A rational design of synthetic peptide vaccine with a built-in adjuvant

A modular approach for unambiguity

JEAN-PHILIPPE DEFOORT, BERNARDETTA NARDELLI, WOLIN HUANG and JAMES P. TAM
The Rockefeller University, New York, NY, USA

Received 24 April, accepted for publication 3 May 1992

We describe a peptide vaccine model containing a built-in adjuvant. This model used a multiple antigen peptide system (MAPS) to amplify peptide antigens and a lipopeptide, tripalmitoyl-S-gluceryl cysteine (P3C), as a built-in adjuvant. An 18-residue peptide antigen (B2) derived from the third variable domain (amino acid 312-329) of the glycoprotein gp120 of type I human immunodeficiency virus (HIV-1) was used in this model. This peptide antigen is a suitable target since it consists of neutralizing, T-helper, and T-cytotoxic epitopes. The peptide antigen in a tetravalent MAPS format (B2M-P3C) with a lipophilic attachment was synthesized by two routes for comparison: a direct stepwise approach and an indirect modular approach. In the stepwise approach, each residue was sequentially added to the peptide resin to give B2M-P3C and the P3C was incorporated to the side chain of a carboxyl terminal lysine as Fmoc-Lys(P3C). In the modular approach, a module containing a chloroacetylated core matrix of MAPS (M-P3C) with a carboxyl tetrapeptide bearing Lys(P3C) and a second module containing the peptide antigen B2 with a cysteine at its terminus were synthesized and purified separately, and then coupled to each other to form B2M-P3C. In the modular approach, the molecular ion of B2M-P3C was unambiguously identified by ion-spray mass spectrometry. B2M-P3C, administered in liposomes without any adjuvant such as Freund’s complete adjuvant, was used to immunize mice and found to induce gp120-specific antibodies in vitro, and prime cytotoxic T lymphocytes in vivo. In contrast, the tetravalent B2 on the MAPS (B2M) without a built-in adjuvant was non-immunogenic and required Freund’s complete adjuvant to elicit a low-titered antibody response. Our results show that our model with a built-in adjuvant may lead to useful designs of vaccines suitable for humans.

Key words: AIDS vaccine; cytotoxic T-cell lymphocytes; liposome; multiple antigen peptide system; solid-phase peptide synthesis; vaccine AIDS

Dedicated to Professor Bruce Merrifield on the occasion of his 70th birthday

A rational design in our laboratory for the development of the peptide-based vaccine for humans (1-6) has been focused on an unambiguous presentation of peptide antigens in a scaffolding as a macromolecule and with a built-in adjuvant. Such a vaccine would have the advantages of simplicity, safety, and unambiguity over the conventional approaches of using peptide antigens conjugated to a protein carrier (7) or by polymerization (8).

To achieve the purpose to produce a macromolecule based on a linear monomeric peptide, our laboratory has developed a two-stage approach. In the first stage, we introduce a simple scaffolding known as the multiple antigen peptide system (MAPS) to amplify the monomeric peptide antigen as a macromolecule (1-6). This system uses a simple core matrix which consists of a low number of sequential levels of a branching trifunctional amino acid such as lysine for attaching peptide
antigens. For example, a core matrix of 2–3 levels of branching would result in a MAPS that amplifies covalently antigens 4–8 fold in a defined manner. Furthermore, this approach is flexible and allows preparation of immunogens consisting of various epitopes in different arrangements (4–6). Because of the close packing of these antigens in a scaffolding, MAPS may have the advantage of providing a tertiary structure that allows stabilization of secondary structures of peptide antigens. In the second stage, a lipophilic membrane-anchoring moiety at the carboxyl terminus of MAPS enables further noncovalent amplification by a liposome or micellar form. Such a noncovalent amplification of MAPS in a lipid matrix to many thousand fold has been called the macromolecular assemblage approach of MAPS (9).

To achieve the purpose of a peptide antigen with a built-in adjuvant, we choose a lipophilic membrane-anchoring moiety with known adjuvant activity. Similar to other peptide antigens conjugated to protein carriers, MAPS preparations without a built-in adjuvant usually require Freund's complete adjuvant (FCA) to elicit a high titered antibody response. FCA, which is derived from the extraction of mycobacteria cell wall, produces many side effects (14). FCA is pyrogenic and induces arthritis in rats and anterior uveitis in rabbits. To eliminate these undesirable side effects, extensive structure-activity relationships of FCA have been carried out and significant progress has been made to identify the minimal active structure derived from the mycobacterial cell wall essential for adjuvant effects. Noteworthy examples include muramyl dipeptide analogs (14) which represent the minimal active structure derived from the mycobacterial cell wall essential for adjuvant effects. The resulting clear solution was evaporated to dryness several times with toluene. The oily residue was dissolved in ethylacetate and washed sequentially with 5% KHSO4, 5% NaHCO3 and H2O. The organic phase was dried over Na2SO4, evaporated to dryness and purified by silica gel chromatography to give 0.344 g Fmoc-Lys(P3C)-OPac with a yield of 55%. Rf = 0.2 (hexane/EtOAc 7/3). FAB mass spectrometry: calculated (M + H)+ = 587, found (M + H)+ = 587.

**EXPERIMENTAL PROCEDURES**

**Fmoc-Lys(Boc)-OPac, 1**

Fmoc-Lys(Boc) (0.5 g, 1.07 mmol) was dissolved in 15 mL of 95% ethanol. The solution was adjusted to neutrality with a solution of cesium hydroxide. The resulting clear solution was evaporated to dryness several times with toluene. This cesium salt of the Fmoc-Lys(Boc) was then allowed to react with 0.233 g bromoacetophenone (1.173 mmol) in 10 mL DMF. After 3 h, DMF was removed under vacuum. The oily residue was dissolved in ethylacetate and washed sequentially with 5% KHSO4, 5% NaHCO3 and H2O. The organic phase was dried over Na2SO4, evaporated to dryness and purified by silica gel chromatography to give 0.344 g Fmoc-Lys(Boc)-OPac with a yield of 55%. Rf = 0.2 (hexane/EtOAc 7/3). FAB mass spectrometry: calculated (M + H)+ = 587, found (M + H)+ = 587.

**Fmoc-Lys(P3C)-OPac, 2**

Boc-Lys(Boc)-OPac (0.334 g, 0.569 mmol) was treated with 10 mL of 1 N HCl/AcOH for 30 min to remove the Boc protecting groups. After completion of the reaction, acetic acid was removed under vacuum several times with toluene. The oily residue was kept under vacuum overnight. Then, P3C (0.569 g, 0.625 mmol) was coupled to the HCl salt of Boc-Lys-OPac via the DCC method in the presence of triethylamine (0.87 μL, 0.626 μmol) in CHCl3. After evaporation of the CHCl3, the crude product was purified by silica gel chromatography to give 0.465 g Fmoc-Lys(P3C)-OPac with a yield of 59%. Rf = 0.25 (hexane/EtOAc 7/3). FAB mass spectrometry: calculated (M + H)+ = 1379, found (M + H)+ = 1379.

**Fmoc-Lys(P3C), 3**

Boc-Lys(P3C)-OPac (0.452 g, 0.327 mmol) was dissolved in 18 mL AcOH, 0.45 mL H2O and a minimum amount of CHCl3 for complete dissolution. Then, zinc powder was introduced into the flask and was stirred for 4 h. Zinc was removed by filtration, acetic acid was evaporated and the desired product was extracted in...
Synthesis of B2M-P3C, 4 by the direct approach
Synthesis was accomplished by a stepwise solid phase procedure (21) on 0.39 g Fmoc-Ala-OCH2-resin (0.297 mmol/g) (22, 23). After removal of the Fmoc group by 20% piperidine in DMF, the Ala-resin was coupled to 0.115 mmol of 3 via DCC/HOBt in CH2Cl2. Two consecutive serines were coupled to the Lys(P3C)-Ala-resin prior to the addition of the two levels of N\(^{Fmoc}\)-N\(^{Fmoc}\)-Fmoc-lysine to give a tetrabranched [Fmoc-Lys(Fmoc)]\(_2\)-Lys-Ser-Ser-Lys(P3C)-Ala-resin. In one preparation, Fmoc Lys (P3C)-Ala-resin formed during its synthesis. The protecting group N\(^{-}\)amino group and the tert.-butyl side chain protect the resin by a 4 h treatment with TFA/dimethylsulfide/3% anisole/ethanedithiol 95:2:2:1. The crude peptide was treated with thiolphenol in DMF for 12 h at 60\(^\circ\)C in order to reduce sulfoxide which might have been formed during its synthesis. The protecting group scheme for the synthesis was as follows: Fmoc for the N\(^{Fmoc}\)-amino group and the tert.-butyl side chain protecting group for trifunctional amino acid except for Arg(Pmc) and Lys(Boc). Three equivalents of Fmoc-amino acid were used for each coupling by the DCC/HOBt method. Couplings were monitored by the qualitative ninhydrin test (24). Deprotection by 20% piperidine in DMF (10 min) was preceded by a 2-min pre-wash. After completion of the synthesis, Fmoc protecting group was removed prior to the cleavage of peptide resin by a 4 h treatment with TFA/dimethylsulfide/anisole/ethanedithiol 95:2:2:1. The crude peptide was washed several times in cold tert.-butyl methyl ether and then lyophilized from a 5% acetic acid solution. Amino acids analysis was carried out after hydrolysis in 5.7 N HCl at 110\(^\circ\)C for 24 h. Amino acid analysis results: Thr 3.97 (4), Ser 5.06 (6), Gly/Gln 4.17 (4), Pro 3.99 (4), Gly 12.85 (12), Ala 5.25 (5), Val 4.06 (4), Ile 12.44 (12), Phe 4.61 (4), Lys 12.37 (12), Arg 10.19 (12).

Cys-B2 monomeric peptide, 5
The synthesis of the monomeric linear peptide of B2 containing an additional amino terminal Cysteine (Cys-B2) was accomplished by the Fmoc/tert.-butyl strategy (22, 23). The cysteine residue on the amino terminus of the peptide was protected with the tert.-butyl protecting group. Amino acid analysis: Thr 0.98 (1), Ser 0.79 (1), Glu/Gln 1.06 (1), Pro 1.1 (1), Gly 3.19 (3), Ala 1.09 (1), Val 1.07 (1), Ile 2.92 (3), Phe 1.04 (1), Lys 1.98 (2), Arg 2.72 (3). Ion-spray mass spectrometry: calculated (M + H)\(^+\): 2087.52, found (M + H)\(^+\): 2087.9.

Chloroacetylated core matrix containing P3C(CI-M-P3C, 6)
Chloroacetic acid was coupled via the symmetric anhydride method to Lys\(_2\)-Lys-Ser-Ser-Lys(P3C)-Ala-resin (96 mg). The core matrix with chloroacetyl and P3C groups (CI-M-P3C) was cleaved from the resin (38 mg) by a treatment with 96% TFA containing 2% dimethylsulfide and 2% anisole for 2 h. The product 6 was very hydrophobic and could not be suitably analyzed by the reverse-phase HPLC even those with C4 RP columns. As a result, the crude product was purified by repeated precipitation in cold tert.-butyl methyl ether, redissolved in glacial acetic acid, and lyophilized to a powder to give 6 (7 mg). Amino acid analysis: Ser 1.55 (2), Ala 1.00 (1), Lys 3.99 (4).

Synthesis of B2M-P3C, 7 by the indirect approach
All solvents used for the synthesis were distilled and purged with nitrogen. Cys-B2 5 (22.4 mg, 8.12 \(\mu\)mol) was dissolved in 1 mL N-methylpyrrolidone in diisopropylethylamine (25 \(\mu\)L, 100 \(\mu\)mol) and tributylyphosphine (3.48 \(\mu\)L, 50 \(\mu\)mol) and stirred for 24 h at room temperature. After 24 h, 300 \(\mu\)L \(\text{H}_2\text{O}\) were added to clarify the solution. The product, B2M-P3C 7, was precipitated by the addition of ether to the aqueous NMP solution lyophilized and purified by gel filtration on a Sephadex G75 (column 30 x 2 cm) and eluted with a 5% acetic acid solution with a flow rate of 21 mL/h. Three peaks (A, B, and C) in the order of their elution from Sephadex G75) were obtained. Peak A was an oligomeric form of 7, peak B the monomeric form, and peak C a mixture of the reduced and oxidized form of the starting material. Amino acid analysis results: Peak A: Thr 3.72 (4), Ser 5.7 (6), Glu/Gln 4.08 (4), Pro 3.68 (4), Gly 12.49 (12), Ala 5.72 (5), Val 4.28 (4), Ile 11.58 (12), Phe 4.99 (4), Lys 11.1 (12), Arg 11.32 (12), Peak B: Thr 3.27 (4), Ser 5.49 (6), Glu/Gln 4.09 (4), Pro 3.90 (4), Gly 14.04 (12), Ala 5.36 (5), Val 4.03 (4), Ile 11.71 (12), Phe 4.83 (4), Lys 10.86 (12), Arg 11.78 (12). Results of the ion spray mass spectrometry were reported in Table 1.

Preparation of positively charged liposome
Liposomes were prepared as described by Gregoriadis et al. (30). Briefly, 56 mg of egg lecithin, 8.4 mg cholesterol and 1.8 mg stearylamine were solubilized in CHCl3 in a 100 mL round bottom flask. The organic solvent was removed under vacuum using a rotary evaporator to form a thin film of lipid on the wall of the flask. After drying, nitrogen was kept flushing in the flask for 10 min. PBS (2 mL) 10 mM at pH 7.4 containing 2.5 mg of peptide was added into the flask. Shaken manually for 10 min, the suspension was then allowed to stand at room temperature for 2 h. The resulting milky solution was sonicated 45 min (Laboratory Supply Company) until the solution became opalescent. After sonication, free B2M-P3C was separated from the liposomes on Sepharose 6 B, liposomes were filtered on a 0.45 \(\mu\)m filter and kept under nitrogen.

Immunoassay procedure and ELISA
BALB/c and CD1 mice (5 per group), 6–8 weeks old, were immunized intraperitoneally four times with 1–100 \(\mu\)g B2M-P3C at 2–3 week intervals and bled a
Ion spray mass spectrometric analysis of the Peak B of B2M-P3C*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ion</th>
<th>M.W.</th>
<th>Average</th>
<th>Calc. Value</th>
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<tr>
<td></td>
<td>(M + 14H) +</td>
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<td></td>
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<td>(X + 9H) +</td>
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</table>

* Peaks A and B were obtained from gel permeation chromatography of B2M-P3C 7 and showed similar amino acid composition. Peak B showed two compounds in ion-spray mass spectrometry, A and B: Compound A is the expected product and compound B contains an additional mass unit of 96.

RESULTS

Direct stepwise approach

The direct approach uses a stepwise solid phase synthesis beginning with the Fmoc-Lys(P3C)-Ala-resin (21). P3C was linked to the side chain of Fmoc-Lys and incorporated to the Ala-resin as Fmoc-Lys(P3C). The synthetic strategy of Fmoc/tert.-butyl chemistry (22) in combination with a hydroxymethylphenyl (HMP) resin (Wang resin) (23) were chosen since P3C was not stable towards HF but it was sufficiently stable to trifluoroacetic acid used for the peptide cleavage step in the Fmoc chemistry.

As shown in Fig. 1, P3C was synthesized in six steps as described in Weismuller et al. (24). Fmoc-Lys(P3C) 3 was prepared in three steps with Fmoc-Lys(Boc) as the starting material (Fig. 2). Since it was possible to

**TABLE 1**

AIDS vaccine
esterify Fmoc-amino acid as its cesium salt (20), the cesium salt of the Fmoc-Lys(Boc) was reacted with the bromoacetophenone to give the Fmoc-Lys(Boc)-OPac 1. After removal of the Boc protecting group, P3C was coupled to the ε-amino group of lysine via the DCC method to give the Fmoc-Lys(P3C)-OPac 2. Removal of the phenacyl ester with zinc in 90% acetic acid gave Fmoc-Lys(P3C)3. The preformed Fmoc-Lys(P3C) was then used as an amino acid derivative for the stepwise solid phase of B2M-P3C on Ala-HMP-resin with a sequential addition of Fmoc-Lys(P3C), two consecutive serines as a spacer, the core matrix with two levels of Fmoc-Lys(Fmoc), and then the peptide antigen (B2). The sequence B2 (KSIRIQRGPGRAVFTIGK), a 18-residue peptide, is derived from the V3 loop of gp120 of human immunodeficiency virus (HIV-1) and contains a neutralizing (26), T-helper (27), and T-cytotoxic epitope (28). The synthesis was performed using DCC/HOBt chemistry and each coupling reaction was monitored to completion.

At the completion of the synthesis, the Fmoc protecting group was removed prior to the TFA cleavage of the peptide from the resin. The TFA cleavage, with the appropriate scavengers, was performed for a prolonged period of 3-4 h to allow the complete removal of the Pmc protecting groups on the Arg. Amino acid analysis of the B2M-P3C 4 (Fig. 3) showed that it gave good agreement with the calculated ratios. The integrity of P3C was confirmed by mitogenic assay using mouse spleen cells (data not shown). Although the quality of B3M-P3C prepared by the direct approach was very high, an alternative indirect approach using two purified segments or modules was developed to provide an unambiguous structure identifiable by the mass spectrometric analysis.

**Indirect modular approach**

The indirect approach of linking a peptide antigen through the side chain of cysteine to the core matrix for the synthesis of MAPS has already been described by us (6) and by others (29). The main idea of this approach (Fig. 4) is to synthesize two modules separately, one module containing a peptide antigen containing a Cys and a second module containing an activated chloroacetylated core matrix. The modules are then reassembled to form a chemically unambiguous MAPS. The thiol alkylation to the core matrix by the peptide antigen is sufficiently specific at the neutral pH to allow the use of an unprotected peptide antigen. We synthesized and purified the two modules, the chloroacetylated core matrix bearing on its carboxyl terminus (P3C, ClM-P3C 6) and the monomeric peptide (B2) with an additional cysteine at its amino terminus to form Cys-B2 5. Because of the hydrophobic nature of the chloroacetylated core matrix, ClM-P3C 6, the thiol alkylation could not be achieved in an aqueous solution, but it was possible to proceed in a basic aqueous N-methylpyrrolidone solution. Tributylphosphine was used as a reducing agent to prevent the oxidation of the sulphydryls.

The resulting B2M-P3C 7 was purified by gel permeation chromatography and the excess monomeric peptide was separated and recycled. Three major peaks, A, B and C in their order of elution, were obtained. Peak C was shown to be a mixture of reduced and oxidized linear peptides. Amino acid analysis and mass spectrometry analysis showed that peak A and B gave similar composition. Because Peak A elutes earlier than Peak B and both contain hydrophobic lipid moieties, it appears that Peak A is a multimeric aggregate of peak B. The mass spectrometric analysis (Fig. 5 and Table 1) of peak B showed further that it contained two components with the first component at a higher intensity than the second. The first component was found to
forcing the thiol alkylation to completion. Similar experiments without tributylphosphine led to the formation of a B2M-P3C bearing only three peptide antigens instead of four. This was confirmed by mass spectrometry analysis which showed a compound with a MW of 8,086 Daltons corresponding to the mass ion of B2M-P3C with three peptide antigens attached to the core matrix. The difference of 2087 Daltons could be accounted for by the MW of the monomeric peptide and a chlorine moiety. This result showed that a prolonged basic treatment and tributylphosphine could lead to the hydrolysis of the chloroacetyl moiety and result in the formation of a tripeptide antigen analog of B2M-P3C.

**Humoral and CTL responses of B2M-P3C**

Mouse spleen cells were used to demonstrate that the mitogenic activity of the P3C was retained in B2M-P3C (data not shown). The mitogenicity was dose-dependent with increased incorporation of 3H-thymidine in spleen cells with escalated concentrations of B3M-P3C. B2M without P3C did not show any mitogenic activity.

The ability of B2M-P3C in liposome or free, without any adjuvant, to induce humoral response was studied in mice. Moderate-titered antibodies were found in sera from animals immunized four times with both preparations and reacted with B2M, or gp120 in an ELISA assay (Table 2). Control mice immunized with a noncovalent mixture of B2M, P3C, and liposome developed only low-titered antibodies against B2 or gp120.

Spleen cells of mice immunized with B2M-P3C free or liposome was assayed for their ability to lyse target cells preincubated with B2M and was shown to elicit CTLs against B2 peptide-coated syngeneic P815 cells. The CTLs responses were similar in both B1M-P3C preparations.
immunogenic when administered alone, in liposome, or in Freund's complete adjuvant. Second, the design of P3C and the combination of the lipid matrix allows the vaccination to elicit CTLs which are the primary cell-mediated responses to clear viral infections. CTLs are usually not elicited by the conventional approach of immunization such as those with B2M and Freund's complete adjuvant. Finally, the MAPS with a lipophilic adjuvant such as P3C can be used in the macromolecular assemblage approach of vaccine adjuvant formulation. This approach allows a combination of various MAPS bearing different antigens to assemble on the lipid matrix. In this way, the macromolecular assemblage approach contains a combination of needed peptide antigens, a non-immunogenic core matrix as a carrier, a built-in P3C as an adjuvant, and liposomes as the vehicle, which are all the required components for an effective vaccine adjuvant formulation.

ACKNOWLEDGMENTS

We thank Ms. D. Shiu and Mr. B. Cox for their technical assistance. We also thank Dr. Brian Chait for the mass spectrometric analysis. This work was supported in part by the U.S.PHS grant AI-28701 and AID.

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Address:
James P. Tam
Dept. of Microbiology and Immunology
A5321 Medical Center North
Vanderbilt University
Nashville
TN 31322
USA