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### Permalink

<https://escholarship.org/uc/item/6tr4w04h>

### Journal

Clinical and vaccine immunology : CVI, 21(8)

### ISSN

1556-679X

### Authors

Venditto, Vincent J  
Wieczorek, Lindsay  
Molnar, Sebastian  
et al.

### Publication Date

2014-08-28

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Peer reviewed

# Chemically Modified Peptides Based on the Membrane-Proximal External Region of the HIV-1 Envelope Induce High-Titer, Epitope-Specific Nonneutralizing Antibodies in Rabbits

Vincent J. Venditto,<sup>a</sup> Lindsay Wieczorek,<sup>b,c</sup> Sebastian Molnar,<sup>b,c</sup> Fernando Teque,<sup>d</sup> Gary Landucci,<sup>e</sup> Douglas S. Watson,<sup>a\*</sup> Donald Forthal,<sup>e</sup> Victoria R. Polonis,<sup>b</sup> Jay A. Levy,<sup>d</sup> Francis C. Szoka, Jr.<sup>a</sup>

Departments of Bioengineering and Therapeutic Sciences, School of Pharmacy, University of California, San Francisco, San Francisco, California, USA<sup>a</sup>; Laboratory of Vaccine Immunology, Walter Reed Army Institute of Research, Military HIV Research Program, Silver Spring, Maryland, USA<sup>b</sup>; The Henry M. Jackson Foundation, Bethesda, Maryland, USA<sup>c</sup>; Department of Medicine, University of California, San Francisco, San Francisco, California, USA<sup>d</sup>; Division of Infectious Diseases, Department of Medicine, University of California, Irvine, Irvine, California, USA<sup>e</sup>

**Broadly neutralizing monoclonal antibodies (bNAbs) 2F5 and 4E10 bind to the membrane proximal external region (MPER) of gp41 and also cross-react with phospholipids. In this study, we investigated if chemical modifications on the MPER adjacent to 2F5 and 4E10 epitopes using mimetics of inflammation-associated posttranslational modifications to induce 2F5- and 4E10-like bNAbs can break tolerance. We synthesized a series of chemically modified peptides spanning the MPER. The serine, threonine, and tyrosine residues in the peptides were modified with sulfate, phosphate, or nitrate moieties and presented in liposomes for rabbit immunizations. All immunizations resulted in high antisera titers directed toward both the modified and unmodified immunogens. Tyrosine modification was observed to significantly suppress anti-epitope responses. Sera with strong anti-gp140 titers were purified by affinity chromatography toward the MPER peptide and found to possess a higher affinity toward the MPER than did the bNAbs 2F5 and 4E10. Modest neutralization was observed in the H9 neutralization assay, but neutralization was not observed in the TZM-bl cell or peripheral blood mononuclear cell (PBMC) neutralization assay platforms. Although neutralizing antibodies were not induced by this approach, we conclude that chemical modifications can increase the immune responses to poorly immunogenic antigens, suggesting that chemical modification in an appropriate immunization protocol should be explored further as an HIV-1 vaccine strategy.**

A prophylactic vaccine capable of generating protective immunity against HIV-1 has been a major objective for numerous investigators spanning 3 decades of research. The membrane proximal external region (MPER) of the gp41 transmembrane protein in the HIV-1 envelope (1) is an obvious target for vaccine development due to the conserved sequence and identification of MPER-specific broadly neutralizing monoclonal antibodies (bNAbs), 2F5, 4E10, 10E8, m66.6, and Z13 (2–5). Structural studies with these bNAbs have informed a variety of immunization strategies (1, 6–8), but the inability to generate bNAbs toward the MPER in response to vaccination has raised the concern that tolerance mechanisms might be the cause of the weak immune responses (9–11). Recent advances in deep sequencing (12), reverse antibody engineering (13), and rational immunogen engineering (14) have provided details on the immune responses toward specific epitopes in HIV-1, including the MPER sequence, which may ultimately lead to an effective vaccine.

The MPER-specific bNAbs 2F5 and 4E10 possess characteristically long third heavy-chain complementarity-determining region 3 (CDRH3) loops rich in hydrophobic residues (6) and exhibit cross-reactivity with phospholipids (15). These characteristics are reminiscent of those of autoantibodies directed toward self-antigens. This finding led to the hypothesis that the neutralization capability of these antibodies lies in the improved affinity or avidity of the antibody due to the potential to interact with the viral envelope and the MPER domain (9). These data also suggest that, although obtained from HIV-infected patient serum, tolerance mechanisms lead to the paucity of such antibodies in the rest of the patient population. More recently, however, a bNAb,

10E8, identified from patient serum has been shown to bind the MPER at an epitope overlapping the 4E10 epitope but lacks the phospholipid cross-reactivity observed with the other bNAbs (3). Moreover, 27% of HIV-1-positive patient sera were found to contain MPER-specific antibodies, while 8% contained 10E8-like antibodies (3). The presence of 10E8-like bNAbs in patient samples does not rule out the possibility that these antibodies are regulated by tolerance mechanisms but does suggest that cross-reactivity to phospholipids is not necessary for neutralization.

Recent studies focusing on tolerance mechanisms have identified specific proteins capable of interacting with bNAbs 2F5 and 4E10 (16, 17), leading to the notion that although lipid cross-reactivity exists, tolerance is actually induced through deletion of protein-specific B cells (17). Immunoprecipitation of whole-cell extracts with 2F5 and 4E10 identified two potential autoantigens that may be the cause of tolerance: kynureninase (KYNU) and

Received 12 May 2014 Returned for modification 23 May 2014

Accepted 24 May 2014

Published ahead of print 28 May 2014

Editor: H. F. Staats

Address correspondence to Francis C. Szoka, Jr., [szoka@cgl.ucsf.edu](mailto:szoka@cgl.ucsf.edu).

\* Present address: Douglas S. Watson, Merck & Co., Elkton, Virginia, USA.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/CVI.00320-14>.

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doi:10.1128/CVI.00320-14

splicing factor 3b subunit 3 (SF3B3), respectively (17). While SF3B3 and the MPER do not have any sequence homology, KYNU contains a sequence identical to the 2F5 epitope (ELDKWA). The authors suggested that the sequence homology between the self-protein and the MPER of HIV-1 might lead to immunological tolerance mechanisms that impair MPER-specific humoral immune responses. In this regard, immunized opossums, which have a mutation in the ELDKWA motif of KYNU, are capable of generating antibodies with higher titers than those of C57BL/6 mice, but neutralization was not reported with these sera (17).

Various methods to enhance the immunogenicity of the MPER sequence have been attempted with little success (18). We previously hypothesized that immunization with immunogens covalently anchored in a liposomal membrane would improve immunogenicity (19, 20) but failed to achieve neutralizing antibodies. We then hypothesized that we could break tolerance and induce bNAbs by immunizing with posttranslational modification mimetics of the MPER peptides (21). This hypothesis stems from the ubiquitous nature of posttranslational modifications during the inflammatory immune response (22), the fact that posttranslational modification mimetics have been shown to break tolerance in model systems (23, 24), and the altered binding of posttranslationally modified peptides in the major histocompatibility complex (MHC), with the subsequent induction of T cell responses (25). In our earlier study (21), we showed that partial MPER immunogens bearing chemically modified side chains can induce high anti-MPER antibody titers in rabbits. Although we failed to elicit neutralizing antibodies, we confirmed that these modifications do not alter the affinities of bNAbs 2F5 and 4E10 toward their cognate epitopes. We also demonstrated that the longer MPER lipopeptide immunogens (25 amino acids) induced stronger humoral immune responses in rabbits than the shorter N-terminal sequence of HIV-1 MPER containing the 2F5 epitope (N-MPER) or the C-terminal sequence of HIV-1 MPER containing the 4E10 epitope (C-MPER) (16 or 19 amino acids).

In this account, we have extended our previous investigation by synthesizing a series of full-length MPER lipopeptides (31 amino acids) with chemical modifications, including sulfonation, phosphorylation, and nitration. These peptides were presented in a liposomal bilayer and used in a subcutaneous and intramuscular rabbit immunization strategy in an effort to improve the humoral immune responses to induce broadly neutralizing anti-HIV antibodies.

## MATERIALS AND METHODS

Amino acids, resins, and coupling agents were obtained from Novabiochem (Darmstadt, Germany), AnaSpec (San Jose, CA), or ChemPep (Miami, FL). Cholesterol, dimyristoylphosphatidylcholine (DMPC), and dimyristoylphosphatidylglycerol (DMPG) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesteryl hemisuccinate (CHEMS) (no. C6512) and monophosphoryl lipid A (MPL) derived from *Escherichia coli* (no. L6638) were obtained from Sigma-Aldrich (St. Louis, MO). Peptides and lipopeptides were synthesized and formulated in liposomes as described previously (19). Phosphorylated, nitrated, and sulfonated residues were incorporated by the addition of amino acids with side chains derivatized with *O*-benzyl-protected phosphate, 9-fluorenylmethoxy carbonyl (Fmoc)-3-nitro-tyrosine-OH (Bachem, Torrance, CA) or *N*-Fmoc-L-tyrosine neopentyl sulfate, respectively. *N*-Fmoc-L-tyrosine neopentyl sulfate was synthesized according to a previous report (26). HiTrap streptavidin HP columns were procured from GE Healthcare Life Sciences

(Pittsburgh, PA). Avastin (bevacizumab) was obtained from the University of California Medical Center.

**Rabbit immunizations.** All rabbit studies were done in compliance with the Covance Research Products, Inc., Denver Site Institutional Animal Care and Use Committee (IACUC) guidelines. Rabbit studies were performed by Covance Inc. (Denver, PA). Eighteen (6 groups of 3 rabbits each) specific pathogen-free New Zealand White (NZW) rabbits received subcutaneous immunizations on days 0, 21, 42, and 63. Blood samples were collected from the ear vein on days 0, 52, and 73. Injections on day 1 contained 500  $\mu$ g lipopeptide, 250  $\mu$ g monophosphoryl lipid A, and 20  $\mu$ mol lipid vehicle in 500  $\mu$ l sterile phosphate-buffered saline (PBS) emulsified in 500  $\mu$ l Freund's complete adjuvant. Subsequent subcutaneous injections contained 500  $\mu$ l of the formulation described above emulsified in 500  $\mu$ l Freund's incomplete adjuvant. Alving and coworkers have demonstrated that liposomes are compatible with oil-in-water emulsions and that the addition of alum may not improve the humoral immune response in NZW rabbits (27, 28). After blood draw, cells were removed by centrifugation, and sera were sent to the University of California, San Francisco (UCSF) on dry ice and stored at  $-80^{\circ}\text{C}$ .

**Epitope-specific antibody purification and concentration determination.** Polyclonal sera were affinity purified using a HiTrap streptavidin HP column and a biotinylated MPER peptide. A solution of biotinylated peptide was prepared in PBS at a final concentration of 0.2 mg/ml and added to the column at a flow rate of 0.5 ml/min followed by an additional 20 ml of PBS. Then, 6 ml of rabbit serum diluted to 24 ml in PBS was passed through the column followed by 25 ml PBS to remove any non-specific binding. Next, 4 ml of 0.2 M glycine (pH 2.8) was passed through the column, and 0.2-ml fractions were collected in 60  $\mu$ l of 1 M Tris (pH 8.0). The total antibody concentration was determined by the Bradford Coomassie assay (Pierce), according to the manufacturer's protocol using Avastin (bevacizumab) as the IgG standard.

**Enzyme-linked immunosorbent assays.** Reciprocal endpoint titers of antisera were determined by enzyme-linked immunosorbent assays (ELISA) as previously described (21). The titer was defined as the reciprocal dilution of antisera yielding an optical density twice that of the background. Samples were assayed in duplicate. The statistical significance was assessed by analysis of variance (ANOVA) with Bonferroni posttest analysis, and results were considered significant when  $P < 0.05$ . Data analyses were performed using Prism (GraphPad Software, Inc.). The affinity-purified antibodies were analyzed by an ELISA toward the immobilized MPER peptide or gp140 protein as previously described (21); however, known concentrations of antibody were analyzed using 11 10-fold dilutions from  $10^{-2}$  to  $10^{-12}$  mg/ml total antibody.

**Cell culture and virus neutralization assay.** Neutralization assays were performed with H9 cells (29), TZM-bl cells (30), or peripheral blood mononuclear cells (PBMC) (31, 32) as previously described. For all assays, neutralization was calculated as the reduction of infection observed in the presence of postimmunization sera compared with that for preimmunization sera or in the presence of purified antibody compared with that for virus alone. All sera were heat inactivated (30 min,  $56^{\circ}\text{C}$ ) before use. Sera were screened at a 1:20 (H9 cells and PBMC with primary isolates) or 1:40 (TZM-bl cells and PBMC with infectious molecular clones) dilution, and purified antibodies were titered in 6-fold dilutions starting at 25  $\mu$ g/ml. The data are reported as averages from duplicate experiments.

Briefly, the H9 assays were performed using H9-adapted HIV-1 strain MN (X4-tropic). The virus was diluted to yield a supernatant concentration of  $1 \times 10^4$  pg p24/ml at day 4 postinfection. The sera and virus were incubated together for 1 h prior to the addition of  $1 \times 10^5$  H9 cells. The sera, virus, and cells were then incubated for 24 h at  $37^{\circ}\text{C}$ . The cells were washed three times and incubated for an additional 72 h at  $37^{\circ}\text{C}$ . The supernatants were lysed, and the p24 concentration was determined by an antigen-capture ELISA (Advanced BioScience Laboratories, Rockville, MD).

The TZM-bl assays were performed using the replication-deficient pseudoviruses SF162 and BZ167 (R5-tropic). The pseudoviruses were di-

luted to yield  $>1 \times 10^5$  relative light units (RLU) at day 4 postinfection. The sera and virus were incubated together for 30 min prior to the addition of  $1 \times 10^4$  TZM-bl cells containing DEAE-dextran. The sera, virus, and cells were then incubated for 48 h at 37°C. The cultures were lysed, and britelite plus substrate (PerkinElmer Life Sciences, Shelton, CT) was added, following the manufacturer's instructions. The endpoint was determined by quantifying the RLU of cell-derived luciferase using the Envision luminometer (PerkinElmer Life Sciences).

PBMC were isolated from fresh buffy coats, using Ficoll-Hypaque gradient separation. The resulting PBMC were stimulated with 0.5% phytohemagglutinin (PHA) for 3 days, washed in Hanks' balanced salt solution (HBSS), and resuspended in HUT medium (RPMI 1640 supplemented with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine) with Polybrene (6 µg/ml) at 36°C for 30 min. PBMC were then resuspended to  $10^6$  cells/ml in HUT medium with 100 U interleukin 2 (IL-2), 500 µL was plated, and an additional 1 ml HUT medium with 100 U IL-2 was added. PBMC neutralization assays were performed using the primary viral isolates SF162 and SF2 at a 1:4 dilution of virus stock in RPMI medium and a 1:10 dilution of serum samples in HUT medium. The sera and virus solutions were combined 1:1 (100 µl:100 µl), kept at room temperature for 30 min with shaking, and then diluted 1:5 in HUT medium containing 100 U IL-2. An aliquot of the resulting solution (400 µl) was added to the plated PBMC in duplicate wells to total 1.9 ml/well. Cells were passaged on days 4, 7, and 10 postinfection and 500 µL from each well was saved for reverse transcriptase (RT) and p24 assays.

PBMC assays were also performed using replication-competent, *Rennilla reniformis* luciferase (LucR)-expressing HIV-1 reporter infectious molecular clones (IMC) SF162 and Bal (R5-tropic). IMC were diluted to yield  $>1 \times 10^5$  RLU at day 4 postinfection. Sera and virus were incubated together for 60 min prior to the addition of  $1 \times 10^5$  PHA/IL-2-stimulated PBMC. Sera, virus, and cells were incubated overnight at 37°C. Fresh medium was added, and the culture was incubated at 37°C until day 4. The cultures were lysed, and the LucR substrate was added, following the manufacturer's instructions (PerkinElmer Life Sciences, Shelton, CT). The endpoint was determined by quantifying the RLU of virus-derived luciferase using the Envision luminometer (PerkinElmer Life Sciences).

**Antibody-dependent cell-mediated viral inhibition.** The target cells for the antibody-dependent cell-mediated virus inhibition (ADCVI) assay were prepared by infecting Polybrene-treated CEM.NKR-CCR5 cells (National Institutes of Health AIDS Research and Reference Reagent Program) with a clinical strain of HIV-1 (92US657; clade B, R5-tropic) at a multiplicity of infection of about 0.05. After 48 h, the target cells were washed to remove cell-free virus. The effector cells (PBMC from healthy donors) were added to the target cells at an effector-to-target cell ratio (E:T) of 10:1. The test sera were added to the target and effector cells to achieve a final dilution of 1/100. Seven days later, the supernatant fluid was collected, and p24 was measured by an ELISA (ZeptoMetrix). The percent inhibition due to ADCVI was calculated relative to the pooled HIV-negative sera as follows: percent inhibition =  $100(1 - [(p24p)/(p24n)])$ , where (p24p) and (p24n) are concentrations of p24 in supernatant fluid from wells containing a source of HIV-positive or -negative antibodies (Ab), respectively.

## RESULTS AND DISCUSSION

The failure to generate robust humoral immune responses toward the membrane proximal external region (MPER) of gp41 of HIV-1 upon immunization has been attributed to the inability to present the epitopes in the proper conformation, poor overall immunogenicity, or immunological tolerance. We hypothesized that the addition of chemical modifications adjacent to the 2F5 and 4E10 epitopes using the full-length sequence of the MPER would break the immune tolerance with mimetics of inflammation-associated posttranslational modifications by generating neopeptides capable of inducing more robust immune responses. Support for this hypothesis is 4-fold. First, posttranslational mod-

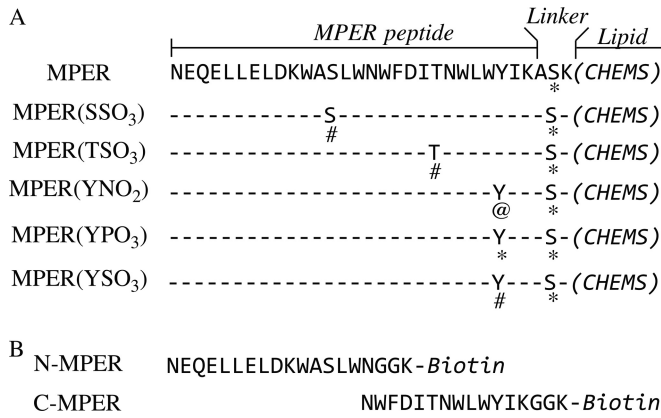
ifications have been shown to arise due to inflammation and cell stress-induced apoptosis (22). Second, HIV-1 infection leads to CD4 T cell death through pyroptosis causing an inflammatory immune response (33). Third, protein kinases have been shown to be dysregulated during acute HIV-1 infection (34). Fourth, post-translational modifications have been observed to induce humoral and innate immune responses toward previously unrecognized self-proteins (23, 24, 35, 36). To test this hypothesis, we investigated the roles of chemical modifications using sulfate, phosphate, or nitrate on the immunogenicity of the MPER.

**Design of chemically modified MPER peptides and lipopeptides.** Previously, we reported the synthesis and evaluation of chemically modified MPER-based peptides containing phosphorylation and nitration (21). We showed that the modified peptides bound with the same affinity to monoclonal antibodies as did the native sequences. When implemented in NZW rabbit immunizations, we observed higher antisera titers directed toward the immunogens when we used longer peptides (25 amino acids versus 16 or 19 amino acids) and phosphorylation of threonine-676 induced high epitope-specific antibody titers in two of three rabbits. These results directed our path forward in designing the next library of immunogens.

We prepared lipopeptides with three new design characteristics. First, we extended the peptide sequence to encompass the entire MPER motif containing the 2F5 and 4E10 epitopes with flanking residues shown to improve binding (37, 38), and we added an additional short linker sequence for conjugation to a lipid anchor. Each peptide used in this study has 31 residues spanning the full MPER peptide containing both the 2F5 and 4E10 epitopes compared to the peptides used in the previous study, which were 25 amino acids (N-MPERext and C-MPERext) 19 amino acids (N-MPER), and 16 amino acids (C-MPER). Second, we included sulfate, nitrate, and phosphate chemical modifications at serine, threonine, and tyrosine residues, which can undergo posttranslational modifications *in vivo*. Finally, since these peptides would be presented in a liposomal bilayer we conjugated cholesteryl hemisuccinate on the C-terminal lysine as we have done in the past; however, in this case we introduced a phosphoserine one residue N-terminal to the lysine in an effort to inhibit the hydrophobic peptide from being buried in the liposomal bilayer. Each of these components is highlighted in Fig. 1.

The stability of the sulfate modification was of special concern due to the pH sensitivity of sulfate-containing amino acids. To address the concerns regarding the loss of the chemical modification during peptide cleavage and deprotection after Fmoc-based solid-phase peptide synthesis, noncleavable sulfonates were used in place of sulfates. Cysteic acid was employed in the peptide as an analogue of sulfoserine and sulfothreonine to prepare MPER(SSO<sub>3</sub>) and MPER(TSO<sub>3</sub>). Sulfotyrosine was synthesized according to a previous protocol (26) through use of an acid-stable neopentyl protecting group on sulfotyrosine. Neopentyl-protected sulfotyrosine resists both acid and base hydrolysis conditions experienced during peptide synthesis and cleavage, resulting in the neopentyl-protected peptide. The neopentyl group is removed by ammonium acetate at 37°C. The synthesis and installation of this neopentyl-protected amino acid enables the successful preparation of MPER(YSO<sub>3</sub>).

**Effects of side chain modifications on antibody responses to MPER lipopeptides in NZW rabbits.** Six MPER lipopeptides were evaluated in groups of three rabbits to determine the effects

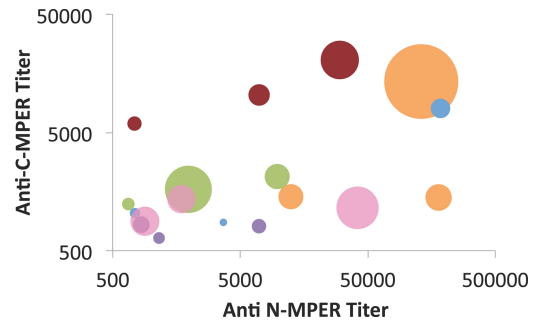


**FIG 1** MPER lipopeptides. (A) MPER peptides containing the nominal epitopes of monoclonal antibodies 2F5 and 4E10 with additional flanking sequences previously reported to improve binding (37, 38). The C terminus is appended with a three-residue linker that is phosphorylated to improve exposure in the liposomal bilayer and a lysine for on-resin lipid conjugation of the cholesteryl hemisuccinate lipid anchor (CHEMS). Residues that were modified with sulfate (#), phosphate (\*), or nitrate (@) are indicated. (B) N-MPER and C-MPER biotinylated peptides used for ELISAs.

of side chain modifications on the immunogenicity of the MPER lipopeptides. All peptides contained the C-terminal serine phosphate adjacent to cholesteryl hemisuccinate and were then characterized by any additional modification. The peptides used for the rabbit immunizations included an unmodified peptide (MPER), three peptides with tyrosine modifications C-terminal to both the 2F5 and 4E10 epitopes [sulfate-MPER(YSO<sub>3</sub>), phosphate-MPER(YPO<sub>3</sub>), and nitrate-MPER(YNO<sub>2</sub>)], one peptide containing a sulfate adjacent to the 2F5 epitope [MPER(SSO<sub>3</sub>)], and one peptide containing a sulfate within the 4E10 epitope [MPER(TSO<sub>3</sub>)] (Fig. 1A). Each immunogen was evaluated in six groups of three NZW rabbits.

High anti-immunogen responses were observed in all 18 rabbits as determined by ELISA subsequent to a 73-day immunization that included four subcutaneous immunizations (days 0, 21, 42, and 63). The sera collected at days 0, 52, and 73 were evaluated for anti-immunogen reciprocal endpoint titers. The MPER peptide provided the highest anti-immunogen geometric mean titer (GMT) of  $2.3 \times 10^5$  followed by MPER(YSO<sub>3</sub>) (GMT,  $1.8 \times 10^5$ ), MPER(YPO<sub>3</sub>) (GMT,  $1.0 \times 10^5$ ), MPER(SSO<sub>3</sub>) (GMT,  $8.7 \times 10^4$ ), MPER(YNO<sub>2</sub>) (GMT,  $3.4 \times 10^4$ ), and MPER(TSO<sub>3</sub>) (GMT,  $2.2 \times 10^4$ ). These titers are higher than those in our previous report of chemically modified peptides, where the group with the largest geometric mean titer was  $5.7 \times 10^3$  (21). This improvement may be attributed to the extended length of the peptides, the C-terminal phosphate, an improved immunization schedule, or a combination of all three.

To further probe the antibody responses directed toward the MPER, we evaluated the titer of each serum sample by an ELISA toward an N-terminal peptide segment of the MPER containing the 2F5 epitope (NEQELLELDKWASLWN) or a C-terminal peptide segment containing the 4E10 epitope (NWFDITNWLWYIK) (Fig. 1B) (epitopes are shown in italics). In general, there was a weak response toward the C-terminal peptide as seen by the reciprocal endpoint titers of  $\leq 1,000$ ; however, animals immunized with MPER(SSO<sub>3</sub>) achieved a GMT of  $1.1 \times 10^4$ , nearly 1 order of magnitude higher than those for all other groups. While a grouped

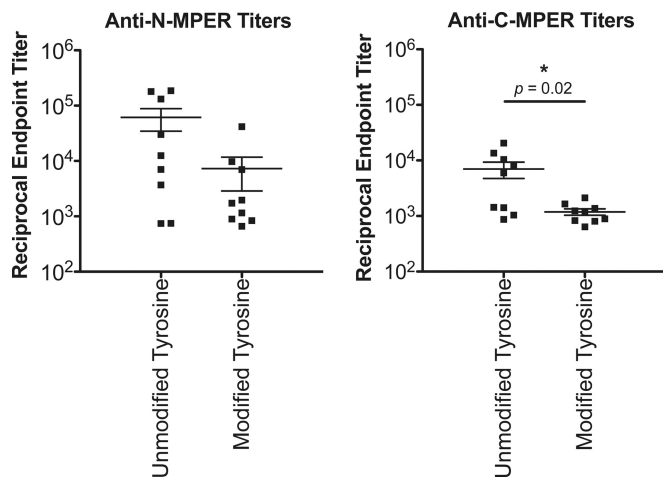


**FIG 2** Reciprocal endpoint titers of individual rabbits. Spots represent individual rabbit titers: anti-N-MPER titers (NEQELLELDKWASLWN) on the x axis and anti-C-MPER titers (NWFDITNWLWYIK) on the y axis. The size of each spot corresponds to the anti-immunogen titers containing the respective chemical modification (NEQELLELDKWASLWNWFDITNWLWYIKASK). The anti-immunogen titers depicted by the spots range from  $9 \times 10^3$  [MPER(TSO<sub>3</sub>)] to  $9 \times 10^5$  (MPER). Groups of rabbits ( $n = 3$  for all groups) are color coded according to their immunogen: orange, MPER; maroon, MPER(SSO<sub>3</sub>); blue, MPER(TSO<sub>3</sub>); pink, MPER(YSO<sub>3</sub>); purple, MPER(YNO<sub>2</sub>); green, MPER(YPO<sub>3</sub>).

analysis of titers using geometric mean averages provides one view of the data, the intricacies of each animal are not properly represented using this analysis.

To accurately represent the data visually, individual animal titers are plotted in Fig. 2, where the x axis corresponds to the anti-N-MPER titers, the y axis corresponds to the anti-C-MPER titers, and the size of the spot corresponds to the anti-immunogen titers. Groups of animals, with three rabbits per group, are color coded. Plotting the data in this manner provides a group perspective and an individual animal perspective of epitope-specific titers. From this graph, it is apparent that most animals had weak responses toward the C-MPER peptide. Animals immunized with MPER(SSO<sub>3</sub>) had the highest anti C-MPER responses as a group ( $6 \times 10^3$ ,  $1 \times 10^4$ , and  $2 \times 10^4$ ), but low to moderate anti-N-MPER responses ( $7 \times 10^2$ ,  $7 \times 10^3$ , and  $3 \times 10^4$ ), respectively. Other animals with elevated anti-C-MPER titers include one animal immunized with MPER(TSO<sub>3</sub>) ( $8 \times 10^3$ ) and one animal immunized with MPER ( $1 \times 10^4$ ), which is also the serum with the highest anti-immunogen titer ( $9 \times 10^5$ ). These data also suggest that residue-specific modifications may direct the immune responses toward specific ends of the peptide. In particular, the animals immunized with the tyrosine-modified peptides failed to induce responses toward the C terminus, and five out of nine rabbits lacking tyrosine modifications possessed elevated anti-C-MPER titers (Fig. 3). Anti-N-MPER titers are more varied within each group. The MPER peptide presentation and orientation in liposomes have been shown to be critical for immunogenicity, especially with tryptophan-680, which is adjacent to this tyrosine (39–41), suggesting that chemical modifications at this residue may change the orientation of the peptide in the bilayer.

Additional trends observed in Fig. 2 are that animals immunized with MPER(YSO<sub>3</sub>) had weak anti-C-MPER responses ( $9 \times 10^2$ ,  $1 \times 10^3$ , and  $1 \times 10^3$ ) and two of three rabbits had weak anti-N-MPER responses ( $8 \times 10^2$ ,  $2 \times 10^3$ , and  $4 \times 10^4$ ), but all three rabbits had elevated anti-immunogen titers ( $1 \times 10^5$ ,  $1 \times 10^5$ , and  $3 \times 10^5$ ). These data indicate one of the following: a sulfate-specific response, a C-terminal phosphate-specific response, a response to the middle of the full-length sequence which

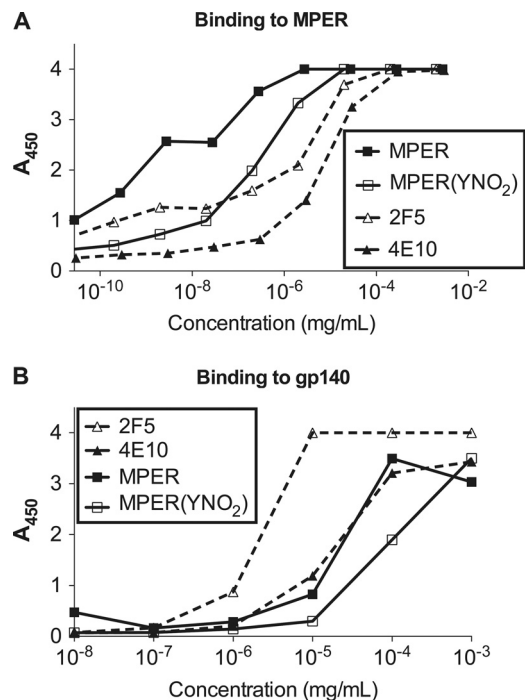


**FIG 3** Anti-N-MPER and anti-C-MPER titers based on the modification of tyrosine. Reciprocal endpoint titers of rabbits immunized with tyrosine or tyrosine unmodified peptides. Rabbits immunized with peptides containing a modified tyrosine fail to respond to the C-terminal peptide (NWFDTINWLWYIK), while five of nine rabbits immunized with unmodified tyrosine responded to the same sequence (anti-N-MPER titers,  $P = 0.86$ ; anti-C-MPER titers,  $P = 0.02$ ).

is not present in the short peptides, a secondary structure induced by the chemical modification, or a combination of effects. ELISA were performed using a series of immobilized peptides of different length and different chemical modifications (see Fig. S1 in the supplemental material). The full-length peptide containing only the C-terminal phosphate produced titers similar to those obtained using MPER( $\text{YSO}_3$ ), providing evidence that the response is not sulfate specific. The titers toward the fully unmodified peptide, however, are 1 to 2 orders of magnitude lower but still higher than the anti-N-MPER responses. This result suggests that, in this animal, the immune response was induced toward the C-terminal phosphate and is epitope specific. It is also likely directed toward an epitope that is either not present in the short peptide sequences or toward a secondary structure induced by the modification.

**Affinity purification and characterization of gp140 specific antibodies.** After characterizing the polyclonal antibody titers and specificity of each rabbit serum sample toward MPER-based peptides spanning the 2F5 epitope, 4E10 epitope, or both epitopes, we determined if these sera also bound to recombinant gp140 using an ELISA. Only two samples possessed positive gp140 binding (Fig. 4A; see also Fig. S2 in the supplemental material); one rabbit was immunized with the MPER and the other was immunized with MPER( $\text{YNO}_2$ ). The rabbit immunized with the MPER possessed the largest anti-immunogen titer ( $9 \times 10^5$ ) and the second and third highest titers toward the C-MPER ( $1 \times 10^4$ ) and N-MPER ( $1 \times 10^5$ ) peptides, respectively. In contrast, the sample from the rabbit immunized with MPER( $\text{YNO}_2$ ) did not bind to the C-MPER ( $8 \times 10^2$ ) peptide and had a weak anti-N-MPER titer ( $7 \times 10^3$ ) and a moderate anti-immunogen titer ( $4 \times 10^4$ ). Each serum sample was then affinity-purified using the MPER peptide. From 6 ml of rabbit serum, 3.3 mg of polyclonal affinity-purified antibodies were obtained from the rabbit immunized with the MPER and 0.8 mg from the rabbit immunized with MPER( $\text{YNO}_2$ ).

The resulting affinity-purified antibodies were then evaluated for binding using an ELISA toward gp140 or the MPER peptide.

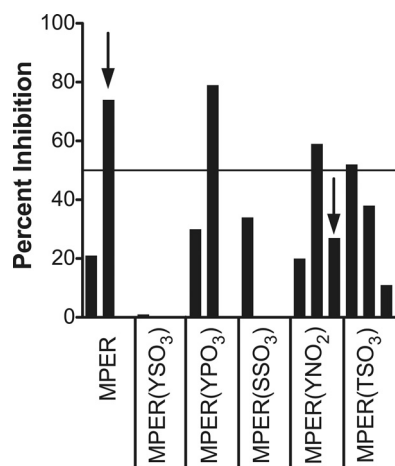


**FIG 4** Characterization and of rabbit sera and affinity-purified antibodies that bind gp140. (A) Binding of epitope-specific purified-antibodies toward the MPER peptide by ELISA. (B) Binding of epitope-specific purified antibodies toward recombinant gp140 protein by ELISA.

Affinity-purified antisera induced with the MPER possessed a titer toward gp140 comparable to that for the 4E10 antibody, while MPER( $\text{YNO}_2$ ) induced antisera possessing a lower titer (Fig. 4B). The titers of both purified serum samples toward the MPER peptide, however, were increased compared with those for 2F5 and 4E10 (Fig. 4C).

**Neutralization with whole sera and affinity-purified antibodies.** A panel of neutralization assays were performed to determine the potencies of the various antisera using H9 cells, TZM-bl cells, and PBMC with five clade B viruses. In the H9 cell neutralization assay, using an H9-adapted MN virus isolate, we observed modest viral inhibition in four samples corresponding to rabbits immunized with the MPER (74% inhibition), MPER( $\text{YPO}_3$ ) (79%), MPER( $\text{YNO}_2$ ) (59%), and MPER( $\text{TSO}_3$ ) (52%) (Fig. 5). In all other rabbit sera, viral inhibition did not exceed 38%. Additionally, the rabbit immunized with the MPER that possessed 74% inhibition corresponded to the same animal that possessed high anti-immunogen titers and a robust titer for gp140. The other serum sample with positive gp140 binding from the rabbit immunized with MPER( $\text{YNO}_2$ ) had a modest 20% inhibition in this assay. Purified serum samples at concentrations of 25  $\mu\text{g/ml}$ , however, failed to neutralize (see Fig. S3 in the supplemental material).

Viral inhibition was also assessed using luciferase reporter-based assays, in TZM-bl cells with SF162 and BZ167 pseudoviruses, in human PBMC with SF162 and Bal infectious molecular clones, and in human PBMC with SF2 primary isolates (see Fig. S4 to S6 in the supplemental material). All serum samples and affinity-purified antibodies failed to inhibit viral infection in these assays. Neutralization observed in the H9 cell assay and the lack of neutralization in all other assays is likely due to the increased neutralization sensitivity of T cell-line adapted viruses (42).



**FIG 5** HIV-1 neutralization using H9 cell targets and rabbit serum antibodies. Neutralization data from 1:20 dilutions of rabbit sera in an H9 cell neutralization assay using the MN viral strain of HIV-1 are shown. Each bar represents an average from duplicate experiments. Arrows denote sera that possessed gp140 binding.

To further explore the potential for neutralization, infected NK cells were incubated with rabbit serum samples and human PBMC to determine the level of antibody dependent cell-mediated viral inhibition (ADCVI). Once again, inhibition of viral infection using the antiserum from each of the rabbits was not observed (see Fig. S7 in the supplemental material). These data, coupled with the failure to neutralize the virus in the H9 cell, TZM-bl cell, and PBMC assays, suggest that although we have achieved potent binding antibodies directed toward the MPER peptide and gp140 using chemically modified epitopes, induction of MPER-specific neutralizing antibodies through vaccination remains an elusive goal.

In conclusion, the focus of our study was to learn if chemical modifications to amino acids in the MPER epitope might induce robust humoral immunity or generate broadly neutralizing antibodies to HIV-1. We followed the hypothesis that neutralizing antibodies targeting the MPER sequence are not generated in animals, including humans, because the sequence is recognized as self and B cells that recognize this sequence have been deleted (17). We hypothesized that chemical modifications within the MPER sequence that mimic posttranslational modifications might present neoepitopes to the immune system, break tolerance, and lead to protective immunity. Using a panel of chemically modified peptides (Fig. 1), we achieved a robust immune response toward the immunogens in all animals (Fig. 2; see also Fig. S1 in the supplemental material), but failed to generate neutralizing antibodies.

While we have succeeded in presenting peptides in a manner that elicits a strong humoral immune response, more precise approaches that activate a smaller subset of epitope-specific B cells with a potential to neutralize HIV may be more appropriate. The epitopes in the liposomal bilayer are presented as short monomeric peptides with little steric hindrance; however, the epitopes on the viral capsid are presented as trimer bundles with a notable amount of steric protection from gp120 above the MPER and the viral membrane below the epitopes. Substantial interest has focused on the development of trimer bundles to induce more nat-

ural epitope-specific responses (43, 44), but such studies, if done in the context of a liposomal vaccine, have not been published.

Rational design of sterically hindered epitopes has not been investigated but may also be achieved using chemical approaches, which append nonimmunogenic polymers from the N terminus of the peptides. Once anchored in the liposomal bilayer, the polymer-conjugated epitopes will then more closely represent the natural state of the epitope, which is shielded from promiscuous antibody interactions. A strategy of sterically hindered, chemically modified epitopes might engage a smaller population of B cells capable of inducing antibodies that neutralize HIV-1. The chemically engineered modified immunogen strategy might be a component of an iterative HIV vaccination approach (45).

Given the robust immune response observed using the chemically modified liposomal immunogens described here, we conclude the following. First, full-length MPER lipopeptides with chemical modifications presented in liposomal formulations induce robust humoral immune responses toward the immunogens (Fig. 2). Second, tyrosine modifications dampen the immune response (Fig. 3). Third, high antibody titer responses toward MPER peptides do not translate to robust binding with gp140, and high titer binding to the purified gp140 does not translate to HIV-1 neutralization (see Fig. S1, S2, and S3 in the supplemental material) as has been shown in other published studies (46). Although we have not yet succeeded in inducing neutralizing antibodies against HIV-1, the chemically modified epitope tactic should be investigated further as a potential component of an HIV-1 vaccine.

#### ACKNOWLEDGMENTS

We thank Anthony DeFranco (UCSF) and Lewis Lanier (UCSF) for helpful discussions and Gary Fujii (Molecular Express, Inc., Rancho Dominguez, CA) for helpful suggestions regarding the liposome formulation. We also thank Nancy Miller (NIH) and Alan Schultz (NIH) for their encouragement and support.

This work was supported by NIH grants R01 GM061851, R21 AI093135, and HHSN27201100016C and by cooperative agreement DAMD17-93-V-3004 between the U.S. Army Medical Research and Materiel Command, the Henry M. Jackson Foundation, and the Division of AIDS, NIAID, NIH. V. J. Venditto was supported by Ruth L. Kirschstein National Research Service Award F32AI095062 from the NIAID, NIH.

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