A CHEMICALLY DEFINED SYNTHETIC VACCINE MODEL FOR HIV-1

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Multiple Ag peptide (MAP) system without the use of a protein carrier was used as a vaccine model in three species of animals. Synthetic peptides from the V3 region of the gp120 of IIIB, RF and MN HIV-1 isolates were used as the Ag. MAP consisting of various chain lengths, from 11 to 24 residues, were prepared in a monopeptid configuration containing four repeats of each individual peptide. In parallel, they were synthesized in a diepitope configuration adding at the carboxyl-terminus of the V3 peptides a conserved sequence, known to be a Th cell epitope of gp120. The antibody response elicited by the monopeptide constructs was species-dependent. Rabbits produced immunity against all nine peptides, whereas mice were strongly reactive mainly to the longest sequence of the IIIB isolate. The immune response of guinea pigs was intermediate to those of rabbits and mice. Diepitope MAPs were immunogenic in all three species and elicited significantly higher titers than those raised by the immunization with the monopeptide MAPs. The response was type specific: the high-titered antibodies were reactive mostly against the isolate from which the peptides were derived, with a small cross-reactivity in ELISA between IIIB and RF strains. The dominant antigenic site of the B cell epitope, IIIB sequence, was located at the amino and central part of the MAP and a sequence overlapping the putative V3 reverse-turn was particularly reactive with the raised antibodies. Moreover, sera from the immunized animals inhibited virus-dependent cell fusion. These results show that MAP, with a chemically defined structure and without the use of a protein carrier, can be potentially useful for the design of synthetic HIV-1 vaccine candidates.

Many methodologic variations have been developed for the conjugation of synthetic peptide Ag to protein carriers to enhance the low immunogenicity of the peptides. Although these methods are useful to generate site-specific antibodies to be used as experimental reagents (1), they have limitations when used to elicit immune responses for the purpose of vaccination. The peptide Ag represents a small portion of the peptide-Ag conjugate, thus the humoral immunity would be directed mostly against other antigenic sites of the complex. The molecular excess of the carrier may suppress the B cell response to the epitope of interest (2). Furthermore, the coupling to the carrier may radically alter immunogenic determinants of the peptide (3).

MAP3 systems for peptide-based vaccines were designed to overcome such limitations. MAP have chemically defined structures and are obtained directly from the solid-phase synthesis of a peptide Ag onto an oligomeric branching lysine core (4). At the surface, the macromolecule presents multiple clusters of the antigenic epitopes and, at the center, a small lysine core matrix that represents only a minimal portion of the total structure. As a result, the core matrix is not immunogenic (4). MAP have been used to generate immune sera that recognize specifically the native proteins and are characterized by titers considerably higher than the sera prepared from the same peptides anchored covalently to keyhole limpet hemocyanin as carrier (5). They have been used in the preparation of experimental vaccines against hepatitis (6), malaria (7), and foot-and-mouth disease (8). This approach has several advantages. MAP are chemically unambiguous, i.e., the exact structure of the antigenic epitope is known. They can be made by the attachment of different antigenic peptides on the same macromolecule, thus allowing to study the effect that the arrangement and the stoichiometry of the multiple epitopes have on the immunogenicity of the whole complex.

In this report, we evaluate the antibody response elicited in different animal species by MAP corresponding to sequences from the V3 region of the envelope glycoprotein gp120 of the HIV-1. Although several virion proteins, such as core protein p17, reverse transcriptase and envelope, elicit antibodies (9–11), the most effective epitope has been mapped to the V3 region of gp120 (12–14). It encompasses aa residues 296–331 (IIIB isolate) and is centered in a disulfide loop formed by two invariant cysteines (15) containing a relatively conserved GPG sequence, flanked by hypervariable aa (16). It has been demonstrated that mutations in this domain generate variants resistant to neutralizing antibodies (17) and with reduced ability to induce cell fusion (18). Mother-to-infant transmission of HIV-1 is found to correlate with the absence of anti-V3 loop antibodies in infected women (19). Furthermore, an Immunodominant cytotoxic T cell epitope has been identified on V3 (20).

The MAP used in this study were synthesized to present multiple copies of the principal neutralizing determinant of divergent HIV-1 isolates (IIIB, RF, and MN). These B

Received for publication July 30, 1991.
Accepted for publication November 4, 1991.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 This work was supported by United States Public Health Service Grant AI-28791.
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3 Abbreviation used in this paper: MAP, multiple Ag peptide; aa, amino acid.
cell peptides were comprised of various chain lengths, spanning from 11 to 24 residues, to define the minimum immunogenic determinant. In addition, the immunogenicity of dipeptide MAP, consisting of tandemly linked B and Th cell epitopes, were evaluated.

MATERIALS AND METHODS

Animals

Outbred CD-1 mice and NZW rabbits were purchased from Charles River, Wilmington, MA. Outbred Dunkin-Hartley guinea pigs were raised and immunized by Hazelleon Biotechnologies Company, Denver, PA.

Synthesis of peptides

Synthetic peptides were prepared manually by a stepwise solid-phase peptide synthesis [5] on t-butyloxycarbonyl (Boc)-Ala-OCH_3 resin or p-alkoxybenzyl alcohol resin. The monoepitope peptides were synthesized by Fmoc-tertbutyl chemistry. The dipeptide epitopes were synthesized with Fmoc-tertbutyl chemistry. The coupling was mediated with dicyclohexycarbodiimide/1-hydroxybenzotriazole in dimethylformamide. After completion of synthesis, each MAP-resin was treated with a deprotecting reagent to remove Nα-protecting groups and cleaved with low-high fluoride (Boc-chemistry) or 95% trifluoroacetic acid (Fmoc-chemistry). The peptides were extracted in 8 M urea in 0.1 M Tris/HCl, pH 8, then dialyzed several times and lyophilized. The purity of the products was confirmed by aa analysis.

Immunization procedure

The animals were injected four times, in 2-wk intervals, using CFA (Sigma Chemical Co., St. Louis, MO) for the first immunization, and IFA for the booster injections. CD-1 mice (five for each peptide) received i.p. 50 μg of the peptides each immunization. Rabbits (two for each peptide) were injected intradermally with 400 μg (first immunization) and 200 μg (second immunization) and subsequently intramuscularly with 200 μg of the peptides. Guinea pigs (three for each group) received s.c. 100 μg of the peptides the first and second immunization and 50 μg the last two boosts. Mice and rabbits were immunized with all the MAP peptides synthesized, guinea pigs with the nine monoepitopes and the dipeptides from IIIB and RF isolates. The animals were bled immediately before each immunization. The sera were used for the reported experiments were obtained 15 days after the last boost.

Antibody solid-phase immunoassays

ELISA. Mouse and rabbit antisera were analyzed by standard direct ELISA using flat-bottomed microplates (Maxisorp, Nunc, Denmark) coated with 5 μg/well of each peptide or 0.1 μg/well of purified recombinant gp120 from IIIb isolate (Repligen, Cambridge, MA). The plates were blocked for 90 min at 37°C with the diluent buffer (PBS + 1% calf serum). The incubation with the antisera, serially diluted in the same buffer, was carried out for 2 h at 37°C and was followed by three washes with 0.05% Tween 20 in PBS. Phosphatase conjugate goat secondary antibody (Sigma), diluted 1/1000, was then added for 2 h at 37°C. After additional three washes, the substrate p-nitrophenyl phosphate (1 mg/ml, Sigma), in diethanolamine buffer pH 9.8, was added and the bound secondary antibody was detected at 405 nm. A different procedure was used for analyzing guinea pig antisera. Plates were coated with V3 synthetic peptides RP135 (IIIB), RP 139 (RF), or RP 142 (MN) [12]. For detection, either protein A- or goat antibody anti-guinea pig horseradish peroxidase conjugate was used with a substrate solution of 2.2’-azinobis(3-ethylbenzthiazoline sulfonic acid) and hydrogen peroxide; absorbance was read at 405 nm. The antibody titer was calculated as the reciprocal of the serum dilution at which the absorbance produced by the immune serum is 0.3. The OD obtained with preimmunization sera was always lower than 0.1 U.

PEPSCAN. The Pepscan was performed as described [21]. Briefly, overlapping eight-residue long peptides corresponding to the B1 sequence (IIIb isolate) were synthesized on the tip of polyethylene rods. The rods were assembled into a holder with the format of a microtiter plate. All the subsequent reactions were carried out at the tip of the rods using a microtiter plate. Nonspecific binding was avoided by the incubation for 1 h at room temperature with the diluent buffer. The rods were then incubated with 1/5000 dilution of the antisera for 1 h at 4°C, washed four times in 0.05% Tween 20 in PBS and incubated with 1/1000 dilution of goat antirabbit IgG coupled to alkaline phosphatase for 1 h at room temperature. The presence of the conjugate antibody on the tips, after a further washing procedure, was detected by the reaction with the p-nitrophenyl phosphate solution described above.

Assay for Proliferative Response of Ag-Specific T Cells

BALB/c mice were immunized at the base of the tail with 50 μg of MAP in 50 μl of CFA emulsion. Ten days later the draining lymph node cells were removed and cultured for 4 days in microtiter at 2 x 10^6 cells/well in 0.2 ml (final volume) of RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 5 x 10^-5 M 2-ME, and antibiotics. Various concentrations of the Ag to be tested were added to the wells. The optimal proliferation occurred at 10 μg/ml (final concentration). The cells were pulsed for the last 18 h of incubation with 1 μCi/well of [3H]Tdr (NEN, Boston, MA) and then harvested. The proliferative response was calculated as stimulation index ratio of experimental cpm mean to control cpm mean without Ag.

Synctiyta Inhibition Assay

CD4+ CEM cells (American Type Culture Collection, Rockville, MD) were infected with recombinant vaccinia viruses expressing HIV-1 gp160, at a multiplicity of infection of 1. The viruses expressing IIIb and RF envelope genes were provided by Dr. Bernard Moss (NIH). The recombinant virus expressing the V3 region of HIV_m, was constructed as described (22). The tested immune sera were added to the cultures 1 h postinfection and syncytia were counted 24 h postinfection. The fusion inhibition titer for an immune serum was defined as the reciprocal of the dilution reducing the number of syncytia to 10% of the number obtained in the presence of a normal serum control.

RESULTS

Antibody Response to Monoepitope MAP

Nine peptides, each individually synthesized in MAP format, from the V3 regions of isolates IIIb, RF and MN were used as B cell epitopes. As shown in Figure 1, parallel groups of three peptides were made for each isolate adding increasing fewer aa residues to both sides of the central tripeptide (GPG). MAP was used in a tetravalent configuration because it has been shown previously to be as effective as the octavalent configuration...
Mice, rabbits, and guinea pigs were immunized with the three groups, each consisting of three monoepitope MAP. The antisera were analyzed for antipeptide reactivity in an ELISA assay and different immune responses were observed in these three species.

In mice, the degree of immune response correlated closely to the length of the IIIB peptide used for the immunization (Fig. 2). The longest B1 peptide (aa 308-331) induced the strongest response, followed by the intermediate B2 peptide (aa 312-328), and the shortest B3 peptide (aa 315-325) was the least immunogenic with responses in only two of five mice. The same pattern of immunoreactivity was observed against the native gp120 protein. The enhanced antibody response of B1 over B3 suggests that the B1 sequence contains a Th determinant. Evidently, this Th epitope is lost when the chain is reduced to only 11 aa in the B3 peptide. This was confirmed by the induction of specific proliferative response in the lymph nodes of mice immunized with the B1 peptide and not with the B3 peptide (data not shown).

The presence of a T stimulatory epitope within the same V3 loop in a IIIB peptide (residues 303-321) has been observed by others in goats (23). Unlike the peptides of IIIB, the parallel series of peptide Ag derived from RF and MN did not elicit significant antibody production in mice. A plausible explanation is that these sequences do not contain an epitope able to bind the murine Th receptor. In contrast, all the sequences from the three isolates were able to elicit immunity in rabbits (Table I). Antibody titers varied from the highest values of about $1.2 \times 10^6$ induced by immunization of B2 and B8 peptides to the lowest of about $2.3 \times 10^5$ induced by injection of the shortest B6 peptide. The responses of the guinea pigs were uneven (Table II). Although all the sequences of the IIIB isolate produced immunity, only the two longer of the sequences of the RF and MN isolates were able to elicit response in the monoepitope format.

**Antibody Response to Diepitope MAP**

To test the effectiveness of the addition of a known Th peptide to our constructs, the animals were immunized with diepitope MAP containing at the carboxyl-end of each B cell peptide a homologous Th cell determinant (BT-MAP). Several such epitopes of HIV-1 have been predicted and a 16-aa peptide, located in the fourth conserved domain of gp120 (IIIB isolate, residues 429-443), was selected because it displayed a helper activity in mice (24), humans (25) and goats (23).

As summarized in Table I, the presence of the T cell peptide in the diepitope MAP constructs used for the immunization proved to be very effective for the induction of the mouse antibody response. When mice were injected with the longest MAP of the IIIB sequence (B1T), the Th epitope increased only marginally the antibody level that was already high after the immunization with the monoepitope construct. However, a much improved titer level was observed in the mice injected with B2T peptide than in those receiving the intermediate length monoepitope B2. A strong antibody induction was observed using B3T in contrast to the low response obtained with the short 11-residue B3. Similar findings were also observed in the other two parallel series of peptides: totally nonimmunogenic monoepitope MAP, from the RF and MN series, became highly effective in stimulating humoral immune responses in the diepitope MAP configurations.

To show that the additional T epitope at the carboxy end of the B cell sequence did not function as a spacer to move the epitope from the MAP core and consequently increase its immunogenicity, a T cell proliferation assay was performed using B1T- and B3T-MAP peptides. Lymph node cells from Ag-primed BALB/c mice were cultured in vitro with the same BT construct used for the immunization or with the corresponding B epitope. As shown in Table III, lymph node cells from mice immunized with B1T proliferated when cultured with B1 peptide (because it contains an helper determinant) and even more strongly when the T peptide was used for the in vitro stimulation. Also the B3T-primed cells exhibited high level of proliferation following stimulation with the diepitopic B3T peptide; in contrast, only a background level $[^3H]TdT$R uptake was observed when stimulated with the B3 peptide.

In rabbits, antisera elicited by each of the diepitope BT-MAP showed greater immunoreactivity than antisera raised against the corresponding monoepitope constructs (Table I). The enhancement of immune responses in rabbits was less marked than in mice because, in rabbits, the monoepitope MAP constructs alone were able to elicit antibody response. Furthermore, results obtained from immunizing guinea pigs with the mono and diepitope peptides of IIIB and RF isolates supported the general trend observed (Table II); an increase of the humoral reactivity followed the immunization with the diepitopic MAP.
**Characterization of Antibody Response to MAP Constructs**

**Specificity.** To characterize the specificity of the B cell response to the MAP the rabbit antisera were assayed for their reactivity to B and T peptides (Table IV). The immune serum elicited by the immunization with B1-MAP contained antibodies only to the B cell epitope and its binding to the B1-coated plate was completely inhibited by the preincubation with B1 peptide. In contrast, the antisera induced by the diepitope B1T-MAP was able to react strongly also with the resin-bound T peptide, and no competition was observed in this case after the addition of the B1 peptide.

**Eptipe mapping.** To define the antigenic sites of the B peptides (IIIB isolate) the pepscan analysis was performed. Seventeen octapeptides, each sequentially overlapping the previous one by seven aa, were synthesized on plastic pins to cover the full length of the B1 epitope. These octapeptides would also cover the B2 epitope (peptides 5–14) and the B3 epitope (peptides 8–11). Rabbit antisera elicited by the three IIIB-MAP peptides were analyzed for their reactivity to the pins. A major antigenic region, distal to the core matrix, was identified. As shown in Figure 3, in the three sera analyzed the peak reactivity was located at the tip of the V3 loop corresponding to the octapeptide QRGPGRAF. The same kind of reactivity was observed also in murine sera anti-B1 peptide (data not shown).

**Cross-reactivity among HIV-1 isolates.** The rabbit antibodies were further analyzed for their ability to bind isolates other than their own. The results (Table V), showed a low level of cross-reaction between the IIIB and RF isolates. The anti-B1 sera (IIIB strain) was able to bind the homologous B4 peptide from RF, although 10-fold less strongly than B1 peptide. Conversely, 84 antisera reacted with B1 peptide. In contrast, no significant cross-reactivity with IIIB and RF peptides was observed in the rabbits immunized with the homologous B4 peptide of the MN isolate. The marginal cross-reactivity between IIIB and RF peptides is likely due to the close sequence homology of the B1 and B4 peptides at their N-terminus. Seven consecutive aa (position 308–314), preceding the conserved tripeptide GPG, are the same in these two sequences. Indeed, the pepscan analysis of the B4 antisera revealed a strong binding to one octapeptide (aa 308–315) of the B1 sequence (data not shown). However, the cross-reactivity did not extend to the binding to the putative protein gp120 of the IIIB strain. Interestingly, the serum from one rabbit immunized with the shortest peptide...
Assays were performed using cells expressing recombinant gp160 genes derived from either the IIIB, RF, or MN isolates. A majority of animals developed antiserum that was active against the isolate corresponding to the immunogen (Table VI). In general the best inhibition titers were obtained from the longest peptide Ag and the diepitope MAP with BT arrangements were more effective than the corresponding monoepitope MAP. A correlation between inhibition titer and immunogenicity was observed. However, antiseras raised against the IIIB-derived peptides did not cross-inhibit the MN-expressing cells, and vice versa RF and MN-induced immune sera were not reactive against IIIB-expressing cells. We observed just one exception to this type-specific reactivity: a guinea pig immunized with B7-MAP developed antiserum able to neutralize, at a very low titer, the syncytia formation in CEM cells infected by recombinant IIIB vaccinia virus. Although the highest titers were elicited by the MN-derived peptides, the MN assay seems to be more sensitive than the corresponding IIIB and RF assays (22).

**DISCUSSION**

A primary goal in the design of a synthetic vaccine is the development of an approach for producing an entity containing strongly immunogenic and essential epitopes but lacking suppressive or irrelevant determinants. In the search for such an approach to engineer a peptide-based vaccine, a relevant role can be played by the MAP system. In this study we demonstrate the effectiveness of the MAP in eliciting antibody response against sequences of gp120 V3 region, without the need for conjugation to a protein carrier. The efficacy of MAP over linear peptides has previously been reported in comparative immunizations (8). A preliminary experiment showed that a short linear sequence from the V3 loop (aa 312–328) did not elicit immune response in mice, whereas humoral immunity could be developed by the same peptide in a MAP format (B. Nardelli and J. P. Tam, unpublished observations). Therefore, we sought to determine for the V3 B cell epitope the minimal structural requirement sufficient to elicit high-titered antibodies using MAP configurations. It was found that the immunogenicity is dependent on the animal species. After immunization with the monoepitope constructs, containing peptides from the V3 regions of divergent HIV-1 isolates, rabbits respond to all the peptides. Mice are low or non-responders to the RF and MN peptides whereas their ability to develop antibody response to the IIIB peptides is proportional to the length of the chain. The immune response of guinea pigs appears to be intermediate between that of rabbits and mice. The explanation of these results is open to several hypotheses. Although it is clear that a murine Th epitope is absent in the shortest sequence (B3), it is possible that the smallest peptide contains T cell sites recognized by rabbits.

The effect of the T-cell epitope (aa 429–443) to enhance the immunogenicity and serve as a replacement for a protein carrier (26, 27) is clearly confirmed by the use of diepitope MAP which elicit immune responses in all of the animals tested. The diepitope BT configurations overcome the poor immunogenicity of the monoeptope B constructs in mice and give strong and enhanced immune responses in rabbits and guinea pigs. Similar tandem arrangements of diepitope MAP have been previously reported to raise high antibody titers to a determinant of the S region of hepatitis B surface Ag (6) and to elicit
protective immunity against the malaria challenge in mice (7).

An advantage of the MAP is that it is a clearly defined system that allows studies in the relationship of polarity to immunogenicity and the determination of the antigenic site in a molecule. In a tandemly linked dipeptide arrangement, the polarity between the B and T peptides may have a profound effect on the immunogenicity, in the case when both B and T peptides are linked in a linear format, there is no conclusive evidence to favor either polarity. Cox et al. (28) have found that a Tb peptide enhances antibody response when linked to the C-terminus of an adjacent B peptide and it is inactive if linked in opposite orientation. However, others have reported an inverse TB configuration is more efficient in stimulating humoral response (29). Unlike the linear peptide Ag format, there is evidence showing the effectiveness of a BT arrangement in a MAP configuration. We have found that when a Th peptide attached to the carboxyl-end of the B peptides. It results in the generation of a strong and specific immunity (6, 7). This preference of polarity may be useful as a general approach to test different B epitopes with an universal T epitope and is particularly relevant in view of the conserved occurrence of the Th epitope in gp120. A major difference between a linear peptide format and a MAP construct is in their molecular assembly. Although a linear peptide has two flexible ends, the asymmetry of a MAP, with a conformational restricted core matrix at the carboxyl-end and a flexible amino-end, creates a preference for polarity. Analysis of antigenic sites of our monoepitope MAP constructs shows that the dominant antigenic sites are located at the amino and central part of the B cell epitope. Interestingly, the major site of antigenicity in the rabbit B1B-immune sera is found to be generated by a sequence QRGPGRAF overlapping the conserved tip of the V3 loop. This finding is consistent with previous work showing that, in experimental immune sera raised against V3 peptides, the neutralizing epitope is located in the same central part of the sequence (30–32). The epitope is also recognized by the sera of chimpanzees experimentally infected with HIV-1, IIIB isolate (13).

In summary, our results show that immunization with the MAP induces a strong antipeptide antibody response that recognizes epitopes of the native protein and is capable of inhibiting viral infection. The in vivo protection afforded by immunization with the MAP remains to be demonstrated.

Other synthetic vaccine approaches based on V3 sequences have recently been published. Similar to our study, successful attempts have been used to replace the conventional method of peptide immunization with protein carriers. Hart et al. (33) reported the induction of neutralizing antibodies and T cell proliferative response in monkeys immunized with carrier-free synthetic peptides consisting of T and B cell epitopes from gp120 (33). Neurath et al. (34) were able to elicit immunity in rabbits using a long V3 peptide (aa 306–338) without its conjugation to a carrier.

The major problem posed by the immunization with the highly variable V3 loop is that the resulting immunity is subtype specific. Such strain restriction is consistently found in works using V3 peptides (13, 23, 35, 36). We observed no evidence for a pool of broadly reactive antibodies that recognizes the relatively conserved sequence QRGGRAF, although such a pool is occasionally elicited (31). Failure to observe wider crossreactive antibodies in this work may be due to the small number of animals immunized. However, because the MAP approach has the potential ability to incorporate a combinatorial library of epitopes, we are currently exploring the design of multivalent vaccine prototypes that may overcome the isolate-restricted antibody response to HIV-1.

Acknowledgments. The authors acknowledge the helpful contributions of Drs. S. Silver and J. Boyd.

REFERENCES

MANY ANTIGEN PEPTIDE SYSTEM AS VACCINE MODEL


