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Immunogenicity Comparison of a Multi-antigenic Peptide Bearing V3 Sequences of the Human Immunodeficiency Virus Type 1 with TAB9 Protein in Mice

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Abstract: The multiple antigenic peptide system (MAP) has been proposed as a novel and valuable approach for eliciting antibodies for peptides and developing synthetic vaccines. Multi-epitope polypeptides (MEP) have also been developed as an alternative to the recombinant approach for vaccines. The V3 loop from the HIV type 1 (HIV-1) external glycoprotein (gp120) contains the principal neutralization domain (PND). Antibodies against this region neutralize HIV-1 *in vitro* and *in vivo*. In this work, a novel presentation of di-epitope MAP was synthesized. A monomeric MAP carrying two identical JY1 V3 sequences as B-cell epitopes and the 830–843 region of tetanus toxoid as a T-helper cell epitope was synthesized. This basic structure was covalently linked to produce a four-JY1-branched homodimer (JY1-MAP4). Additionally, six different monomeric MAPs, bearing four copies of V3 from isolates LR150, JY1, RF, MN, BRVA and IIIB, were synthesized. These monomers were conveniently linked among themselves to produce homodimeric and heterodimeric MAPs of eight V3 branches (V3-MAP8). JY1-MAP8 elicited higher antibody titers in Balb/c mice than JY1-MAP4. The immunogenicity of two different, hexavalent V3-MAP8 mixtures and the MEP TAB9, which tandems the same six V3 sequences in a single molecule, were compared. The antibody response against the mixtures of the heterodimeric MAP showed a wider recognition pattern of the V3 region, while the homodimeric cocktail showed an intermediate pattern. Antibodies elicited by TAB9 recognized only the JY1, LR150 peptides. These results emphasize the influence of V3 epitope presentation upon the characteristics of the antibody response generated. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Boc chemistry; HIV-1; multiple antigen peptide; synthetic peptides; V3 loop

INTRODUCTION

Synthetic peptides have been widely used to generate anti-peptide antibodies with predetermined specificity. A major disadvantage is that peptides are generally poorly immunogenic, and therefore must be linked to a carrier protein [1,2]. The multiple antigen peptide system (MAP) was designed to overcome this problem [3,4]. It uses solid-phase synthesis technology to generate several variations of branched peptides, which differ only in the

Abbreviations: Boc, *t*-butyloxycarbonyl; DIPC, 1,3-diisopropylcarbodiimide; HOBT, 1-hydroxybenzotriazole; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DCM, dichoromethane; TFA, triflouroacetic acid; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronuim tetrafluoroborate; HF, hydrogen fluoride; OPD, orthophenylenediamine; BSA, bovine serum albuminin; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; DTT, dithiotreitol.

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design of the lysine core, to form a cascade of one, two or three lysine branches. This core represents a minor portion of the total MAP structure, and is not immunogenic by itself. These synthetic macromolecules may contain either B- or T-cell epitopes in tandem, preferably in a T–B orientation [5]. MAP represents a valuable approach for developing synthetic vaccines. They have been used in the preparation of experimental vaccines against hepatitis B [5], malaria [6,7], HIV [8] and foot-and-mouth disease [9,10].

The multi-epitope polypeptides (MEP) concept as vaccine against HIV has been developed by Duarte *et al.* [11,12]. This alternative was designed to generate a broadly reactive and neutralizing antibody response against the third variable region (V3 loop) of HIV type 1 (HIV-1). MEPs are chimeric proteins bearing various V3 sequences from different HIV-1 strains. The V3 loop of the HIV-1 external glycoprotein gp120 contains the principal neutralizing domain (PND) and has been extensively used as a model for synthetic peptide vaccines against AIDS [13,14].

In the present work we studied the immunogenicity of a novel version of MAP containing a 2:1 B-cell:T-cell epitope ratio. The B- and T-cell epitopes were linked by a cathepsin-like enzyme cleavage site. The T-cell epitope was placed contiguous to the lysine core. Four- and eight-V3-branched homodimeric MAPs were constructed. The sequence from the central V3 region of HIV-1 JY1 isolate was selected as the B-cell epitope, while the region comprising amino acids 830–843 of the tetanus toxoid constituted the T-cell helper epitope (Th).

The chimeric protein TAB9 is an MEP containing six copies of the central region of the V3 loop from HIV-1 isolates LR150, JY1, RF, MN, BRVA and IIIB. The immunogenicity of this chimeric protein was compared with two different hexavalent combinations of V3-MAP8.

MATERIALS AND METHODS

Materials

Boc-protected amino acids used for the synthesis of the MAP were purchased from Bachem (Switzerland). The 4-methylbenzhydrylamine (MBHA) resin was from Fluka and had a substitution level of 0.1 mmol/g. Solvents were obtained from Merck (Germany).

Synthesis of MAP

The synthesis of MAP was accomplished manually by a stepwise solid-phase procedure [15], using *tert*-butoxicarbonyl/benzyl chemistry. The protecting group scheme for synthesis was as follows: the Boc group for the α-NH_2 terminus and side-chain protection groups for the trifunctional amino acids were: 4-methoxybenzyl (Mob) for Cys, benzyl (Bzl) for Ser and Thr, benzyl ester (OBzl) for Asp and Glu, tosyl (Tos) for Arg, benzyloxymethyl (Bom) for His, dichlorobenzyl (Cl_2-Bzl) for Tyr and chlorobenzyloxycarbonyl (Cl-Z) for Lys. The lysine core was assembled using Boc-Lys(Boc) via TBTU. Cleavage of the Boc group was carried out with 40% TFA in DCM. The TFA salt was neutralized by 5% DIPEA in DCM. The coupling reactions were mediated with DIPC/HOBT in DMF and monitored by the qualitative ninhydrin test [16]. Double couplings with tenfold excess of Boc-amino acids were carried out in most cases. The capping step was performed with 4% acetic anhydride and 1% DIPEA in DMF. After the last coupling cycle, each MAP–resin system was cleaved from the resin with HF and the corresponding mixture. The MAPs were extracted into 8 M urea and 100 mM Tris–HCl buffer (pH 8.0). The dimerizations by disulfide bonds of the monomer MAP were carried out, at basic pH, directly in the extraction buffer. The reaction was followed by Ellman's test [17]. The homodimer MAPs were then dialyzed in 2 M urea and 100 mM Tris–HCl buffer, pH 8.0 for 24 h. Finally, the MAPs were dialyzed in 1 M HOAc, to remove all the urea, and were then lyophilized.

Dimerization of Heterodimeric V3-MAP8

Equivalent amounts (1 μmol) of the six individual homodimeric V3-MAP8 were mixed and reduced using 10 mM DTT, 6 mol/l guanidium chloride and 100 mM Tris–HCl at pH 8.5 for 2 h at 37°C. Next, the monomer MAP mixtures were dialyzed in 6 mol/l guanidium chloride and 100 mM Tris–HCl buffer, pH 8.0, to remove all the DTT. The dimers were then formed randomly by oxidizing to disulfide bonds during dialysis against 3 mol/l guanidium chloride and 100 mM Tris–HCl buffer, pH 8.0–8.5. The reaction was also followed by Ellman's test. Finally, the heterodimeric V3-MAP8s were dialyzed in 1 M HOAc and lyophilized.

Chromatography

The MAPs were analyzed by reversed-phase, high-performance liquid chromatography (RP-HPLC) on a

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Pharmacia LKB (Sweden) dual pump chromatograph model 2150, coupled with an UV detector at 226 nm (Knauer Variable Wavelength Monitor). A C-8 wide-pore column (100 mm × 4.6 mm, Baker, USA) was used with a linear gradient (10–70% B in 40 min) of acetonitrile (mobile phase B) in water (mobile phase A), containing 0.05% and 0.1% trifluoroacetic acid, respectively, at a flow of 0.8 ml/min. Data were processed by the BioCrom program (CIGB, Cuba).

Reduction and *S*-Carboxymethylation

The lyophilized homodimeric MAPs were dissolved in an adequate volume of the reduction and carboxymethylation buffer (300 mM Tris–HCl, (pH 8.5), 6 mol/l guanidium chloride and 3 mM EDTA) at a concentration of 5 mg/ml. A twentyfold molar excess of DTT over the number of cystein residues was added, the solution was flushed with nitrogen and incubated for 2 h at 37°C. A twofold 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 further purified by RP-HPLC as described above.

Amino Acid Analysis

Samples of *S*-carboxymethylated MAP were hydrolyzed for 24 h in vacuum-sealed ampoules, with 6 mol/l HCl containing 0.1% phenol and 0.1% 2-mercaptoethanol. After evaporation, free amino acids were dissolved in the adequate buffer and loaded onto an automatic amino acid analyzer, Alpha Plus 4151 (Pharmacia-LKB, Sweden), using a sodium buffer system for amino acid separation and *ortho*-phthalaldehyde derivatization for fluorescence detection. All samples were analyzed in three replicates.

Design of Four and Eight-JY1-Branched Homodimer MAP, JY1-MAP4 and JY1-MAP8

Two homodimeric JY1-MAP variants were used for a preliminary immunogenicity experiment. These variants differed in the number of B- and T-cell epitopes. The JY1-MAP4 molecule consists of four B- and two Th-epitopes (Figure 1A), while the JY1-MAP8 carried eight and four epitopes, respectively (Figure 1B). The B-cell epitope comprises the central 15 amino acids (317–331) from the V3 loop of the gp120 HIV-1 JY1 isolate [18]. The Th epitope comprises the region 830–843 of tetanus toxoid [19]. B- and Th-epitopes were linked by a Lys-Lys cathepsin-like cleavage site [20].

Design of V3-Branched MAP Mixtures

Design of homodimeric V3-MAP8. The dimerization reaction was carried out using monomers with the same V3 sequence. After this, equivalent amounts of the six individual dimers were mixed (non-covalent).

Design of heterodimeric V3-MAP8. The dimers were formed randomly, starting from an equivalent mixture of six different monomers presented in the MEP TAB9.

TAB9 is a recombinant MEP, including the V3 region from six divergent HIV-1 isolates LR150, JY1, RF, MN, BRVA and IIIB, in this order; fused to the

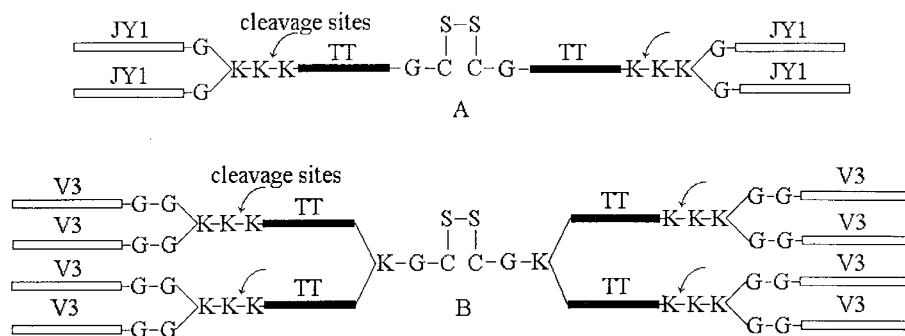

Figure 1 JY1-MAP4 and V3-MAP8 variants. Both structures include two monomers linked through a disulfide bond and with 2:1 epitope ratio. A cathepsin-like enzyme cleavage site (-KK-) was inserted between B- and T-cell epitopes. TT stand for tetanus toxoid. Six different V3-MAP8 constructions bearing divergent V3 HIV-1 sequences LR150, JY1, RF, MN, BRVA and IIIB as B-cell epitopes were synthesized. Monomers were properly assembled for heterodimeric MAP combinations.

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amino terminal (47 amino acids) from P64K protein of *N. meningitidis* [21].

Immunization Protocols

Groups of nine female Balb/c mice (8 weeks old) were inoculated with equimolar amounts of B-cell epitopes.

Comparison of JY1-MAP4 and JY1-MAP8. Balb/c mice were inoculated on day 0 by subcutaneous injection of JY1-MAP4 (17.2 μg) or JY1-MAP8 (16.4 μg) variants (Buffer CH_3COONa, 10 mM) emulsified in a final volume of 0.1 ml in complete Freund's adjuvant (CFA). Incomplete Freund's adjuvant (IFA; Sigma, USA) was used on days 15 and 40. Animals were bled on day 50.

Comparison of homodimeric and heterodimeric V3-MAP8 mixtures and TAB9. Three groups of Balb/c mice were injected subcutaneously with antigen (100 μg) on days 0, 14, 28 and 56 using CFA/IFA. Group B ($n = 9$) was inoculated with a cocktail of homodimeric V3-MAP; group C ($n = 9$) with a mixture of heterodimeric V3-MAP and group A ($n = 8$) with TAB9. The mice were bled on days 40 and 67.

V3 Peptide ELISA

V3 peptide ELISA was performed as described elsewhere [22]. Briefly, 96-well polystyrene microplates (High binding, Costar), were coated with BSA-coupled V3 peptides (100 μl at 10 μg/ml), and incubated for 3 h at 37°C. To evaluate the response against JY1-MAP8 and JY1-MAP4, JY1 or TT peptides conjugated to BSA were used. To compare the response of mice immunized with MAP cocktails and TAB9, all six BSA-conjugated V3 peptides were employed. After three washings with 0.05% Tween 20 in distilled water, plates were blocked with 0.5% BSA in PBS (blocking solution) for 1 h at 37°C. Twofold serial dilutions of sera starting from 1:100 in 0.05% Tween 20, and 5% sheep serum in blocking solution, were added. After three washings, the reaction with an anti-mouse peroxidase conjugate was developed for 1 h at 37°C, with OPD substrate as previously described. Cut-off values were calculated for each plate, at twice the absorbance value obtained for a pool of pre-immune mice sera. The last dilution that gave absorbance values higher than the cut-off was regarded as the serum titer.

Table 1 Amino Acid Sequences of the V3-HIV-1 Isolates

Peptide	Sequence
JY1	RQSTPIGLGQALYTT
BRVA	RKRITMGPGRVYYTT
IIIB	SIRIQRGPGRAFVTI
MN	RKRIHIGPGRAFYTT
LR150	SRGIRIGPGRAILAT
RF	RKSITKGPGRVIYAT
TT	QYIKANSKFIGITE

RESULTS AND DISCUSSION

Synthesis and Characterization of the MAP

Six peptides from the V3 regions of isolates LR150, JY1, RF, MN, BRVA and IIIB were used as B-cell epitopes, and tetanus toxoid as the Th-cell epitope (Table 1). The assembly of the MAP construction containing B- and T-cell epitopes placed in tandem was achieved using solid-phase synthesis. The coupling reaction of the protected amino acid was verified using the ninhydrin test according to the Kaiser procedure. The cysteine residue was added during the synthesis in order to allow the respective MAP to be dimerized via disulfide bonds. The degree of purity of MAP, assessed by HPLC, was at least 85%. The main peaks from seven MAPs were separated by RP-HPLC and their amino acid compositions were determined after 24 h of acid hydrolysis. Amino acid analysis of three samples of each MAP showed they had the expected composition. A summary of these results is given in Table 2.

Humoral Response in Mice Immunized with JY1-MAP4 and JY1-MAP8

In this study, we examined the characteristics of the humoral response induced by chemically-defined synthetic polymers, MAP, containing B- and T-epitopes. Earlier studies showed that the inclusion of a Th-cell epitope is necessary to raise antibodies against synthetic peptides. For instance, an antibody response against the B-epitope of the *P. Berghei* CS protein can be induced after immunization with MAP containing T- and B-epitopes (BT4), but not with MAP containing only the B-epitope (B4) [23]. Furthermore, the V3 region of HIV-1 has shown poor immunogenicity in mice, but the inclusion of a conserved Th-epitope in V3 loop MAP has enhanced the antibody response against non-immunogenic V3 in mice [24].

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In this report, the immunogenicity of two MAPs with different B-cell epitope densities was compared. These molecules were assembled using a 2:1 epitopic ratio of B- (JY1) and Th-cell (Tetanus toxoid) epitopes, and administered to Balb/c mice in FCA. The antibody responses elicited by these MAPs were titered in ELISA against BSA-JY1 and BSA-TT conjugates. Both antigens were highly immunogenic and generated specific antibodies against the JY1 epitope. The JY1-MAP8 variant elicited higher antibody titers, fifteenfold more strongly, than JY1-MAP4 after three doses ($p < 0.05$). This result is compatible with previous experiments in which octameric constructions (high B-epitope density per molecule) were more immunogenic than monomeric peptides or peptides conjugated to a protein carrier (low B-epitope density) [25]. In contrast, other authors using MAP synthesized on a polylysine core did not observe an increase in the immunogenicity when the number of branches were raised from four to eight [26].

The frequency of antibody responses against the Th-epitope did not differ between variants after four inoculations: 6/9 for JY1-MAP4 and 5/9 for JY1-MAP8. Additionally, OD values were also similar for both MAPs, which indicates that the antibody response against the Th-epitope was independent of the MAP structure. On the other hand, the levels of antibodies against the B-cell epitope were remarkably higher than those generated against the T-epitope (data not shown). Finally, suppression against the B-cell epitope was not observed after repeated boosters with the MAP [23].

Humoral Response in Mice Immunized with TAB9 or with Two Different Hexavalent V3-MAP8 Combinations

Since the JY1-MAP8 structure was the best elicitor of specific antibodies against the JY1 B-cell epitope, we synthesized another five V3-MAP8. They were combined in two ways. The homodimeric combination consisted of an equimolar mixture (non-covalent) of six different homodimeric V3-MAP8, where each molecule was a dimer of two identical monomers linked by a disulfide bond. The heterodimeric combination also consisted of an equimolar hexavalent mixture, but dimers contained different monomers (Figure 1B).

After three immunizations, all sera were positive at 1:100 dilution versus the cognate antigen (data not shown). To study the recognition of the V3 peptides, a pool of sera from each group was tested. The heterodimeric mixture (group C) showed higher absorbance values than the other two groups (Figure 2).

After four immunizations, sera from all groups were individually assayed for their reactivity to V3 peptides. A strong immuno-dominance towards JY1

Table 2 Amino Acid Composition of the MAP

Amino acid	JY1-MAP4	JY1-MAP8	BRVA-MAP8	IIIB-MAP8	MN-MAP8	LR150-MAP8	RF-MAP8
Asx	1.1 (1)	2.5 (2)	3.1 (2)	2.6 (2)	2.9 (2)	2.7 (2)	2.8 (2)
Thr	6.4 (7)	11.7 (14)	12.2 (14)	5.8 (6)	9.2(10)	5.8 (6)	8.6 (10)
Ser	2.3 (3)	5.6 (6)	3.3 (2)	4.6 (6)	2.8 (2)	4.8 (6)	5.8 (6)
Glx	6.7 (6)	12.2 (12)	6.2 (4)	8.9 (8)	5.6 (4)	5.6 (4)	5.6 (4)
Gly	8.7 (8)	16.3 (19)	17.4 (19)	20.6 (19)	21.3 (19)	23.7 (23)	20.8(19)
Ala	2.7 (3)	5.4 (6)	2.4 (2)	5.5 (6)	6.4 (6)	8.9(10)	5.8 (6)
Val	–	–	3.6 (4)	4.0 (4)	–	–	3.5 (4)
Met	–	–	0.6 (4)	–	–	–	–
Ile	5.2 (5)	9.7 (10)	10.6 (10)	19.0 (18)	15.4 (14)	18.3 (18)	14.3(14)
Leu	4.1 (4)	7.8 (8)	–	–	–	3.4 (4)	–
Tyr	3.3 (3)	5.9 (6)	10.5 (10)	2.0 (2)	5.2 (6)	2.2 (2)	6.3 (6)
Phe	0.9 (1)	3.1 (2)	2.2 (2)	6.2 (6)	5.5 (6)	2.9 (2)	2.8 (2)
His	–	–	–	–	2.0 (4)	–	–
Lys	6.2 (6)	13.7 (11)	13.9 (15)	13.6 (11)	15.7 (15)	13.7 (11)	21.1(19)
Arg	2.1 (2)	4.6 (4)	10.0 (12)	10.9 (12)	10.6 (12)	10.7 (12)	7.1 (8)
L-Cys	1.1 (1)	1.1 (1)	0.9 (1)	0.8 (1)	1.0 (1)	0.7 (1)	0.9 (1)

Data is expressed as number of residues per molecule. Experimental values represent the mean of three independent determinations. The expected values calculated from MAPs sequences are presented in parentheses.
Asx, aspartic acid plus asparagine; Glx, glutamic acid plus glutamine.
Proline is not detected under these conditions.

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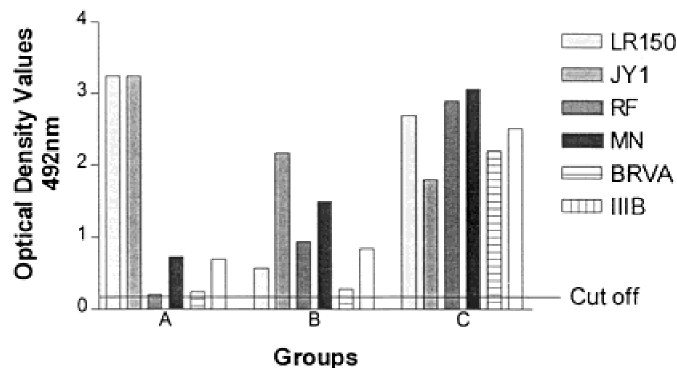

Figure 2 Reactivity against V3 peptides. After three inoculations, sera of each group were pooled and samples were duplicated for analysis. Group A received TAB9 protein, group B received homodimeric mixture, and group C received heterodimeric MAP. Plates were coated with BSA-V3 conjugated as specified in the text. Optical densities are expressed as the mean values.

and LR150 epitopes in mice immunized with TAB9 was evident. MN was only weakly recognized by three sera with OD values very close to the cut-off and the other three peptides (RF, BRVA and IIIB) were not recognized at the dilution assayed. In contrast, mice immunized with the heterodimeric MAP mixture showed a more uniform pattern of reactivity against all V3 epitopes. Finally, antibodies induced by homodimeric MAP showed an intermediate pattern, not as narrow as TAB9, but not as wide as the heterodimeric MAP mixture (Figure 3).

The best frequency of response was observed in mice immunized with the heterodimeric mixture (group C). This group differed statistically ($p < 0.05$) from mice immunized with the homodimeric mixture (group B), but not from those that received TAB9 protein (group A) (Figure 3).

The main problem posed by immunization with the highly variable V3 region of HIV-1 is that the resulting immunity is subtype specific. The MAP approach has the potentiality to overcome this isolate-restricted antibody response. Previous work has shown how a non-covalent hexavalent mixture of octameric V3 peptides accelerated the kinetics for development of a broadened neutralizing activity response in guinea pigs [27]. Other authors have shown that antibodies against multiple-chain peptides recognize antigenic determinants different from those present on the monomeric peptides coupled to a carrier protein [25].

The results described in this paper demonstrate that the heterodimeric V3-MAP8 mixture administered to mice induced a broader range of antibodies than the homodimeric variant. This suggests that V3 sequences presented in an heterodimeric context activate B-cell clones with a higher cross-reactive potential than those in the homodimeric context. The two V3-MAP8 mixtures were also able to modify the immuno-dominance of the JY1 and LR150 sequences seen in the TAB9 context. It has been shown that neither the epitope order nor the mice's genetic background have any influence in the immuno-dominant pattern of the V3 regions in TAB9 protein [unpublished results]. Interestingly, the immuno-dominance observed for JY1 and LR150 epitopes in TAB9 in mice has not been observed in rabbits and macaques [accepted for publication]. Our observations emphasize the importance of the form in which V3 epitopes are presented in the characteristics of the antibodies generated.

CONCLUSIONS

Up to now, the strategy of MEP has been used to induce antibody-mediated responses against HIV [11]. For this purpose, the MAP has been widely used against diverse diseases such as hepatitis B virus [5], human malaria [28], blue tongue virus [29], and HIV-1 [8]. The findings of those studies and those described in this paper indicate that MAP are highly immunogenic and might provide a good framework to develop broad recognition of V3 peptides. Thus, MAP are good candidates for the development of vaccines against microbial diseases in which circulating antibodies play a protective role.

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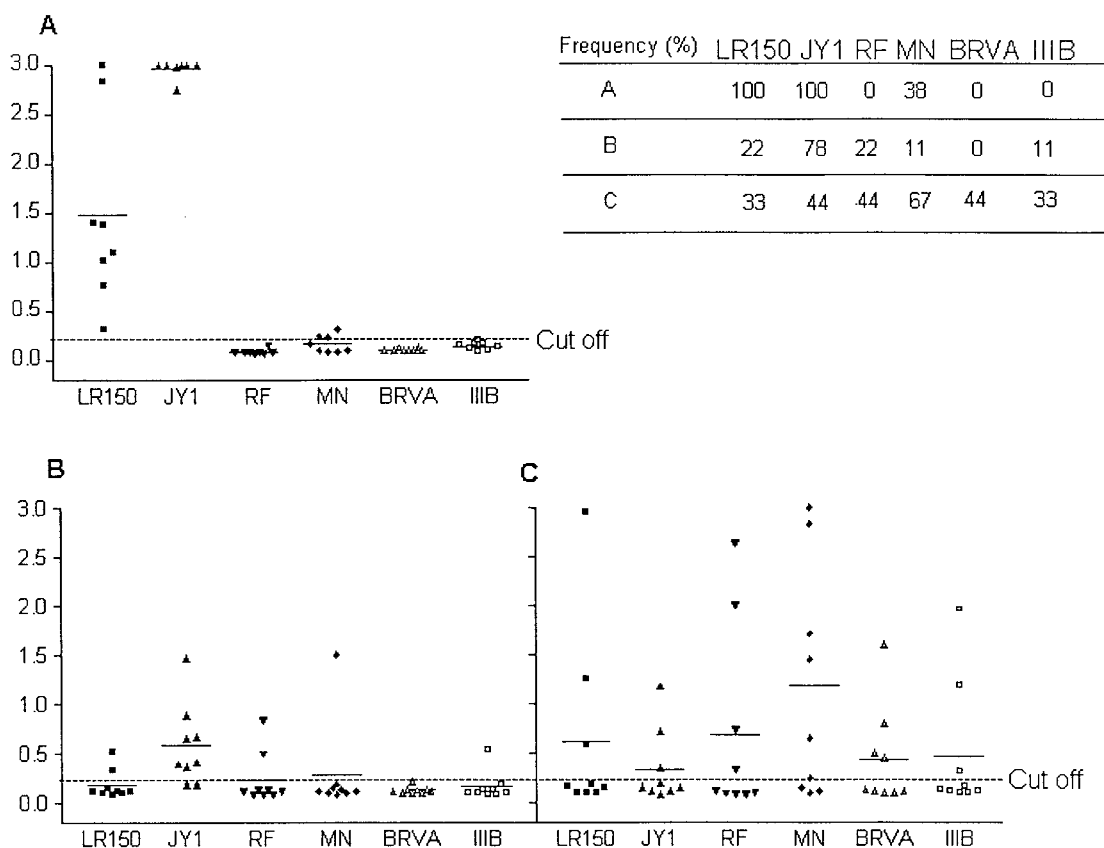

Frequency (%)	LR150	JY1	RF	MN	BRVA	IIIB
A	100	100	0	38	0	0
B	22	78	22	11	0	11
C	33	44	44	67	44	33

Figure 3 Reactivity against V3 peptides and frequency of reactivity. After four doses, sera of mice were individually assessed (1/100) for the recognition of V3 peptides. A: group immunized with TAB9; B: with the homodimeric combination; and C: received the heterodimeric mixture. The table shows the frequency of reactivity after four inoculations, values are expressed as percent of recognition of BSA-V3 conjugated.

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REFERENCES

1. Schutze MP, Leclerc C, Jolivet M, Audibert F, Chedid L. Carrier induced epitopic suppression a major issue for the future synthetic vaccines. *J. Immunol.* 1985; **135**: 2319–2322.
2. Liu F-T, Zinnecker M, Hamaoka T, Katz DH. New procedures for preparation and isolation of conjugates of proteins and synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. *Biochemistry* 1979; **18**: 690–697.
3. Tam JP. Synthetic peptide vaccine design: synthetic and properties of a high-density multiple antigenic peptide system. *Proc. Natl. Acad. Sci.* 1988; **86**: 5409–5413.
4. Tam JP. High-density multiple antigen-peptide system for preparation of antipeptide antibodies. *Methods Enzymol.* 1989; **168**: 7–15.
5. Tam JP, Lu YA. Vaccine engineering: enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T- and B-cell epitopes. *Proc. Natl. Acad. Sci.* 1989; **86**: 9084–9088.
6. Tam JP, Clavijo P, Lu YA, Nussenzweig V, Zavala P. Incorporation of T and B epitopes of the circumsporozoite protein in a chemically defined synthetic vaccine against malaria. *J. Exp. Med.* 1990; **171**: 299–306.
7. Wang R, Charoenvit Y, Corradin G, Porrozzi R, Hunter RL, Glenn G, Alving CR, Church P, Hoffman SL. Induction of protective polyclonal antibodies by immunization with a *Plasmodiumyoelii* circumsporozoite protein multiple antigen peptide vaccine. *J. Immunol.* 1995; **154**: 2784–2793.
8. Ahlers JD, Dunlop N, Pendleton CD, Newman M, Nara PL, Berzofsky JA. Candidate HIV type 1 multi-determinant cluster peptide-P18MN vaccine constructs elicit type 1 helper T-cells, cytotoxic T-cells, and

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neutralizing antibody, all using the same adjuvant immunization. *AIDS Res. Human Retroviruses* 1996; **12**: 259–272.
9. Francis MJ, Hastings GZ, Brown F, McDermed J, Lu YA, Tam JP. Immunological evaluation of the multiple antigen peptide (MAP) system using the major immunogenic site of foot-and-mouth disease virus. *Immunology* 1991; **73**: 249–254.
10. Volpina OM, Yarov AV, Zhmak MN, Kuprianova MA, Chepurkin AV, Toloknov AS, Ivanov VT. Synthetic vaccine against foot-and-mouth disease based on a palmitoyl derivative of the VP_1 protein 135–159 fragment of the A_{22} virus strain. *Vaccine* 1996; **14**: 1375–1380.
11. Duarte CA, Montero M, Seralena A, Valdés R, Jiménez V, Benítez J, Narciandi E, Madrazo J, Padrón G, Sánchez G, Gjilliam G, Pearson C, Domínguez MC, Wharen B, Menéndez A. Multiepitope polypeptide of the HIV-1 envelope induces monoclonal antibodies against V3 loop. *AIDS Res. Human Retroviruses* 1994; **10**: 235–243.
12. Montero M, Menéndez A, Domínguez MC, Navea L, Vilarubia OL, Quintana D, Izquierdo M, Jiménez V, Reyes O, Lobaina L, Noa E, Duarte CA. Broadly reactive antibodies against a gp120 V3-loop Multi-Epitope Polypeptide neutralize different isolates of Human Immunodeficiency Virus type 1 (HIV-1). *Vaccine* 1997; **15**: 1200–1208.
13. Javaherian K, Langlois AJ, McDanal C, Ross K, Eckler LI, Jellis CL, Profy AT, Rusche JR, Bolognesi DP, Putney SD, Matthews TJ. Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *Proc. Natl. Acad. Sci.* 1989; **86**: 6768–6772.
14. Moore J, Nara P. The role of the V3 of gp120 in HIV infection. *AIDS* 1991; **5**: 521–533.
15. Houghten RA, Graw ST, Bray MK. Simultaneous multiple peptide synthesis: the rapid preparation of large numbers of discrete peptides for biological, immunological and methodological studies. *BioTechniques* 1996; **4**: 522–526.
16. Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in solid-phase synthesis of peptides. *Anal. Biochem.* 1970; **34**: 595–598.
17. Beales D, Finch R, McLean AE, Smith M, Wilson ID. Determination of penicillamine and other thiols by combined high-performance liquid chromatography and post-column reaction with Ellman's reagent: application to human urine. *J. Chromatogr.* 1981; **226**: 498–503.
18. Yourno J, Josephs SF, Reitz M, Zagury D, Wong-Staal F, Gallo RC. Nucleotide sequences analysis of the env gene of a new Zairian isolate of HIV-1. *AIDS Res. Human Retroviruses* 1988; **4**: 165–173.
19. Panina-Bordingnon P, Tan G, Termijtelen A, Demotz S, Corradin G, Lanzavecchia A. Universally immunogenic T-cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T-cells. *Eur. J. Immunol.* 1989; **19**: 2237–2247.
20. Borras-Cuest F, Fedon Y, Pellit-Camordan A. Enhancement of peptide immunogenicity by linear polymerization. *Eur. J. Immunol.* 1988; **18**: 199–202.
21. Silva RR, Sosa MSH, Nieto GG, Martinez LSH, Mas JRF, Pérez LIN, Inés L, Morales JG, Morera V, González S, Santos B, Menéndez E, Acosta A, Couzeau E, Cruz S, Musacchio A. Nucleotide sequence coding for an outer membrane protein from *Neisseria meningitidis* and its use in vaccine preparations. European Patent Application, EP 0474 313 A2, Erhard-strasse 27, D-8000, München 2, Germany, 1992.
22. Gomez CE, Lopez-Campistrous AE, Duarte CA. An immunoassay with bovine serum albumin coupled peptides for the improved detection of anti V3 antibodies in HIV-1 positive human sera. *J. Virol. Methods* 1998; **71**: 7–16.
23. Romero PJ, Tam JP, Schlesinger D, Clavijo P, Gibson H, Barr PJ, Nussenzweig RS, Zavala F. Multiple T-helper cell epitopes of the circumsporozoite protein of *Plasmodium berghei*. *Eur. J. Immunol.* 1988; **18**: 1951–1957.
24. Nardelli B, Lu YA, Shiu DR, Delpierre-Defoort C, Profy AT, Tam JP. A chemically defined synthetic vaccine model for HIV-1. *J. Immunol.* 1992; **148**: 914–920.
25. Chai SK, Clavijo P, Tam JP, Zavala F. Immunogenic properties of multiple antigen peptide systems containing defined T and B epitopes. *J. Immunol.* 1992; **149**: 2385–2390.
26. Kelder HC, Schlesinger D, Valentine FT. Immunogenic and antigenic properties of an HIV-1 gp120-derived multiple-chain peptides. *J. Immunol.* 1994; **152**: 4139–4148.
27. Wang CY, Looney DJ, Li ML, Walfield AM, Ye J, Hosen B, Tam JP, Wong-Staal F. Long-term high-titer neutralizing activity induced by octameric synthetic HIV-1 antigen. *Science* 1991; **254**: 285–288.
28. Collins WE, Sullivan JS, Morris CL, Galland GG, Jue DL, Fang S, Wohlhueter R, Reed RC, Yang C, Hunter RL, Lal AA. Protective immunity induced in squirrel monkeys with a multiple-antigen construct against the circumsporozoite protein of *Plasmodium vivax*. *Am. J. Trop. Med. Hyg.* 1997; **56**: 200–210.
29. Li JKK, Yang YY. Mapping of two immunodominant antigenic epitopes conserved among the major inner capsid protein, VP7 of five blue-tongue viruses. *Virology* 1990; **178**: 552–559.

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