Vaccination of Rhesus Monkeys with Synthetic Peptide in a Fusogenic Proteoliposome Elicits Simian Immunodeficiency Virus–specific CD8⁺ Cytotoxic T Lymphocytes

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Summary

An effective vaccine against the human immunodeficiency virus should be capable of eliciting both an antibody and a cytotoxic T lymphocyte (CTL) response. However, when viral proteins and peptides are formulated with traditional immunological adjuvants and inoculated via a route acceptable for use in humans, they have not been successful at eliciting virus-specific, major histocompatibility complex (MHC) class I-restricted CTL. We have designed a novel viral subunit vaccine by encapsulating a previously defined synthetic peptide CTL epitope of the simian immunodeficiency virus (SIV) gag protein within a proteoliposome capable of attaching to and fusing with plasma membranes. Upon fusing, the encapsulated contents of this proteoliposome can enter the MHC class I processing pathway through the cytoplasm. In this report, we show that after a single intramuscular vaccination, rhesus monkeys develop a CD8⁺ cell-mediated, MHC class I-restricted CTL response that recognizes the synthetic peptide immunogen. The induced CTL also demonstrate antiviral immunity by recognizing SIV gag protein endogenously processed by target cells infected with SIV/vaccinia recombinant virus. These results demonstrate that virus-specific, MHC class I-restricted, CD8⁺ CTL can be elicited by a safe, nonreplicating viral subunit vaccine in a primate model for acquired immune deficiency syndrome. Moreover, the proteoliposome vaccine formation described can include multiple synthetic peptide epitopes, and, thus, offers a simple means of generating antiviral cell-mediated immunity in a genetically heterogeneous population.

The simian immunodeficiency virus (SIV)/macaque monkey model of AIDS provides an important system for evaluating HIV vaccines. Protective immunity against a cell-free AIDS virus infection has previously been demonstrated in this model using inactivated whole virus vaccines (1, 2). However, perhaps because inactivated virus vaccines are not likely to induce CTL, this approach has resulted in only partial protection of monkeys from cell-associated SIV challenge (3). More recently, Hu et al. (4) have demonstrated protection against SIV using a combination of a live recombinant SIV/vaccinia virus followed by recombinant SIV env protein. Although live vectors can induce CTL (5), there may be a safety risk involved in the large scale use of some vaccines of this type (6, 7). Thus, we sought to develop a strategy for inducing SIV-specific MHC class I-restricted CTL through the use of a nonreplicating subunit vaccine.

MHC class I antigen presentation generally occurs only for antigens synthesized and processed within a cell (8). However, early studies had shown that viral antigens of replication-inactivated fusogenic enveloped viruses could be presented by MHC class I molecules (9). More recently it has been demonstrated that exogenous protein antigens can be presented by MHC class I molecules if these antigens are introduced directly into the cytoplasm without entering the endosomal/lysosomal degradation pathway (10, 11). Our laboratory has developed procedures for preparing large unilamellar liposomes with biologically active Sendai or influenza glycoproteins incorporated into the liposome bilayer (12). The glycoproteins enable these proteoliposomes to deliver drugs, proteins, and nucleic acids directly into the cytoplasm of cells either in vitro or in vivo (13). These proteoliposomes can therefore introduce encapsulated antigen directly into the cytoplasm for subsequent presentation by MHC class I molecules. Previously we have shown that SIVmac-infected rhesus
monkeys sharing a particular MHC class I molecule, *Mamu-A*¹⁰, develop a gag-specific CD8⁺ CTL response that recognizes a nine-amino acid epitope of gag p27, and is restricted by *Mamu-A*¹⁰ (14). In the present study, we have assessed whether a 12-amino acid synthetic peptide (peptide 11C) containing this CTL epitope induces a MHC class I-restricted CTL response in rhesus monkeys when noncovalently encapsulated within a proteoliposome capable of fusing to cellular membranes.

**Materials and Methods**

**Animals.** Heparinized blood was obtained from immunized rhesus monkeys (*Macaca mulatta*) every other week.

**Cell Lines.** Rhesus monkey B-lymphoblastoid cell lines (B-LCL) were generated by incubating 10⁷ Ficoll-Diatrizoate-isolated PBMC with 100 µl of SS94. SS94 is a cell line productively infected with the transforming herpesvirus *Herpes papio*. The B-LCL were maintained in antibiotic-supplemented RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10% FCS (Flow Laboratories, McLean, VA).

One-dimensional (1-D) IEF. This technique has been completely described (16). Briefly, 5 × 10⁶ B-LCL from a group of rhesus monkeys were metabolically labeled and MHC class I molecules were immunoprecipitated with a human MHC class I-specific mAb. These precipitates were phase separated using Triton X-114 extraction and then treated with neuraminidase (type VI; Sigma Chemical Co., St. Louis, MO) to remove sialic acid residues. Finally, the precipitates were subjected to 1-D IEF under reducing conditions and then treated with neuraminidase (type VI; Sigma Chemical Co., St. Louis, MO) to remove sialic acid residues. Finally, the precipitates were subjected to 1-D IEF under reducing conditions and an autoradiograph was prepared.

**Immunogen Formulations.** Unmodified liposomes: large unilamellar phosphatidyl serine/cholesterol (9:1 weight ratio) vesicles containing buffer, with or without peptide 11C, were prepared by direct addition of EDTA to lipid cochleates (15). Sendai proteoliposomes: the envelope glycoproteins and lipids of Sendai virus (parainfluenza type I) were extracted, converted to lipid protein cochleates, then to proteoliposomes by direct addition of EDTA (15). Vaccinations contained 100 µg of peptide 11C (EGCT-PYDINQML) encapsulated in 1.5 mg lipid. The peptide 11C used to immunize was synthesized using a stepwise solid-phase method and subsequently HPLC purified.

**In Vitro Restimulation of T Cells.** 5–12 × 10⁶ Ficoll-Diatrizoate-isolated PBMC were placed in 2 ml of RPMI containing 20% FCS and 10 µg/ml of peptide 11C (Multiple Peptide Systems, San Diego, CA) in a 12-well plate. On day 3, 2 × 10⁴ mitomycin C (Sigma Chemical Co.)-treated autologous 7–10-d-old Con A (Sigma Chemical Co.)-activated rIL-2 (Hoffman-La Roche, Inc., Nutley, NJ)-expanded cells were pulsed with 50 µg/ml peptide 11C in 300 µl and then added to the cultures along with 2 ml of RPMI/20% FCS and 40 U/ml of rIL-2. Cells were maintained for a total of 9–11 d before the assay. Dead cells were removed by Ficoll-Diatrizoate centrifugation.

**Cytotoxicity Assay.** Target cells were either autologous (Mm 90 and Mm 211) or *Mamu-A*¹⁰ + allogeneic H. *pepo*-immortalized B-LCL incubated at 37°C with 50 µg/ml of peptide 11B (ALSEGCTPYDIN) or 11C and 0.50 mCi/ml Na₂¹⁵CrO₄ (ICN Laboratories, Irvine, CA) for 16 h and then washed twice. Target cells were plated at 10⁴ cells per well and effector cells were added at various E/T ratios. These cell mixtures were incubated for 4 h at 37°C. Percent peptide 11C-specific lysis is calculated as percent specific lysis of peptide 11C-pulsed target cells minus the percent specific lysis of peptide 11B-pulsed target cells. Percent specific lysis is calculated as: 100x (experimental release – spontaneous release)/(maximum release – spontaneous release). All experimental values were calculated in duplicate while control releases were done in quadruplicate.

**CD8⁺ Cell Depletion.** 2 d before the assay, CD8⁺ cells were isolated by incubating the cultured cells with an anti-CD8 mAb (7PT3F9, 1:400 dilution of ascites; S. Schlossman, Dana Farber Cancer Institute, Boston, MA) followed by immunomagnetic bead separation (Dynal, Oslo, Norway) using a bead-to-cell ratio of 40:1. Just before the assay the magnetic beads were removed from the CD8⁺ cells. The live cells were then isolated by Ficoll-Diatrizoate density centrifugation. The CD8-depleted population contained <5% containing CD8⁺ cells as assessed by flow cytometry (data not shown).

**Results and Discussion**

As peptide 11C-specific CTL are restricted by the MHC class I molecule *Mamu-A*¹⁰, the MHC class I phenotype of a group of rhesus monkeys was assessed in order to select potential responder animals for peptide 11C immunization. 1-D IEF was used to identify thses monkeys expressing *Mamu-A*¹⁰ by comparing the migration of immunoprecipitated

![Figure 1](https://example.com/figure1.png)

**Figure 1.** 1-D IEF analysis of the MHC class I molecules of a group of rhesus monkeys. Lysates were subjected to electrophoresis over a pH gradient of 5–7. The first seven lanes represent animals expressing the MHC class I molecule *Mamu-A*¹⁰, with Mm 177 having been previously defined; the last two lanes represent two *Mamu-A*¹⁰⁻ animals. The positions of *Mamu-A*¹⁰ and β₂-microglobulin are indicated with arrows.
MHC class I molecules from B-LCL of a cohort of animals to that of the previously characterized SIV-infected, Mamu-A*01 + rhesus monkey Mm 177. Six Mamu-A*01 + animals were identified for immunization studies (Fig. 1). These results were confirmed by demonstrating the capacity of peptide 11C-specific CTL from Mm 177 to lyse peptide 11C-pulsed B-LCL derived from the six monkeys (data not shown).

Initially, two Mamu-A*01 + rhesus monkeys (Mm 91 and 138) were immunized three times intramuscularly with 100 μg of peptide 11C encapsulated in unmodified liposomes, while two Mamu-A*01 + monkeys (Mm 59 and 347) were given unmodified liposomes without peptide according to a similar schedule. As shown in Fig. 2 A, left, peptide 11C-restimulated cultures of PBMC from these animals did not develop any detectable peptide 11C-specific lytic activity. Similarly restimulated PBMC from the SIV-infected animal Mm 177 reproducibly demonstrated peptide 11C-specific CTL activity (data not shown).

The immunogenicity of peptide 11C encapsulated within fusogenic liposomes containing the attachment and fusion glycoproteins of Sendai virus was assessed in the same monkeys that previously failed to respond to peptide 11C encapsulated in unmodified liposomes. The PBMC from monkeys receiving 100 μg of peptide 11C in the Sendai proteoliposome formulation demonstrated a peptide 11C-specific lytic response, whereas those animals receiving the empty proteoliposomes did not (Fig. 2 A, right). These results were confirmed by immunizing a second group of naive animals, two Mamu-A*01 + (Mm 8 and 196) and two Mamu-A*01 - (Mm 90 and 211), with identical peptide 11C/Sendai proteoliposome formulations (Fig. 2 B). All four peptide 11C/proteoliposome-immunized Mamu-A*01 + monkeys developed peptide-specific lytic activity within 2 wk of primary immunization. The peptide-specific lytic activity was demonstrable >2 mo after the final immunization and was consistently demonstrated over the period in which the assays were performed (data not shown).

The peptide-specific effector cells generated in the peptide 11C/proteoliposome-immunized Mamu-A*01 + animals were CD8+, MHC class I-restricted CTL. Rhesus monkeys not
expressing *Mamu-A*01 failed to generate a lytic response after two immunizations with the immunogenic peptide 11C/proteoliposome (Fig. 2 B). In addition, PBMC from peptide 11C/proteoliposome–immunized *Mamu-A*01+ monkeys were able to lyse either peptide 11C–pulsed *Mamu-A*01+ rhesus monkey allogeneic B-LCL or a human B cell line stably transfected with *Mamu-A*01, but not cell lines that were *Mamu-A*01− (Table 1). Finally, effector cell populations depleted of CD8+ cells by antibody/immunobead selection demonstrated no peptide 11C–specific lytic activity, while the purified CD8+ cells exhibited enriched activity (Fig. 3).

The capacity of this vaccine to generate CTL that would recognize virally infected cells was assessed using target cells infected with vaccinia recombinants containing the SIVmac251 gag gene. As shown in Fig. 4, PBMC from all four peptide/proteoliposome–immunized *Mamu-A*01+ animals were able to lyse target cells expressing the SIVmac gag epitope as a result of vaccinia/SIVmac gag recombinant infection and endogenous antigen processing. These effector cells did not lyse target cells infected with a control vaccinia construct. The gag-specific lytic activity was not present in p11C-restimulated PBMC of these animals before immunization (data not shown).

The induction of CD8+, MHC class I–restricted CTL specific for SIVmac gag using the relatively hydrophilic SIVmac gag peptide 11C requires that the peptide be encapsulated within liposomes containing the attachment and fusion proteins of Sendai virus integrated into the liposome bilayer. This observation together with previous demonstrations of preparations that induce CD8+ CTL (15–19) suggest that the minimal immunogenic formulation for the elicitation of CD8+ CTL includes: (a) a peptide that represents a MHC class I epitope; (b) a component that enhances uptake by the MHC class I–presenting cells of the reticuloendothelial system; and (c) properties that can compromise the integrity of a lipid bilayer, facilitating delivery of the antigen directly into the cytoplasm. The observation that an OVA peptide can induce CD8+ CTL in mice after intravenous administration in saline appears to be an exception to this paradigm (20). However, the amino terminus of this OVA peptide epitope is highly hydrophobic and has special membrane-perturbing characteristics that may subserve all of these postulated immunogenic requirements. We have had similar results inducing CD8+ CTL in mice using a strongly amphipathic HIV peptide epitope encapsulated in liposomes without the inclusion of Sendai glycoproteins (our unpublished observations).

**Table 1. Peptide 11C/Proteoliposome–induced CTL Are Restricted by the Rhesus Monkey MHC Class I Molecule Mamu-A*01**

| Effector cells$^5$ | E/T ratio$^6$ | 11B | 11C | 11B | 11C | 11B | 11C | 11B | 11C |
|---------------------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Mm 138 (p11C Imm.) | 40:1        | 11  | 40  | 6   | 27  | 12  | 32  | 1   | 3   | 5   |
|                     | 20:1        | 8   | 23  | -4  | 19  | 6   | 21  | 5   | 9   | 4   |
|                     | 10:1        | 3   | 11  | 0   | 8   | 4   | 12  | 6   | 6   | 3   |
| Mm 8 (p11C Imm.)   | 40:1        | 7   | 41  | 5   | 23  | 15  | 42  | 3   | 8   | 12  |
|                     | 20:1        | 2   | 26  | 8   | 19  | 4   | 39  | 2   | 5   | 8   |
|                     | 10:1        | 2   | 17  | 7   | 12  | 7   | 20  | 0   | 2   | 5   |
| Mm 59 (control Imm.) | 40:1  | 1   | -3  | 3   | 4   | 10  | 8   | 1   | 1   | 1   |
|                     | 20:1        | -2  | 0   | 2   | 2   | 6   | 4   | 0   | 0   | 0   |
|                     | 10:1        | -5  | -1  | 3   | 1   | 5   | 3   | 0   | 0   | 0   |
| Mm 177 (SIV infected) | 8:1   | 0   | 51  | 2   | 50  | -2  | 41  | -5  | 3   | 2   |
|                     | 4:1         | -2  | 48  | 1   | 39  | -1  | 31  | 0   | 1   | 0   |
|                     | 2:1         | 0   | 33  | 1   | 27  | -1  | 23  | -2  | 0   | 1   |

* Target cells were *Mamu-A*01+ rhesus monkey allogeneic B-LCL (Mm 164 and 210), a *Mamu-A*01-transfected human EBV-immortalized B cell line .221(A1)(14), the untransfected .221 (25), and a *Mamu-A*01− rhesus monkey allogeneic B-LCL (Mm 90). All target cells were either pulsed with peptide 11B or 11C.

$^1$ E/T cell ratio

$^5$ Effector cells were peptide 11C–restimulated PBMC from peptide 11C/proteoliposome–immunized animals Mm 138 and 8 obtained at 41 and 11 wk after primary immunization, respectively; from proteoliposome control immunized animal Mm 59 at 41 wk; and from the *Mamu-A*01+, SIVmac251-infected animal Mm 177 at 2 yr postinfection.

$^6$ Percent specific release.
Figure 3. Peptide 11C-specific lysis is mediated by CD8+ cells. PBMC from all four Mamu-A*01+ peptide-immunized rhesus monkeys were re-stimulated in vitro and were separated into CD8+ (circles) and CD8− (triangles) populations. Target cells were a Mamu-A*01+ allogeneic rhesus B-LCL pulsed with peptide 11C (filled symbols) or 11B (open symbols).

However, the majority of CTL peptide epitopes do not have such unusual properties and, thus, will require an appropriate formulation to reliably induce CD8+ CTL. The proteoliposome formulation defined in the present study meets this objective without toxicity or inflammation in multiply immunized rhesus monkeys and with the ease of intramuscular administration.

Although recombinant HIV gp160 has been used to generate virus-specific CTL in mice (16), this study represents the first example of a viral subunit vaccination inducing CTL in an established model for HIV infection of humans. The use of peptide immunogens as a vaccination against HIV is attractive, as only those epitopes contributing to a beneficial immune response can be included, eliminating the possibility that immunization might enhance viral infectivity or induce autoreactivity (21, 22). Although the immunogenicity of a given peptide is dependent on the MHC of an individual, peptides corresponding to many defined epitope/MHC combinations (23) can be easily synthesized. Moreover, hypervariable epitopes can be accommodated by synthesizing multiple peptides corresponding to divergent virus strains. The Sendai proteoliposome encapsulation technique that we have employed is quite suitable for encapsulating cocktails of unmodified viral peptides and, thus, provides a simple method of generating MHC class I-restricted cellular immunity in outbred populations. In addition, these proteoliposome formulations can induce humoral immunity when B and T helper cell epitopes are covalently linked to phospholipids incorporated within the lipid bilayer (24). These flexible features of peptide/proteoliposomes as immunogens support the potential utility of this vaccination approach for the control of HIV and other infections in humans.

We thank D. Panicali (Therion Biologics Corp., Cambridge, MA) for providing the recombinant SIV/vaccinia constructs.

This work was supported by the National Institutes of Health grants AI-20729, CA-50139, DK-43351, AI-26507, and NCR R-00168, and funds provided by Medimmune, Inc.
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