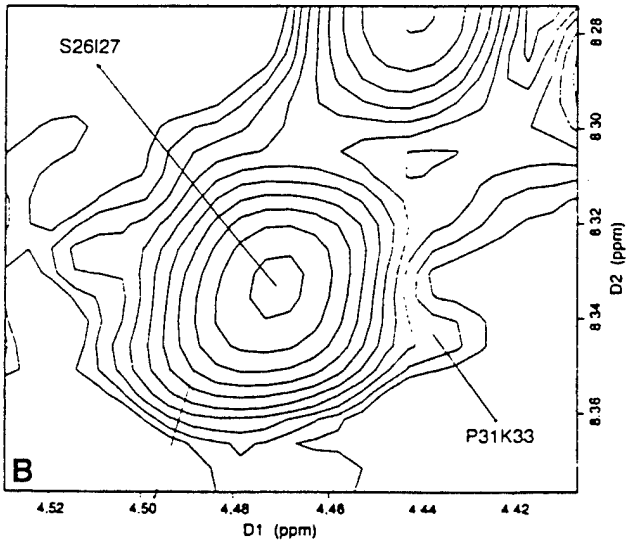


FIGURE 5: NOESY spectrum (300 ms mixing time) showing medium-range NOEs. (A) Two dNN (i, $i+2$) cross-peaks that arise from Ile^{2}–Met^{6} and Gly^{11}–Ala^{13} correlations which are indicative of a nascent helical conformation. (B) The dαN (i, $i+2$) NOE between Pro^{31} and Lys^{33} is shown here as part of the evidence for a type II β turn at the tip of the V3 loop.

$i+1$) NOE between the third (Gly^{32}) and fourth (Lys^{33}) residues of the turn shown in Figure 4, a medium to strong dαN (i, $i+1$) NOE between the second (Pro^{31}) and third (Gly^{32}) residues shown in Figure 1, and a weak dαN (i, $i+2$) NOE between the second and fourth residues (Pro^{31}–Lys^{33}) shown in Figure 5B. This specific combination of NOEs is diagnostic of a β turn (Dyson *et al.*, 1988a). The nature of the (i, $i+2$) NOE between the second and fourth residues of a β turn indicates its type. In this case, the fact that a dαN (i, $i+2$) NOE is observed together with the unambiguous absence of a dδN (i, $i+2$) between Pro^{31} and Lys^{33} suggests that the sequence GPGK comprises a type II β turn. Immediately C-terminal to this turn is an extended region from Phe^{35} to Thr^{37} which is followed by a nascent helical or turn conformation at the C-terminal end of C4–V3 CAN0A as suggested by a weak dαN (i, $i+2$) NOE between Thr^{37} and Gly^{39}. The overall composite sequence of observed conformations for the T1-SP10CAN0(A) peptide is illustrated in Figure 6 along with the corresponding preferred conformations reported earlier for the C4–V3 RF peptide (de Lorimier *et al.*, 1994).

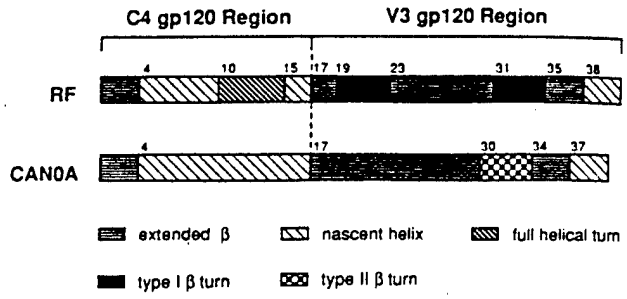


FIGURE 6: Secondary structural conformer comparison between the T1-SP10 C4–V3 peptides from HIV CAN0A and RF gp120. Numbers indicate the first amino acid residues from a specified conformational region.

DISCUSSION

Previous studies have shown that the chimeric C4–V3 peptides designated T1-SP10 induce anti-HIV neutralizing antibodies for HIV grown in T cell lines and induce anti-HIV T helper lymphocytes and cytotoxic T lymphocytes (Palker *et al.*, 1989). These peptides have also recently been characterized for their effectiveness as a multicomponent, polyvalent immunogen (Haynes *et al.*, 1995a). Results indicate differential antigenicities from each component with respect to priming and boosting responses as well as cross-reactivity characteristics toward antibody production aimed at specific HIV-1 strains. In particular, the T1-SP10CAN0-(A) and T1-SP10MN(A) C4–V3 peptides appear to be more broadly reactive than either T1-SP10RF(A) or T1-SP10EV91-(A) peptides. Our interest in studying conformational characteristics for these peptides represents an initial effort to establish likely conformations for related immunogenic sequences from C4–V3 peptides and to compare conformational features between particular peptides in this series to gain insight into structural contributions to specific immunogenic behavior. We have previously reported a conformational analysis of the T1-SP10RF(A) peptide (de Lorimier *et al.*, 1994), to which the results of this study of T1-SP10CAN0(A) are compared and discussed below in the context of immunogenic differences between the two peptides (Haynes *et al.*, 1995a).

The T1 segments of the C4–V3 CAN0A and C4–V3 RF peptides are taken from the fourth constant domain of HIV-1 gp120 which is a component of the HIV gp120 CD4 binding site (Capon & Ward, 1991) and also is a potent T helper epitope (Cease *et al.*, 1987; Palker *et al.*, 1989). The T1 regions in the C4–V3 peptides from HIV CAN0A and RF are identical in primary amino acid sequence and are shown here to be similar in their solution conformations. They both exhibit evidence for nascent helix conformations as indicated by the presence of dNN (i, $i+2$) and dαN (i, $i+2$) NOEs (Figure 3; de Lorimier *et al.*, 1994). The observation of dαN (i, $i+3$) and d$\alpha\beta$ (i, $i+3$) NOEs within this region of C4–V3 RF indicates a full helical turn conformation from Val^{10} to Met^{14}. Such NOEs could not be unambiguously assigned in the case of C4–V3 CAN0A due to the degeneracy of chemical shift values.

The SP10 portions of the C4–V3 CAN0A and C4–V3 RF peptides are derived from the V3 loop regions of gp120 in HIV-1 CAN0A and HIV-1 RF, respectively. This loop

is highly variable in sequence in various strains of HIV-1 gp120 and consists of 35 amino acids flanked by two cysteine residues that form a disulfide bridge (Leonard *et al.*, 1990). It contains a potent neutralizing determinant of gp120 (Javaherian *et al.*, 1989; Zwart *et al.*, 1991). Consequently, it has been a target for numerous attempts at designing vaccines against HIV-1 (Emini *et al.*, 1992; Haynes *et al.*, 1995a). Parallel efforts have been made to elucidate structural features of this region to better understand the basis for its reactivity. The predicted structural elements of the V3 loop of HIV-1 gp120 based on a neural network approach is Cys-β strand-reverse β turn-β strand-α helix-Cys in that order (La Rosa *et al.*, 1990). According to this prediction, the reverse β turn is located roughly at the center of the loop and includes a highly conserved Gly-Pro-Gly tripeptide. X-ray crystal structures of the corresponding GPGR sequence from the HIV-1 MN isolate when bound to Fab fragments of antibodies, 50.1 (Rini *et al.*, 1993) and 59.1 (Ghiara *et al.*, 1994), indicated a type II β turn. Nuclear magnetic resonance studies of free peptides derived from this region of the HIV-1 MN, IIIB, and RF strains in aqueous solution also suggested a reverse β turn within this sequence at the tip of the V3 loop (Chandrasekhar *et al.*, 1991; Zvi *et al.*, 1992; de Lorimier *et al.*, 1994).

We report here that conformational preference for a reverse β turn also exists in the V3 loop of HIV-1 CAN0A gp120. The corresponding GPGK sequence in the SP10 portion of the C4−V3 CAN0A peptide tends to form a type II β turn from Gly^{30} to Lys^{33} as indicated by a combination of dαN (Pro^{31}−Gly^{32}), dαN (Pro^{31}−Lys^{33}), and dNN (Gly^{32}−Lys^{33}) NOEs. For HIV RF, due to a dδN (i, i+1) NOE between Pro^{32} and Gly^{33} (second and third residues of the turn), de Lorimier *et al.* (1994) suggested a type I β turn for the sequence GPGR. Thus, while the preferred type of reverse turn and therefore the ϕ and ψ main chain bond angles may vary in the solution conformers observed for this domain of different HIV-1 strains, it appears that both free peptides tend to form reverse turn conformations consistent with the general topology found in the HIV MN V3 loop peptide complexed with Mab 50.1 (Rini *et al.*, 1993). This turn conformation may be important for direct antibody and B cell receptor recognition and also may provide an effective method of presenting hydrophobic residues required for interaction and binding (Rini *et al.*, 1993).

An extended β strand conformation N-terminal to the reverse turn is observed for C4−V3 CAN0A from Thr^{17} to Met^{29} as illustrated in Figure 6. This segment of the V3 loop of C4−V3 RF, which is also generally extended in conformation, exhibits evidence for an additional type I β turn within residues Arg^{19}-Pro^{20}-Asn^{21}-Asn^{22}. The asparagine residue at position 21, which is correlated with a high propensity for β turns (Dyson *et al.*, 1988a), is replaced by histidine in C4−V3 CAN0A (TRPNNN in C4−V3 RF versus TRPHNN in C4−V3 CAN0A). This substitution may contribute to the abrogation of a reverse β turn at this position and will likely have influence on the immunogenicity of this B7-restricted CTL epitope of HIV gp120 (Safrit *et al.*, 1994). The C-termini of the SP10 regions of both C4−V3 CAN0A and C4−V3 RF show NOEs characteristic of an extended β conformation immediately following the central turn and end with a three-amino acid stretch of nascent helix.

Taken together, the NMR-derived solution conformations of the C4−V3 CAN0A and C4−V3 RF peptides show similarities in the C4 regions but differ considerably in the V3, SP10 peptide segments. The solution conformation of the SP10 portion of C4−V3 CAN0A V3 loop includes an extended β segment from Thr^{17} to Met^{29}, a type II β turn from Gly^{30} to Lys^{33}, a second extended structure from Ala^{34} to Tyr^{36}, and a nascent helix from Thr^{37} to Gly^{39}. By comparison, the C4−V3 RF peptide exhibits two sequences where type I β turns are preferred conformations, the second of which is some nine residues N-terminal from the central turn (de Lorimier *et al.*, 1994). Furthermore, the V3 portion of the C4−V3 RF peptide shows preferred conformations in the sequence SIT that are not detected in the corresponding region of C4−V3 CAN0A.

Initial studies of the immunological activities of C4−V3 RF and C4−V3 CAN0A peptides have determined the ability of each peptide to prime and/or boost antibody responses to itself and other peptides in the polyvalent mixture in Balb/c mice (Haynes *et al.*, 1995a). In addition, [^{3}H]thymidine incorporation into splenocytes from immunized mice was assayed to evaluate the relative proliferative responses induced by each peptide *in vitro*. Results indicated that sera from mice immunized with the C4−V3 CAN0A peptide induced an anti-HIV CAN0A antibody titer that was 20 times greater than that induced by the C4−V3 RF peptide for corresponding anti-HIV RF antibodies. Mice primed and boosted with C4−V3 CAN0A peptide also stimulated significant antibody production directed toward C4−V3 RF and other C4−V3 HIV peptides, whereas the C4−V3 RF peptide immunogen alone produced antibodies almost exclusively to HIV RF and not to HIV CAN0A (Haynes *et al.*, 1995a). The fact that the V3 region of RF peptide exhibits conformations not detected in CAN0A or MN (unpublished data; Chandrasekhar *et al.*, 1991) suggests that the immunogenic specificity of RF arises from these conformational features. Studies are underway to test this hypothesis.

It seems unlikely that the T1 regions alone contribute differently to the overall immunogenicities of the T1-SP10 peptides since their primary amino acid sequences are identical and the NMR-detected conformational features of this segment are very similar between the C4−V3 CAN0A and C4−V3 RF peptides (de Lorimier *et al.*, 1994). It is possible however that both sequence and conformational variations of the two SP10 V3 segments of the C4−V3 peptides determine their differential immunogenic behavior and are responsible for different levels of antibody production and T cell proliferation induced by HIV CAN0A and RF C4−V3 peptides. Indeed, it has been suggested that the type specificities of the antibodies generated to various HIV strains are defined by residues flanking the reverse β turn in the V3 loop that vary from one strain to another (Meloen *et al.*, 1989).

To better understand the interplay of primary and secondary structural elements of C4−V3 HIV peptides, we have constructed molecular models of the antibody-binding sites from the SP10 domains of CAN0A and RF C4−V3 peptides on the basis of NMR results as outlined above. The models were subjected to molecular dynamics for a total of 300 ps followed by energy minimization. The space-filling CPK models generated for the tips of the V3 loops of HIV CAN0A and RF are depicted in Figure 7. By developing the conformational model for a single short domain of the peptide, we avoid any implication that other regions of the

FIGURE 7: CPK models of the tips of the V3 loops of CAN0A and RF. Important basic residues are shown in gray, and the nonpolar residues of the apolar patches are darkened. Note the protrusion of a positively charged lysine residue in the middle of the hydophobic region in RF which differentiates it from CAN0A.

molecule have time-correlated conformers. Indeed, for largely unstructured biopolymers, the conformational elements of independent domains experimentally observed in solution NMR studies may not be present at the same time in the same molecules. Crystallographic studies of the cocrystal of the comparable V3 segment (16 residues) of gp120 from the HIV-1 MN strain complexed with its monoclonal antibody 50.1 show extensive contacts between the antigen-binding pocket of Fab 50.1 and the side chains of residues located immediately N-terminal to the tip of the V3 loop (Rini *et al.*, 1993). For MN, these residues are Ile (P314), Ile (P316), Gly (P319), and Pro (320) and appear to form a continuous hydrophobic surface that not only interacts with the Fab fragment but also is required for high-affinity binding in mutagenesis studies. In the C4−V3 CAN0A and C4−V3 RF peptides compared here, the amino acids corresponding to these four hydrophobic residues are I, M, G, and P and I, K, G, and P, respectively, shown darkened in Figure 7. Like the X-ray crystal structure of the antibody-bound MN sequence, C4−V3 CAN0A exhibits an apolar patch that includes residues Ile^{27}, Met^{-9}, Gly^{30}, and Pro^{31}. Our models show that the presence of methionine in place of isoleucine does not alter the geometry of the apolar patch in that it appears to be flat, much like that in the crystal structure of MN (Figure 7; Rini *et al.*, 1993). For the C4−V3 RF peptide, however, this extended stretch of amino acids is interrupted by a positively charged residue, a lysine at the second position of the patch (gray in Figure 7). Thus, the V3 region of HIV CAN0A, but not of RF, may contain a structural motif similar to that of HIV MN. As mentioned above, Haynes *et al.* (1995a) demonstrated that, while HIV MN and CAN0A are immunologically similar to each other in that they both induce cross-reactive antibodies, HIV RF is very type-specific in antibody responses induced. That is, whereas antibodies raised against C4−V3 RF peptide do not recognize antigens from other HIV strains, those raised against C4−V3 CAN0A or C4−V3 MN peptides can cross-react to each other and to the HIV RF V3 loop as well. From these models, we therefore suggest that the selectivity and restriction to cross-reactivity of anti-HIV RF V3 loop antibodies may be due to the presence of the positively charged lysine residue, the side chain of which is conformationally presented in the center of the hydrophobic region. This feature likely induces antibodies specific for its recognition. The presence and continuity of an apolar surface in proximity to the GPGX turn region of MN and CAN0A V3 peptides may be important for antibody cross-reactivity.

ACKNOWLEDGMENT

We acknowledge Drs. Anthony Ribeiro and Ronald Venters for helpful assistance and Dr. David Myers for sharing his preliminary spectra and analysis of CAN0A.

SUPPORTING INFORMATION AVAILABLE

One table of the proton resonance assignments of the T1-SP10CAN0(A) C4−V3 peptide (1 page). Ordering information is given on any current masthead page.

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BI952665X

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 13, Number 8, 1997
Mary Ann Liebert, Inc.

Identification of a Synthetic Peptide That Mimics an HIV Glycoprotein 120 Envelope Conformational Determinant Exposed following Ligation of Glycoprotein 120 by CD4

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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 10 divergent HIV isolates were screened for their ability to bind to 17b and 48d in ELISAs. 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 IIIB V3 peptide, whereas MAb 17b bound none of the peptides tested. Monoclonal antibody 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, or that neutralized HIV primary isolates. These data suggested that the primary sequence of the HIV Can0A V3 loop exists in a conformer that mimicks 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 the C4 region also affect gp120–CD4 binding, including amino acid substitutions in the V3 loop.[10]

Data suggest that the V3 loop may be physically located in

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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 composed 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 α-fluorenylmethoxycarbonyl (Fmoc) chemistry using an Applied Biosystems (Foster City, CA) 431A peptide synthesizer. Peptides were purified by high-performance liquid chromatography (HPLC) and molecular mass was determined by electrospray mass spectrometry performed by R.D. Stevens (Department of Pediatrics, Division of Genetics and Metabolism, Duke University Medical Center, Durham, NC) 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 μl, 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).

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assay (ELISA) plates were coated with 2 μg of peptide antigen per microtiter well in 50 μl of binding buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, 0.02% NaN_3 in distilled water, pH 9.6) per well and incubated overnight at 4°C. 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 μl/well) for 1 hr at 20°C. Wells were washed three times with wash buffer. Alkaline phosphatase-conjugated anti-human IgG was diluted in serum solvent and added (100 μl/well) for 1 hr at 20°C. The wells were then washed as described above. Next, 100 μl of *p*-nitrophenyl phosphate in substrate buffer (0.015 M Na_2CO_3, 0.033 M $NaHCO_3$, 0.02% $MgCl_2$) per well was added and incubated 1 hr at 20°C in the dark. Absorbance was read at 405 nm on an ELISA reader (Anthros; Denley Instruments Co., Durham, NC). Data are expressed as optical density (OD) at 400 nm or as the ratio of experimental (E) OD of 48d MAb binding to control (C) MAb binding (*E/C* ratio).

Addition of soluble peptide

ELISAs 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°C in a separate ELISA plate with 20 μg 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.

Treatment with 8 M urea

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°C and thawed when needed. Then 200 μl of stripping solution (8 M urea, 2% sodium dodecyl sulfate [SDS], 2% 2-mercaptoethanol [2-ME]) was added to each well and incubated for 2 hr at 37°C. Plates were washed 10 times with wash buffer following the incubation. The plates were then blocked again with blocking buffer and frozen at −20°C. On the day of the experiment, the plates were thawed, washed three times with wash buffer, and a second ELISA performed as described above.

Flow cytometry

HSB acute lymphoblastic leukemia T cells (HSB T-ALL cells) were incubated with 2.24×10^{-8} M (100 μg) C4–V3 Can0A peptide in 50 μl of 0.9% saline for 45 min at 4°C, and washed twice with PBS. Cells were incubated with primary antibody for 30 min at 4°C and washed as described above. Finally, cells were incubated with goat anti-human IgG labeled with fluorescein isothiocyanate (IgG–FITC; Organon Teknika Corp., West Chester, PA) for 30 min at 4°C 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 rhe-

TABLE 1. SYNTHETIC PEPTIDE SEQUENCES[a]

Peptide	*Sequence*
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

[a]Sequences taken from *Human Retroviruses and AIDS, 1995: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences* (Myers G, Korber B, Wain-Hobson S, Smith RF, and Pavlakis GN, eds.). Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, New Mexico, 1995.

sus 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 (the number of experiments [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 values of two or greater. Monoclonal antibody 48d reacted strongly with the C4–V3 Can0A peptide (OD [mean ± SEM], 0.970 ± 0.096; $p < 0.01$) and weakly with the C4–V3 MN peptide (OD 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 (Fig. 1B). 48d again reacted well with the V3 Can0A peptide (OD 0.708 ± 0.064; $p < 0.001$), minimally with the V3 MN peptide, and did not react with other V3 peptides tested. The MAb 48d therefore selectively and strongly bound to the V3 portion of the HIV Can0A peptides.

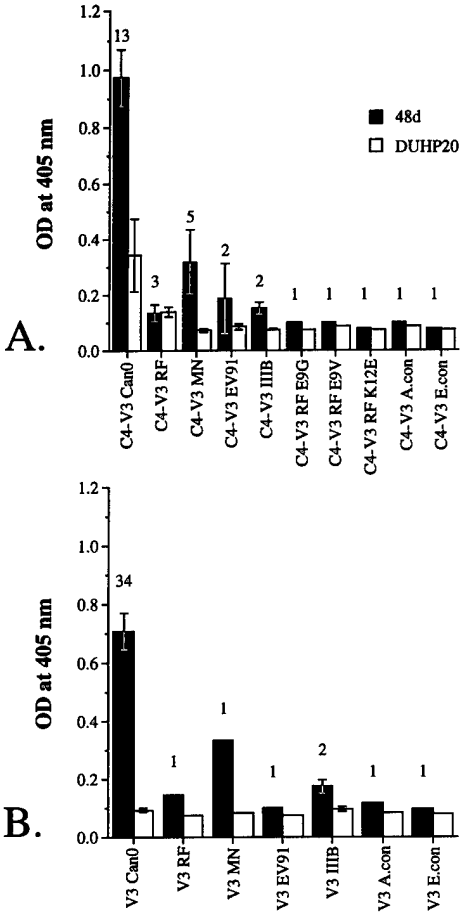

FIG. 1. Binding of monoclonal antibody 48d to C4–V3 and V3 HIV peptides. Shown here is reactivity of 5 μg of monoclonal antibody 48d with 2 μg of C4–V3 peptides (A) or V3 peptides (B) from divergent HIV isolates bound to ELISA plate (number [n] of experiments performed for each peptide is shown above 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 405 nm for MAb 48d or control DUHP20 MAb binding in ELISA.

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 (Fig. 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, 20 μg 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 (Fig. 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 ELISAs 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. Monoclonal antibody 48d reacted with the C4–V3 Can0A pep-

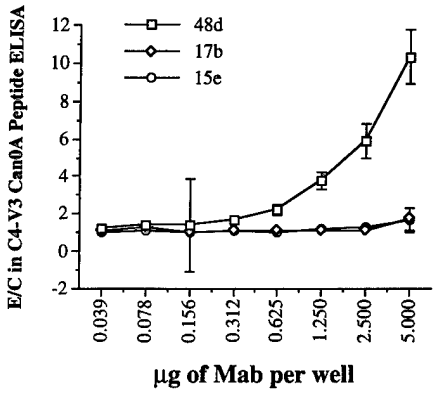

FIG. 2. Binding of monoclonal antibodies 17b, 48d, and 15e to the C4–V3 Can0A peptide in solid-phase ELISA. Shown here is reactivity of increasing amounts of monoclonal antibody to 2 μg of C4–V3 Can0A peptide bound to ELISA plate. Monoclonal antibody 48d reacted well with plate-bound C4–V3 Can0A, whereas monoclonal antibodies 17b and 15e did not react. Data representative of seven experiments.

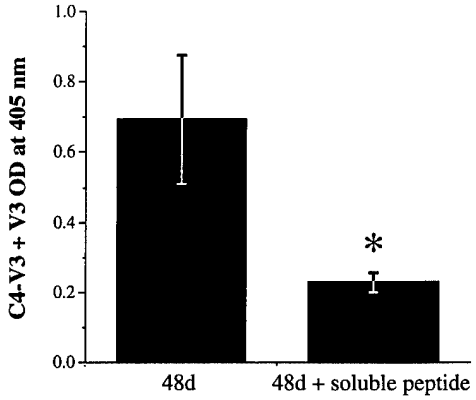

FIG. 3. Soluble V3 Can0A peptide can block monoclonal antibody 48d binding to plate-bound V3 Can0A peptide. Twenty micrograms of soluble C4–V3 Can0A peptide ($n = 3$) or soluble V3 Can0A peptide ($n = 2$) was added to subsaturating amounts of monoclonal antibody 48d at the time of monoclonal antibody 48d binding to Can0A peptide on an ELISA plate. The soluble peptide inhibited the binding of 48d to the plate-bound Can0A peptide ($^*p = 0.043$).

tide, the V3 Can0A peptide, and the C4 + V3 Can0A peptide mixture (Fig. 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 compared to binding of 48d to the mixture of the C4 and V3 Can0A peptides ($p = 0.04$). There was

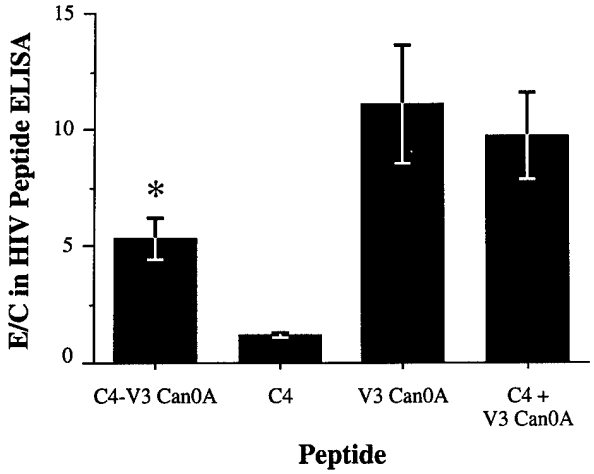

FIG. 4. Monoclonal antibody 48d binds to the V3 sequence of the C4–V3 HIV Can0A peptide. C4–V3 Can0A peptide (4.5×10^{-10} M; 2 μg), the C4 peptide alone, the V3 Can0A peptide alone, and a 4.5×10^{-10} M mixture of each of the C4 and V3 Can0A peptides were each plated in microtiter wells, and a dilution curve of monoclonal antibody 48d compared to 17b and a human paraprotein control antibody was studied in an ELISA. 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 equimolar 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.

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 8 M urea treatment on monoclonal antibody 48d binding to V3 Can0A peptide

Monoclonal antibody 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 8 M urea. Treatment of the C4–V3 Can0A peptide prior to antibody binding with 8 M urea significantly decreased the ability of MAb 48d to bind to the C4–V3 Can0A peptide ($p = 0.0006$), 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 (Fig. 5).

Lack of binding of monoclonal antibody 48d to C4–V3 Can0A peptide bound to cells

HSB T-ALL cells were incubated with 100 μg of C4–V3 Can0A peptide, washed, and then incubated with human MAb 48d, 17b, or anti-V3 human MAb 19b. As shown in Fig. 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. Monoclonal antibody 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.

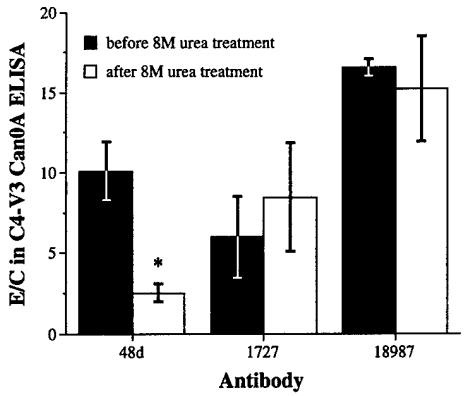

FIG. 5. Effect of urea treatment on monoclonal antibody 48d binding to V3 Can0A Peptide. Monoclonal antibody 48d, the anti-V3 monoclonal antibody (1727), and a rhesus monkey polyclonal anti-GPGRAF serum (18987)[16] were assayed for reactivity to 2 μg of C4–V3 Can0A peptide in solid-phase ELISA, both before and after treatment of the peptide on the plate with 8 M urea. Following treatment for 1 hr with 8 M urea, the plates were washed and the reactivity of the peptide on the plate with the three different antibodies determined. Treatment of the C4–V3 Can0A peptide with 8 M urea significantly decreased the ability of monoclonal antibody 48d to bind to the peptide ($n = 8$; $p < 0.0001$), but had no effect on binding of the anti-V3 monoclonal antibody 1727 ($n = 3$) or polyclonal rhesus antiserum 18987 ($n = 2$).

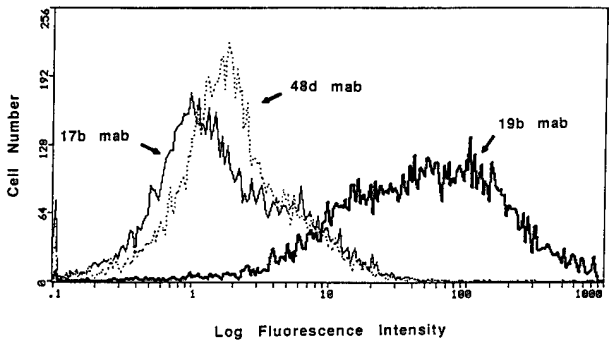

FIG. 6. Monoclonal antibody 19b (anti-V3 human monoclonal antibody) strongly reacted with the C4–V3 Can0A peptide bound to T cells whereas monoclonal antibody 48d did not recognize C4–V3 Can0A peptide bound to T cells. HSB T-ALL cells were incubated with C4–V3 Can0A peptide (100 μg/ml), washed, and incubated with 10 μg 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) was used as a negative control, and had the same binding pattern as the DUHP20 human paraprotein negative control. Data are representative of three experiments.

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 the reciprocal of the serum dilution that 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 peripheral blood mononuclear cells (PBMCs) with V3 loop sequences that were nearly 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 article 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 gp120 following gp120 binding to soluble CD4,[14] the preferred solution conformers of the HIV MN and Can0A peptides have been determined by nuclear magnetic resonance (NMR),[18] and the gp120 epitope to which MAb48d binds has not been completely characterized.

Monoclonal antibodies 17b and 48d were 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 article 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.[19] 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, MAb 48d did not bind well to V3-less gp120 either in the presence or absence of soluble CD4 (sCD4), whereas MAb 17b did bind the same Env mutant protein.[19] Finally, Sodroski and colleagues demonstrated that a large number of Env mutations abrogated MAb 48d binding to gp120, suggesting the 48d epitope to be conformational.[19] Even though MAb 48d binds to the HIV IIIB clone HXB2 envelop protein,[14,19] in our study 48d did not bind to the HIV IIIB V3 loop peptide. Moreover, we and others have found that MAb 48d strongly neutralizes the HIV MN laboratory-adapted HIV strain, but only weakly bound ($p = 0.06$) the HIV MN V3 loop peptide (Fig. 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 ELISAs, it is interesting that treatment of the HIV Can0A C4–V3 peptide with 8 M urea prevented MAb 48d binding to the peptide while not affecting the binding of other known antibodies to gp120 V3 region linear determinants (Fig. 5). The decrease in binding of 48d following 8 M 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 were the HIV MN C4–V3 and V3 peptides. It was disappointing that C4–V3 Can0A-induced antibodies did not neutralize other disparate HIV isolates, or HIV primary isolates grown and assayed in peripheral blood mononuclear cells. Thus, the HIV Can0A V3 peptide epitope reactive with MAb 48d is probably not well expressed on the surface of HIV primary isolates. In other studies, MAb 48d has inconsistently neutralized primary isolates.[20] 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 pattern similar to that of anti-HIV MN murine MAbs.[21] Vu and colleagues have recently identified a structural basis for the similar specificity of HIV MN and Can0A-induced anti-V3 antibodies.[18] 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.[18]

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. Monoclonal antibody 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 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, although, again, this effect could have been due to the relatively low affinity of MAb 48d for the Can0A V3 peptide. 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.

ACKNOWLEDGMENTS

The authors thank John Moore and Thomas Palker for helpful discussions, William Millard for expert technical assistance, and Kim R. McClammy for expert secretarial assistance.

Supported by NCVDG Grant No. AI35351 and DAMD Grant No. 17-94-J-4467. Jason B. Weinberg is a Howard Hughes Medical Institute Medical Student Fellow.

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