Different Immune Response of Mice Immunized with Conjugates Containing Multiple Copies of Either Consensus or Mixotope Versions of the V3 Loop Peptide from Human Immunodeficiency Virus Type 1

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The critical role that antibody responses to the V3 loop epitope play in human immunodeficiency virus type 1 (HIV-1) neutralization has caused this peptide to be used in many HIV-1 vaccine candidates. To enhance cross-reactivity toward several V3 sequences, a database of 50 peptides of the V3 region from HIV-1 subtype A was used to design both a consensus peptide and a combinatorial peptide (mixotope) library representative of these sequences. The two immunogens (consensus and mixotope) were incorporated into multiple antigen peptide (MAP) constructions, conjugated to a recombinant surface antigen from hepatitis B virus (HbsAg) carrier protein, and inoculated to mice in combination with a C4 (CD4-binding) peptide MAP construction, also conjugated to HBsAg. The respective responses and cross-reactivity to several V3 loop sequences of both types of immunogens were compared. Mice inoculated with the V3 consensus-MAP−HBsAg + C4-MAP−HBsAg mixture elicited higher antibody responses than those given the V3 mixotope-MAP−HBsAg + C4-MAP−HBsAg mixture. In addition, pooled serum from the first group of immunogens analyzed at dilution 1:100 had higher cross-reactivity against V3 peptides on cellulose membranes than those from mice given the combinatorial immunogen. Fine epitope mapping of both consensus and C4 peptide by the spot synthesis technique showed that sera of the first group strongly recognized both sequences in their entirety, whereas mice immunized with the mixotope library recognized only the N-terminal region of V3. These results seem to suggest that the V3 consensus peptide is superior to the combinatorial strategy in inducing potent and cross-reactive responses to HIV.

INTRODUCTION

The complexity of the HIV[1] life cycle, with high mutation rates and resulting polymorphism, its different mechanisms of evading the immune system, and the absence of suitable animal infection models, have so far combined to forestall the development of successful vaccines. These difficulties notwithstanding, recent advances in the study of HIV entry mechanisms and of structure/function relationships for envelope proteins gp120 and gp41 (*1*) still offer good possibilities for the development of vaccines with preventive and/or therapeutic properties (*2*). Most vaccine prototypes designed so far involve these two targets (*3*). Such prototypes include recombinant proteins (*4*), naked DNA (*5*), and synthetic peptides (*6*).

Several recombinant multiepitope HIV peptide constructions have been described (*7*). For instance, TAB9, which includes six V3 loop peptides (isolates LR150, JY1, RF, MN, IIIB, and BRVA), was used in a phase I clinical study (*8*). The recombinant protein was immunogenic and induced high levels of antibody neutralization, but the immune response was mainly directed at JY1 and LR150 and only weakly at MN, suggesting the need for considerable improvement (*9*). The problem with chimeric polypeptides of this type is that they adopt unpredictable 3D structures that favor the exposure of some epitopes, at the expense of others that in practice remain silent. Alternative strategies, such as synthetic MAP dendrimer constructs, have been proven more effective in achieving more balanced epitope presentations (*10*).

Naked DNA technology has not progressed as far or as fast as expected, largely because no suitable delivery routes have yet been devised. In addition, the long-range collateral effects associated with this approach have not

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[1] Abbreviations: AAA, amino acid analysis; AcOH, acetic acid; Boc, *t*-butyloxycarbonyl; Bzl, benzyl; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DIPC, *N*,*N*′-diisopropylcarbodiimide; DTT, dithiothreitol; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DIEA, *N*,*N*-diisopropylethylamine; EDTA, ethylenediaminetetraacetic acid; EDAC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; IFA, incomplete Freund's adjuvant; MAP, multiple antigenic peptide; MeCN, acetonitrile; MS, mass spectrometry; PBS, phosphate-buffered saline; RP, reverse phase; TFA, trifluoroacetic acid; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

10.1021/bc049944u CCC: $27.50
Published on Web 08/19/2004

been adequately clarified. On the other hand, plasmid DNA vaccines, initially regarded as promising, have failed to provide a sufficiently protective response against either HIV or simian immunodeficiency virus (SIV) in primates. Viral mutants escaping plasmid DNA-induced CTL (cytotoxic T lymphocytes) responses have recently been described (*11*).

The synthetic peptide approach makes it possible, in principle, to select only the epitopes of interest and thus largely circumvent problems such as viral variability, unwanted immunosuppression, etc., by focusing on selected segments of envelope proteins such as gp41 and gp120, directly involved in HIV entry and widely regarded as ideal targets for blocking the first step of viral replication. One particular envelope protein, gp120, includes the V3 loop, one of the most widely studied motifs, which defines epitopes for both antibodies and lymphocytes (*12*) and also has a crucial role in viral tropism and pathogenesis (*13*). Consequently, the V3 loop is featured in almost all vaccine candidates currently under evaluation. A recent U.S. clinical trial for a mixture of five V3 peptides showed decreased HIV levels even in patients with strains differing in two or three residues from those used for immunization (*14*). Another relevant gp120 motif, the fourth conserved (C4) domain, is a major player in the binding of HIV to its primary receptor, CD4, leading to virus−cell fusion and replication; a single amino acid change in C4 results in loss of infectivity (*15*).

As is well-known, the V3 region of HIV-1, containing the principal determinant of neutralization, is highly variable. To overcome this difficulty, consensus peptides preserving those amino acid positions more consistently repeated in field or clinical isolates have been explored (*16*). A competing approach, aimed at covering a broad spectrum of V3 sequences, is the generation of combinatorial libraries, named mixotopes (*17*−*19*), representing the more variable positions of the V3 loop. Promising results from this type of strategy have recently been reported (*20*, *21*).

In the present study we have decided to compare both types of strategies. To this end, both consensus and mixotope versions of V3 were synthesized as MAP constructs and conjugated to an HBsAg carrier protein to find out which of the two approaches provides better results in terms of immunogenicity and cross-reactivity. To enhance the immune response, the two kinds of V3 conjugates were coadministered with HBsAg conjugates of a MAP version of the C4 (CD4-binding) sequence. The antibody responses generated by the two types of immunogens were analyzed against several V3 representative peptides, and fine epitope mapping of both groups of sera were performed.

EXPERIMENTAL PROCEDURES

Chemicals and Biologicals. Boc-protected amino acids and other reagents for peptide synthesis were purchased from Bachem (Bubendorf, Switzerland). *p*-Methylbenzhydrylamine (MBHA) resin was from Fluka (Buchs, Switzerland). Solvents for peptide synthesis (DCM, 2-propanol, DMF) were obtained from Merck (Darmstadt, Germany). The recombinant surface antigen from hepatitis B virus (HBsAg) was produced at CIGB (Habana, Cuba). Succinic anhydride was from Sigma (Milwaukee, WI).

MAP Synthesis. All MAPs were prepared by manual stepwise solid-phase synthesis methods (*22*) on *p*-methylbenzhydrylamine (MBHA) resin. The protecting group scheme was based on the Boc group for the α-NH_2 terminus, and the side chains of trifunctional amino acids were protected as benzyl (Asp, Glu, Ser, Thr), 2-chlorobenzyloxycarbonyl (Lys), dichlorobenzyl (Tyr), 2,4-dinitrophenyl (His), formyl (Trp), 4-methoxybenzyl (Cys), and tosyl (Arg) derivatives. The three Lys residues making up the tetravalent dendrimer core were incorporated onto the β-Ala-Cys-MBHA resin as Boc-Lys(Boc) derivatives, by use of TBTU/HOBt/DIEA for the coupling. Cleavage of the Boc group was done with 40% TFA in DCM followed by neutralization with 5% DIEA in DCM. Double couplings with a 10-fold excess of Boc-amino acid and DIPC/HOBt or TBTU/HOBt/DIEA were used for most residues of the V3 epitope. When required by the Kaiser test (*23*), capping steps were performed with 4% acetic anhydride and 1% DIEA in DMF. After low−high HF cleavage and deprotection, the MAPs were extracted into 0.1 M Tris-HCl (pH 8.0) containing 8 M urea, dialyzed against 2 M urea in 0.1 M Tris-HCl (pH 8.0) for 24 h, and then stirred in the same solvent in an open vessel to promote dimerization by disulfide bond formation, until a negative Ellman test (*24*) was obtained. The homodimeric MAPs were then dialyzed against 1 M AcOH, to remove all the urea, and lyophilized.

For the V3 mixotope, a similar synthetic scheme was followed, except that at high-variability positions a representative mixture of the corresponding amino acids (in equimolar proportion) was coupled by means of TBTU/DIEA in DMF under substoichiometric conditions for long reaction times, as previously described (*17*).

Characterization of MAPs. For analytical purposes only, the homodimeric MAP constructs were converted to the monomer form. Lyophilized material was redissolved at 5 mg/mL concentration in 0.3 M Tris-HCl, 6 M guanidinium chloride, and 3 mM EDTA (pH 8.5), a 20-fold molar excess of DTT over Cys residues was added, and the solution was flushed with nitrogen and incubated for 2 h at 37 °C. A 2-fold molar excess of iodoacetic acid over DTT was next added and the solution was kept at room temperature for 1 h in the dark. Modified MAPs were characterized by RP-HPLC on Baker C_8 wide-pore (100 × 4.6 mm) columns in a system consisting of a Pharmacia model 2150 dual pump coupled to a Knauer (FRG) variable-wavelength UV detector. Linear gradients of MeCN (+0.05% TFA) into water (+0.1% TFA) were used for the separation, at 0.8 mL/min flow rate. Optical density was monitored at 226 nm. Data were processed with the BioCrom program (CIGB, Cuba). Electrospray MS analysis was done in a hybrid quadrupole time-of-flight (Q-TOF) instrument (Micromass, Manchester, U.K.) fitted with a Z-spray nano flow ion source (*25*). The mass spectrometer was operated with a source temperature of 80 °C and a drying gas flow rate of 50 L/h. RP-HPLC-purified MAPs were dissolved in a 50% (v/v) water/MeCN solution containing 1% acetic acid, to an approximate concentration of 5 pmol/μL. The MAP solution was infused at 3 μL/ min into the mass spectrometer by use of a syringe pump (Harvard Apparatus, Holliston, MA). The capillary and cone voltage were set to 3 kV and 35 V, respectively. Data acquisition and processing were performed with the Micromass MassLynx package.

For amino acid analysis, samples of S-carboxymethylated MAPs were hydrolyzed with 6 M HCl containing 0.1% phenol and 0.1% 2-mercaptoethanol for 24 h in vacuum-sealed ampules. After evaporation, amino acids in the hydrolysate were determined in a Pharmacia-LKB Alpha Plus 4151 analyzer with a sodium buffer system and *o*-phthalaldehyde derivatization for fluorescence detection. All samples were analyzed in triplicate.

Table 1. Immunization Protocols[a]

group	dose (μg)
A: HBsAg	10
B: C4-MAP−HBsAg	10
C: C4-MAP−HBsAg and consensus MAP−HBsAg	5 + 5
D: C4-MAP−HBsAg and library MAP−HBsAg	5 + 5

[a] All immunogens formulated in CFA (100 μL, day 0) and IFA (days 14, 28, 56). Each group consisted of eight mice.

Conjugation of MAPs to Carrier Protein. Homodimeric, octavalent MAPs were coupled to HBsAg as described by Deen et al. (*26*) (see Figure 1 also). Briefly, 5 mg of HBsAg was dissolved in 5 mL of 0.2 M K_2HPO_4 (pH 8.0), solid succinic anhydride (1 mg) was added, and the solution was stirred until all anhydride was dissolved, with occasional dropwise addition of 3 M NaOH as required to keep the pH at 8−8.5. The activated protein was next exhaustively dialyzed against dilute HCl (pH 4.5), mixed with 7 mg of EDAC, and stirred for 10 min at 25 °C. Then, 5 mg of octavalent MAP dissolved in 1 mL of 3 M guanidine hydrochloride in PBS was added and the mixture was gently stirred for 3 h at 25 °C. The conjugates were purified by gel filtration through Sephadex CL-4B (Pharmacia, Uppsala, Sweden) eluted with PBS. The front-eluting fractions were pooled and quantitated by the Coomassie method (*27*).

Immunizations. Groups of eight female Balb/c mice, 6−8 weeks old (Cenpalab, Cuba), were inoculated subcutaneously on days 0, 14, 28, and 56 with 100 μL of a suspension containing 10 μg of antigens (Table 1) emulsified in either CFA (day 0) or IFA (other days). Animals were bled 10 days after the fourth dose.

Enzyme-Linked Immunosorbent Assay against Synthetic Peptides. ELISA of anti-peptide sera was done as previously described (*28*). Briefly, 96-well (Costar high binding) polystyrene plates (Corning Biosciences, Acton, MA) were coated with 100 μL of 10 μg/mL solutions of consensus or C4 peptides (as BSA conjugates) or of the MAP library and incubated overnight at 4 °C. After three washings with 0.05% Tween 20 in PBS, plates were blocked with 2% milk in PBS (blocking solution) for 1 h at 37 °C. Serum samples were diluted with 0.05% Tween 20−5% sheep serum in blocking solution and incubated for 2 h at 37 °C. After four washings, an anti-mouse IgG peroxidase conjugate (Amersham, Little Chalfont, U.K.) was added and the mixture was incubated for 1 h at 37 °C. The color reaction with *o*-phenylenediamine was allowed to develop for 10 min and then stopped with 3 M H_2SO_4 solution (50 μL), and the absorbance at 492 nm was determined in a SensIdent Scan (Merck). Results were expressed as log titer, determined by interpolation of OD values at fixed serum dilutions into a standard curve of a serum with known titer. Titer was defined as the logarithm of the highest dilution giving twice the absorbance of negative control sera. Titers are given as the geometric mean ± SD of the titers of individual sera.

Epitope Mapping by Use of Cellulose-Bound Peptides. The spot synthesis technique previously described by Frank (*29*) was used. The Whatman 540 paper support (Whatman, Maidstone, U.K.) was functionalized by esterification of Fmoc-β-Ala-OH, activated by DIPC and *N*-methylimidazole in dry DMF, at defined positions to give a spot array suitable to the required number of sequence peptides. All subsequent residues in the different V3 loop sequences were incorporated manually by standard Fmoc/tBu chemistry. All peptides were acetylated at the N-terminus at the end of the synthesis program.

Table 2. V3 Loop Sequences of 50 HIV-1 Subtype A V3 Isolates Used to Define the Consensus Sequence

#	Accession number[a].	Amino acid sequence
-	consensus	SVRIGPGQAFYATGDIIGDIRQ
1	AAC37794	--HM---R----------
2	AAC37804	--HM---KT----------
3	AAB87297	-M------T----------
4	AAC37825	-I----------
5	AAB34131	-IHI-P-Q------A-------
6	AAP37302	----------
7	CAA50920	--N----------
8	CAA50937	--H---------R---------
9	CAA50942	-L------T----------
10	AAO20500	--H----------
11	AAC55823	--------T----------
12	AAG48444	GIH----SAI----------
13	CAC15292	GIH-----T-----E---N---
14	AAS18950	--------T-----A-------
15	AAD05114	--------T---A---------
16	AAF67679	----------TR-
17	AAD24257	-IHF----TL----N-------
18	AAD42826	-I---S—TS---------N--E
19	CAA91881	GIH----R------Q-T-----
20	AAC42384	-I----------T-----N---
21	AAQ83975	-IH----------N---
22	AAC55896	-I------T---------N---
23	CAD36394	-I----------T---T-----
24	AAB58169	-IH----------S--------
25	AAK85213	-I---------T----------
26	AA020437	--H----------N---
27	AAA70070	-AN----------E-------
28	AAA70078	GIH-----S-----S-V-N---
29	AAS19003	-I------T-----E---N---
30	AAC16336	G-H----------
31	AAF02185	-I------S-H----------
32	CAB00597	--H----------V------
33	AAF02186	G----------G-------
34	AAK98157	--H-----TS----------
35	AAD03181	-IHL---R----------
36	AAC42562	----------N---
37	CAD87155	--H---------R---------
38	AA048836	-IH---------R----N----
39	AAA44006	--HI----------E-------
40	CAA08527	--------T---------N---
41	AAA44019	--H----K------G-V-----
42	AAA44029	--H----------A---S---
43	AAA43985	-IG-----T---ADN-------
44	AAB58176	--------S----------K-
45	AAS17820	-IS----R—F----------
46	AAA78796	GIHM----IL----S-------
47	AAA78797	-I------V—TN----------
48	AAB34131	-IH-A---------A-------
49	AAB34136	GIHL---------NA-------
50	AAB58169	-IH----------

[a] Accession numbers are from the National Center for Biotechnology Information (BLAST Program).

Paper-Based Peptide Enzyme Immunoassay. Washing steps and incubations were performed at 25 °C with Tris-buffered saline (TBS) containing 0.05% Tween-20. Blocking of free binding sites was performed by overnight incubation with 5% skim milk in TBS. Spots were next incubated with sera for 3 h, washed, and incubated for 2 h with alkaline phosphatase-conjugated anti-mouse IgG (Sigma). Detection of bound antibody was achieved with 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in substrate buffer [0.1 M Tris (pH 8.9), 0.1 M NaCl, and 2 mM $MgCl_2$]. Washing with PBS stopped staining. The peptide-bearing cellulose sheets were regenerated and prepared for the next run as described by Frank (*29*).

Statistical Analysis. All ELISA OD values were transformed to the natural logarithm to get a normal distribution in the immunized groups. An *F*-test was performed to assess variance homogeneity between groups and a Student's *t*-test was done ($p < 0.05$ was considered statistically significant).

Table 3. HIV-1 Amino Acid Sequences Used for the Immunogens

Peptide	Sequence synthesized[a]
C4	KQIINMWQEVGKAMYA
Consensus	KSVRIGPGQAFYATGDIIGDIRQA
Combinatorial mixotope[b]	KXXXIGPGQXFYATGXIIGXIRQA SVR A D D GMH T A N LN S E S IG V G AS I S N Q

[a] In the consensus and mixotope sequences, a non-native N-terminal Lys residue was added to facilitate conjugation to the carrier protein. The N-terminal Lys of the C4 sequence is native. [b] In the mixotope top sequence, the Xs denote combinatorial sites, where mixtures of the residues shown below have been incorporated.

RESULTS

Design and Synthesis of V3 Consensus and Combinatorial MAPs. Fifty sequences (Table 2) from the V3 region of HIV-1, subtype A, with (i) reasonable chemical diversity, (ii) adequate geographical representation, and (iii) demonstrated infectivity were manually selected out of ca. 7000 sequences available at the time at the National Center for Biotechnology Information (BLAST program) and used to design a 24-residue consensus sequence, where the most frequent amino acid among the isolates was chosen for each position. The same database was used to build a mixotope combinatorial library (*18*) in which sites with over 90% conservation among the 50 isolates were represented by a single residue, while at more degenerate positions an amino acid mixture representative of the different mutations was incorporated (Table 3).

The design of our immunogens started (Figure 1A) with the incorporation of either the consensus single peptide, or the mixotope library, or of another HIV peptide sequence (C4) known to play a major role in viral binding to the CD4 primary receptor, into tetravalent MAP platforms. The original approach of Tam (*30*) was followed, with two minor modifications: (i) a Lys residue was added at the N-terminus of both consensus and mixotope sequence to facilitate conjugation to carrier protein (the C4 peptide already has a native Lys at the N-terminus), and (ii) a Cys residue was placed at the C-terminus of the entire MAP dendrimer, to allow dimerization by disulfide formation. The resulting octavalent constructions would next be conjugated to HBsAg (see next section and Figure 1B).

All three MAPs were prepared by Boc-based methods of solid-phase synthesis and, after cleavage from the resin with HF, were solubilized under denaturing conditions and dialyzed to remove synthesis and cleavage byproducts while in situ air oxidation was allowed to proceed. The reaction was monitored by HPLC gel filtration and the Ellman test (data not shown). Once shown to be complete by HPLC (see Supporting Information), the dimeric MAPs were dialyzed against 1 M AcOH and lyophilized. MAP dimers were found to be stable at or below pH 7, room temperature, in aqueous solution, with or without chaotropic additives. To further evaluate their quality, an aliquot was reduced, S-carboxymethylated, and characterized by both reverse-phase HPLC and MS (Figure 2). As expected, the HPLC profile corresponding to the combinatorial MAP, containing an estimated mixture of over 5000 different peptide sequences, was a rather broad peak. For the two single-sequence MAPs, the HPLC profiles showed a main fraction that was analyzed by electrospray MS and found to correspond very well with the expected theoretical mass: 11 318.87 Da for the consensus V3-MAP (found *m*/*z* 11 319.63) and 8824.56 Da for C4-MAP (found *m*/*z* 8824.25). In this latter case, the broad elution band overlapping the main peak was attributed to byproducts resulting from Trp degradation during the HF deprotection step. The MAPs were further characterized by amino acid analysis, which in all three cases showed compositions consistent with the expected structure (Table 4).

MAP−HBsAg Conjugates. To further enhance the immunogenicity of the homodimeric MAPs, they were conjugated to recombinant HBsAg, a polymeric protein of about 2000 kDa (*31*, *32*). Our choice of HBsAg was based on its high molecular weight, proven stability, and high immunogenicity and because it is known to act as an effective and safe vaccine in humans. The conjugations were performed with a succinic acid cross-linking between Lys amino groups of the MAP epitopes and the carrier. Thus, HBsAg was first succinylated under mildly alkaline conditions, followed by dialysis to remove excess succinic anhydride and thus avoid unwanted cross-linking. The carboxyl groups on the modified HBsAg were next activated with EDAC (a soluble carbodiimide) and

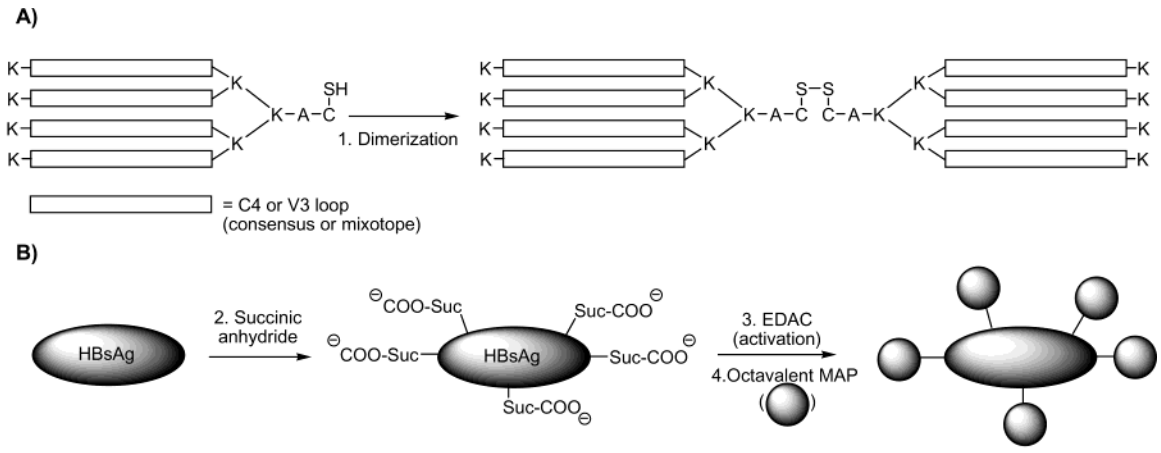

Figure 1. Synthetic strategy for MAP conjugates of C4 and the V3 loop of HIV. (A) Octavalent MAP constructs result from dimerization (1) of synthetic tetravalent MAP through disulfide formation. (B) Amino groups in HBsAg are converted to carboxyls (2) by succinylation and then activated (3) with water-soluble carbodiimide to allow conjugation (4) with the octavalent MAP.

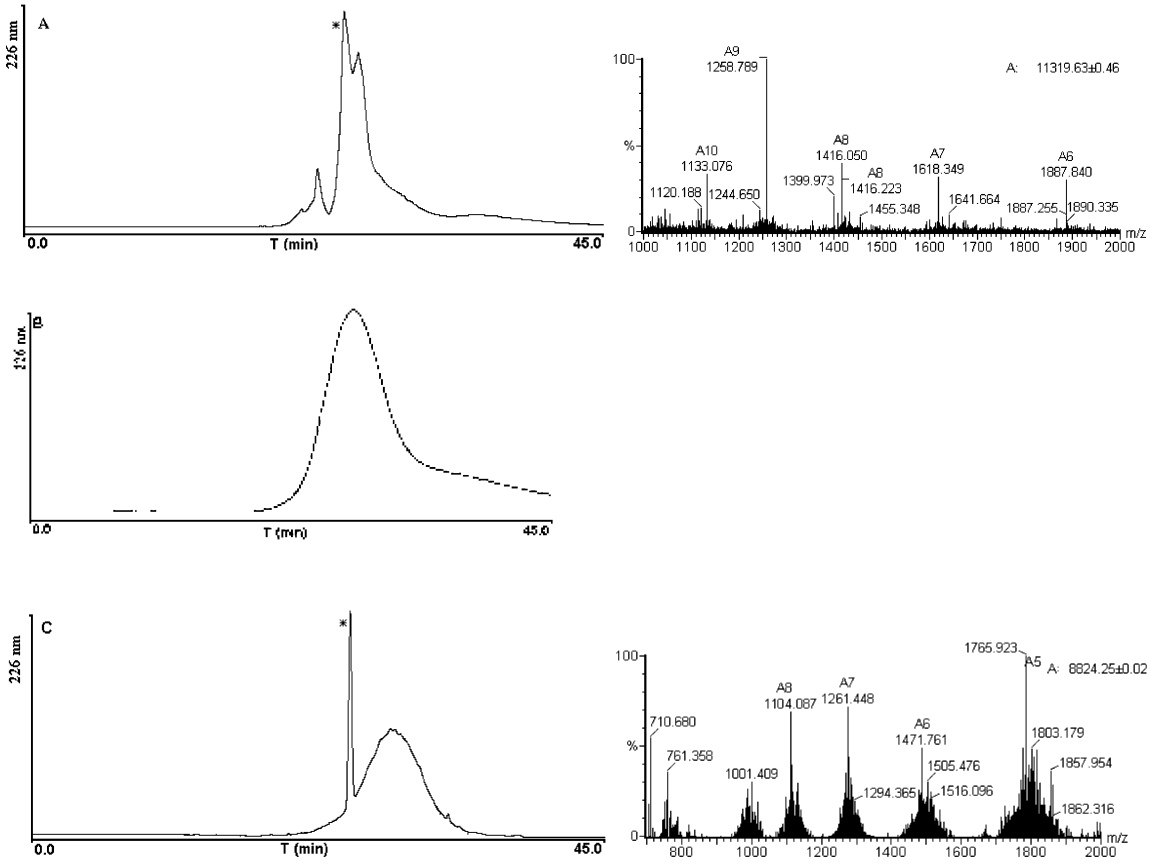

Figure 2. HPLC analysis of tetravalent MAP constructs corresponding to the V3 loop in consensus (A) and mixotope (B) form and (C) to C4. Profiles show the product resulting from reduction and S-carboxymethylation of the synthetic octavalent dimer. In panels A and C, the ESI mass spectrum of the main fraction (marked with an asterisk) is shown at the right of the chromatogram. For the consensus V3 loop MAP, calculated MW 11 318.87 Da; found, *m*/*z* 11 319.63. For the C4 MAP, calculated MW 8824.56 Da; found, *m*/*z* 8824.25.

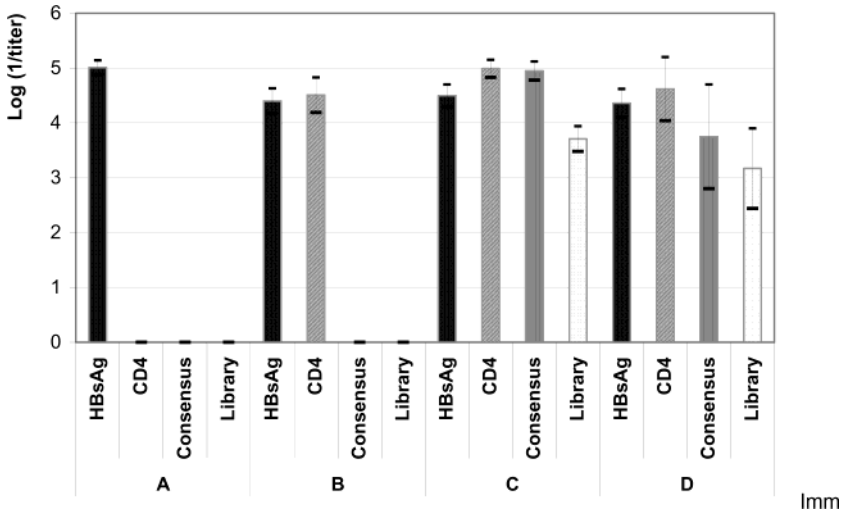

Figure 3. Antibody titers of mice after immunization with four doses of (A) HbsAg, 10 μg; (B) C4-MAP−HBsAg conjugate, 10 μg; (C) mixture of C4-MAP−HBsAg and consensus V3-MAP−HBsAg conjugates, 10 μg; and (D) mixture of C4-MAP−HBsAg and V3 mixotope-MAP−HBsAg, 10 μg. Titers are the geometric mean ± SD of eight mouse antisera.

allowed to react with the free amino grups of each MAP (Figure 1B). The conjugates were purified by gel filtration on Sephadex CL-4B to remove excess carbodiimide and MAP. Since amino acid analysis of HBsAg is notoriously unreliable, no attempt was made to determine MAP-to-HBsAg molar ratios by this method. Instead, a qualitative estimation of the efficiency of epitope incorporation to the conjugates was obtained by ELISA (see Supporting Information).

Analysis of Antibody Response in Mice Immunized with MAP Conjugates. Groups of eight Balb/c mice were inoculated subcutaneously to evaluate the anti-MAP antibody responses against the different immunogens. For coimmunizations, appropriate formulations of each individual conjugate were prepared and mixed before inoculation. All four groups of animals gave anti-HBsAg responses (Figure 3), which were predictably more pronounced in the control group A. Responses to

Table 4. Amino Acid Compositions and Mass Spectrometry of MAPs[a]

amino acid	C4	consensus	mixotope
Asx	3.26 (4)	8.71 (8)	3.27 (4.4)
Thr		3.31 (4)	3.80 (4.8)
Ser		2.56 (4)	4.53 (3.5)
Glx	11.83 (12)	8.19 (8)	8.16 (9.7)
Gly	12.15 (12)	23.99 (24)	27.49 (26.6)
Ala	8.90 (8)	12.88 (12)	6.67 (6.6)
Val	3.16(4)	1.66 (4)	0.72 (1.7)
Met	5.77(8)		0.86 (0.8)
Ile	7.56(8)	15.38 (16)	16.36 (17.8)
Leu			1.07 (0.8)
Tyr	2.60(4)	3.25(4)	3.52 (4)
Phe		3.02(4)	3.30 (4)
His			0.29 (0.8)
Lys	12.71(11)	7.66 (7)	8.47 (7)
Arg		7.10 (8)	1.98 (4.8)
Cys	0.89(1)	0.9(1)	0.94(1)
β-Ala[b]	(1)	(1)	(1)
Trp	3.4 (4)		
Pro		2.08 (4)	3.08 (4)

[a] Data are expressed as number of residues per molecule. Experimental values represent the mean of three independent determinations. The theoretical values calculated from MAP sequences are in parentheses. Asx = Asp + Asn; Glx = Glu + Gln. [b] β-Ala not determined.

C4 were also comparable in the three groups immunized with the corresponding MAP−HBsAg conjugate. Co-immunization of C4-MAP−HBsAg with either the V3 consensus MAP−HBsAg or the V3 mixotope-MAP−HBsAg conjugates produced antibody responses against both (V3 and C4) types of B-cell epitopes. Interestingly, animals receiving the consensus V3 immunogen recognized the mixotope somewhat better than those given the mixotope itself. From Figure 3 it is clear that the antibody responses against all three types of immunogen (C4, consensus, and mixotope) were significantly higher for group C mice (inoculated with consensus V3-MAP−HBsAg + C4-MAP−HBsAg) than for group D (V3 mixotope instead of consensus V3 peptide). From these results we conclude that, at least in this particular case, co-administration of two immunogens is advantageous in terms of obtaining a dual antibody response. Such an approach should be valuable for generating bivalent vaccines when the carrier protein is per se a vaccine immunogen such as HBsAg.

Analysis of Cross-reactivity with Peptides Synthesized on Cellulose Membrane. Cross-reactivity of the different antisera was evaluated (Figure 4) against a panel of 50 cellulose-bound peptides corresponding to different isolates of the V3 loop of HIV-1, subtype A. After the third booster, pooled sera from group D (immunized with C4-MAP−HBsAg and V3 mixotope-MAP−HBsAg) at 1:100 dilution recognized 26% (13/50) of the test peptides, 53% (7/13) of them with high reactivity. In contrast, pooled sera from group C (given C4-MAP−HBsAg and consensus V3-MAP−HBsAg) recognized 72% (36/50) of the same peptide panel same peptides panel, 50% (18/36) with high reactivity. Thus, somewhat counterintuitively with the use of a single sequence, the consensus immunogen gives rise not only to higher titers but also to broader cross-reactivities.

Fine Epitope Mapping of Anti-V3 Sera. Given the substantial differences in cross-reactivity observed for antibody responses to either consensus or mixotope strategies, it seemed appropriate to perform a fine epitope mapping of both types of antisera. For this we resorted again to the spot synthesis technique, using 18 and 10 overlapping heptapeptides to cover the sequences of the consensus and the C4 peptides, respectively (Figure 5). ELISA of pooled sera from groups C and D at a 1:100 dilution showed that the former group (immunized with consensus V3-MAP−HBsAg) strongly recognized the N-terminal (KSVRIGP and RIGPGQA), central (FYATGDII), and C-terminal (IGDIRQA) regions of the immunogen. In contrast, mice immunized with the combinatorial mixotope only recognized heptapeptides from the N-terminal region (KSVRIGP and RIGPGQA). Again, a more satisfactory antibody response was found for mice immunized with the V3 consensus MAP.

Spot	Peptide Sequence	Immunogen group			
		A	B	C	D
1	SVHMGPGRAFYATGDIIGDIRQ				
2	SVHMGPGKTFYATGDIIGDIRQ				
3	SMRIGPGQTFYATGDIIGDIRQ				
4	SIRIGPGQAFYATGDIIGDIRQ				
5	SIHIAPRQAFYATGAIIGDIRQ				
6	SVRIGPGQAFYATGDIIGDIRQ				
7	SVNIGPGQAFYATGDIIGDIRQ				
8	GVHIGPGQAFYARGDIIGDIRQ				
9	SLRIGPGQTFYATGDIIGDIRQ				
10	SVHIGPGQAFYATGDIIGDIRQ				
11	SVRIGPGQTFYATGDIIGDIRQ				
12	GIHIGPGSAIYATGDIIGDIRQ				
13	GIHIGPGQTFYATGEIIGNIRQ				
14	SVRIGPGQTFYATGAIIGDIRQ				
15	SVRIGPGQTFYAAGDIIGDIRQ				
16	SVRIGPGQAFYATGDIIGDTRQ				
17	SIHFGPGQTLYATGNIIGDIRQ				
18	SIRIGSGQTSYATGDIIGNIRE				
19	GIHIGPGRAFYATGQITGDIRQ				
20	SIRIGPGQAFYATGDIIGNIRQ				
21	SIHIGPGQAFYATGDIIGNIRQ				
22	SIRIGPGQTFYATGDIIGNIRQ				
23	SIRIGPGQAFYATGDITGDIRQ				
24	SIHIGPGQAFYATSDIIGDIRQ				
25	SIRIGPGQAFYTTGDIIGDIRQ				
26	SVHIGPGQAFYATGDIIGNIRQ				
27	SANIGPGQAFYATGEIIGDIRQ				
28	GIHIGPGQSFYATGSIVGNIRQ				
29	SIRIGPGQTFYATGEIIGNIRQ				
30	GVHIGPGQAFYATGDIIGDIRQ				
31	SIRIGPGQSFHATGDIIGDIRQ				
32	SVHIGPGQAFYATGDVIGDIRQ				
33	GVRIGPGQAFYATGGIIGDIRQ				
34	SVHIGPGQTSYATGDIIGDIRQ				
35	SIHLGPGRAFYATGDIIGDIRQ				
36	SVRIGPGQAFYATGDIIGNIRQ				
37	SVHIGPGQAFYARGDIIGDIRQ				
38	SIHIGPGQAFYARGDIIGNIRQ				
39	SVHIGPGQAFYATGEIIGDIRQ				
40	SVRIGPGQTFYATGDIIGNIRQ				
41	SVHIGPGKAFYATGGIVGDIRQ				
42	SVHIGPGQAFYATGAIIGSIRQ				
43	SIGIGPGQTFYAADNIIGDIRQ				
44	SVRIGPGQSFYATGDIIGDIKQ				
45	SISIGPGRAFFATGDIIGDIRQ				
46	GIHMGPGQILYATGSIIGDIRQ				
47	SIRIGPGQVFYTNGDIIGDIRQ				
48	SIHIAPGQAFYATGAIIGDIRQ				
49	GIHLGPGQAFYATNAIIGDIRQ				
50	SIHIGPGQAFYATGDIIGDIRQ				

Figure 4. Cross-reactivity analysis of anti-V3 loop-MAP antibodies against heterologous V3 peptides displayed on cellulose membrane. Pooled sera (after initial inoculation + three boosters) from different immunization groups (A−D as in Figure 3) were analyzed. Boxes are coded as follows: white, no recognition; light gray, weak recognition; dark gray, intermediate recognition; black, strong recognition.

DISCUSSION

Different strategies have been proposed to deal with the challenge that highly variable antigenic sites, such as the V3 loop of HIV-1, pose to synthetic peptide-based vaccination schemes (*33*). Most of these strategies have relied on the use of combinations of several representa-

Peptide	Spot	Sequence	A	B	C	D
Consensus	1	KSVRIGP				
	2	SVRIGPG				
	3	VRIGPGQ				
	4	RIGPGQA				
	5	IGPGQAF				
	6	GPGQAFY				
	7	PGQAFYA				
	8	GQAFYAT				
	9	QAFYATG				
	10	AFYATGD				
	11	FYATGDI				
	12	YATGDII				
	13	ATGDIIG				
	14	TGDIIGD				
	15	GDIIGDI				
	16	DIIGDIR				
	17	IIGDIRQ				
	18	IGDIRQA				
C4	19	KQIINMW				
	20	QIINMWQ				
	21	IINMWQE				
	22	INMWQEV				
	23	NMWQEVG				
	24	MWQEVGK				
	25	WQEVGKA				
	26	QEVGKAM				
	27	EVGKAMY				
	28	VGKAMYA				

Figure 5. Epitope mapping of consensus and C4 peptides by the spot synthesis technique. The first 18 overlapping heptapeptides correspond to the consensus V3 loop sequence; the other 10 peptides, to C4. Pooled sera (after initial inoculation + three boosters) from different immunization groups (A−D as in Figure 3) were analyzed. Boxes are coded as follows: white, no recognition; light gray, weak recognition; dark gray, intermediate recognition; black, strong recognition.

tive peptide variants. We had earlier shown that V3 loop-based peptide cocktails, in the form of MAP dendrimers, were a good strategy of epitope presentation (*9*).

In the present study we have compared two alternative approaches to the variability problem, namely, (i) the use of a rationally derived consensus sequence where each position is occupied by the statistically most relevant residue or (ii) the mixotope concept, which not only reproduces a broad spectrum of reported, characteristic mutations, as done before with peptide cocktails (*9*), but additionally merges them in combinatorial fashion. Although our study is confined to basic features of the responses elicited by these two different immunogen types, it provides nonetheless sufficient evidence that the first approach is to be preferred if a strong, cross-reactive, and representative immune response to the V3 loop is wished. Our choice of antigenic presentation, i.e., homodimeric MAPs conjugated to HBsAg, relied on previous data (*32*) demonstrating its enhancing effect on peptide immunogenicity and may have influenced the strength of the immune response (e.g., relative to immunization with free peptides) but would predictably have had parallel effects on both consensus and mixotope formulations and thus little or no effect on the differences we have observed.

In contrast to the report by Resnick et al. (*34*), here we demonstrate that the mixotope strategy did not generate higher levels of cross-reactivity against several V3 peptides than those of the consensus strategy. The fine epitope mapping showed that the anti-peptide antibody response elicited by the consensus MAP was more complete than that generated by the MAP library. The fact that the latter is composed of some 5000 different sequences may account for the substantial reduction in global immune response and may also explain why the antibodies raised against the mixotope library tend to target mainly on the conserved regions of the V3 loop (Figure 5), which are better represented than the variant ones. In conclusion, our data support the hypothesis that, for highly variable antigens, a rationally designed consensus sequence, such as the one derived here for the V3 loop of gp120, may be a valid approach for synthetic vaccine development, providing desirable cross-reactivity and therapeutic advantage.

ACKNOWLEDGMENT

This work was supported by the Center for Genetic Engineering and Biotechnology, La Habana, Cuba. Work at Barcelona was supported by the Spanish Ministry of Science and Technology (Grants PB97-0873 and BIO2002-04091-C03-01, to D.A.), and by the Spanish Agency for International Cooperation (Cuban−Spanish joint research program, to D.A.).

Supporting Information Available: Data on the preparation and stability of MAP dimers and on the purification and characterization of MAP−HBsAg conjugates. This information is available free of charge via the Internet at http://pubs.acs.org.

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BC049944U