Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9448–9452, November 1991
Medical Sciences

Priming of anti-human immunodeficiency virus (HIV) CD8$^+$ cytotoxic T cells *in vivo* by carrier-free HIV synthetic peptides

(immunization/vaccine/retrovirus)

MARY KATE HART*, KENT J. WEINHOLD†, RICHARD M. SCEARCE*, EILEEN M. WASHBURN*, CYNTHIA A. CLARK†, THOMAS J. PALKER*, AND BARTON F. HAYNES*‡

Departments of *Medicine and †Surgery and Duke Center For AIDS Research, Duke University Medical Center, Durham, NC 27710

Communicated by W. K. Joklik, July 29, 1991

ABSTRACT **The generation of antiviral cytotoxic T lymphocytes (CTLs) is a critical component of the immune response to viral infections. A safe and nontoxic vaccine for AIDS would optimally use a carrier-free synthetic peptide immunogen containing only components of HIV necessary for induction of protective immune responses. We report that hybrid synthetic peptides containing either a HIV envelope gp120 T-cell determinant (T1) or the envelope gp41 fusion domain (F) N-terminal to HIV CTL determinants are capable of priming murine CD8$^+$, major histocompatibility complex class I-restricted anti-HIV CTLs *in vivo*. These data demonstrate that carrier-free, nonderivatized synthetic peptides can be used *in vivo* to induce anti-HIV CTL responses.**

For priming of major histocompatibility complex (MHC) class I-restricted, CD8$^+$ antiviral cytotoxic T lymphocytes (CTLs) *in vivo*, viral infection of the host is usually required (1–3). In contrast, viral soluble proteins and synthetic peptides generally do not induce CTLs *in vivo* (2, 4–6). Recently, it has been suggested that viral infection of the host may not always be required for CD8$^+$ CTL generation, as examples of *in vivo* priming of CD8$^+$ CTLs with derivatized or free synthetic peptides have been reported (7–11). For HIV, viral isolate variability may necessitate the ability to include multiple HIV T-cell epitopes in an immunogen for induction of CTLs of disparate MHC haplotypes to many HIV isolates. A general strategy for the construction of multiple synthetic peptides capable of inducing CD8$^+$ CTLs is not known but is essential for the development of a multivalent T-cell immunogen for HIV. We have designed carrier-free synthetic peptides comprised of linear arrays of HIV envelope (env) sequences in various combinations of epitopes to determine peptide sequences capable of priming CD8$^+$ anti-HIV CTLs *in vivo*.

In prior work, we synthesized a HIV env gp120 T-cell epitope (T1) (12) N-terminal to hydrophilic gp120 B-cell epitopes from the V3 loop region (SP10 sequences) (13–15). Amino acid sequences of the gp120 HIV V3 loop (amino acids 303–338) have been found to contain the predominant neutralization determinant to which anti-HIV neutralizing antibodies bind (13–18). The hybrid T1-SP10 carrier-free peptide induced in goats and primates anti-HIV T-helper cell responses via the T1 gp120 region and high titers of anti-HIV neutralizing antibody responses against the SP10 gp120 V3 region (13–15).

To design peptides that potentially would induce anti-HIV CTL responses as well as neutralizing antibody responses, we have now synthesized a series of peptides from the HIV MN and IIIB isolates that include a defined cytotoxic T-cell epitope restricted to the murine MHC class I D^d molecule (19, 20). These new peptides from the MN and IIIB isolates, designated T1-SP10MN(A) and T1-SP10IIIB(A), respectively, extend the SP10 sequences from the HIV gp120 V3 region an additional 5 or 6 amino acids (amino acids 320–324 for MN; amino acids 322–327 for IIIB) to complete the variable D^d CTL epitope (Table 1).

Second, to make a carrier-free synthetic peptide that might insert into the cell membrane of antigen-presenting cells and facilitate processing via the class I pathway, the first 12 amino acids (amino acids 519–530) of the HIV env gp41 fusion domain (F) were synthesized N-terminal to the T1 region or N-terminal to the D^d CTL epitope (Table 1). The fusion domain of gp41 is highly hydrophobic and mediates HIV-induced cell fusion (23–25). In this study, we demonstrate that T1-SP10(A), F-T1-SP10(A), and F-SP10(A) hybrid peptides all prime CD8$^+$ anti-HIV CTLs *in vivo*.

METHODS

Peptides. Peptides were synthesized on an Applied Biosystems 431A peptide synthesizer using *tert*-butoxycarbonyl chemistry. Amino acid coupling efficiencies were monitored using ninhydrin reactions. Synthesized peptides were deprotected and cleaved from the supporting resin with hydrogen fluoride in the presence of 10% anisole, solubilized in 15–25% (vol/vol) glacial acetic acid, and lyophilized. Peptides were analyzed by amino acid analysis, HPLC analysis, and peptide N-terminal sequencing. Peptides were reconstituted in endotoxin-free phosphate-buffered saline (PBS) and dialyzed as described (14, 15) or were HPLC purified and the molecular mass determined by fast atom bombardment mass spectrometry (determined using a JEOL HX110HF double-focusing mass spectrometer by R. B. Van Breeman, North Carolina State University, Raleigh).

Immunizations. For each mouse, 10 μg of dialyzed peptide was brought up in 0.25 ml of PBS and emulsified with 0.25 ml of incomplete Freund's adjuvant except for the first immunization, when complete Freund's adjuvant was used. Mice were immunized subcutaneously in four sites every 2 weeks for a total of three to five immunizations. Spleens were removed from mice, teased into single cell suspensions, and cultured for 7 days *in vitro* with 25 μg of F-T1-SP10MN(A) peptide per ml of RPMI medium/EHAA (1:1) supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, and 10 μg of gentamicin per ml. On day 2, 10% (vol/vol) autologous Con A supernatant was added to the cultures. Con A supernatants were generated by culturing naive spleen cells at 1×10^6 per ml with 10 μg of ConA per ml (Sigma) for 4 days. On day 7, effector cells were washed in 0.3 M *methyl*-α-D-mannopyranoside (Sigma), and 6-hr ^{51}Cr-release

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HIV, human immunodeficiency virus; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte.

‡To whom reprint requests should be addressed at: Box 3258, Duke University School of Medicine, Durham, NC 27710.

Table 1. Variants of T1-SP10 peptides derived from HIV MN and IIIB envelope sequences

Peptide name	Region			
	F	T1	SP10	A
HIV MN				
T1-SP10MN		**KQIINMWQEVGKAMYA**CTRPNYNKRKRIHIGPGRA		
T1-SP10MN(A)		**KQIINMWQEVGKAMYA**CTRPNYNKRKRIHIGPGRA**FYTTK**		
F-T1-SP10MN	AVGIGALFLGFL**KQIINMWQEVGKAMYA**CTRPNYNKRKRIHIGPGRA			
F-T1-SP10MN(A)	AVGIGALFLGFL**KQIINMWQEVGKAMYA**CTRPNYNKRKRIHIGPGRA**FYTTK**			
F-SP10MN(A)	AVGIGALFLGFL.....		CTRPNYNKRKRIHIGPGRA**FYTTK**	
SP10MN(A)			CTRPNYNKRKRIHIGPGRA**FYTTK**	
HIVIIIB				
T1-SP10IIIB(A)		**KQIINMWQEVGKAMYA**CTRPNNNTRKSIRIQRGPG**RAFVTI**		
F-T1-SP10IIIB(A)	AVGIGALFLGFL**KQIINMWQEVGKAMYA**CTRPNNNTRKSIRIQRGPG**RAFVTI**			

Each amino acid is represented by a single-letter code that is the first letter of its name, except for arginine (R), asparagine (N), glutamine (Q), glutamic acid (E), lysine (K), phenylalanine (F), tryptophan (W), tyrosine (Y), and aspartic acid (D). F (fusogenic domain) sequence is amino acids 519–530 from HIVIIIB. T1 sequence is amino acids 428–443 from HIVIIIB. SP10MN sequence is amino acids 301–319 from HIVMN. SP10IIIB sequence is amino acids 303–321 from HIVIIIB. (A) sequence is amino acids 320–324 from HIVMN and amino acids 322–327 from HIVIIIB. HIVIIIB sequences are from ref. 21 and HIVMN sequences are from ref. 22. The dotted line represents no T1 amino acids in the F-SP10MN(A) sequence.

assays were performed in duplicate at an effector:target ratio of 50:1 using 5000 target cells per well and a total volume of 0.2 ml per well. The target cells used were L5178Y ($H\text{-}2^d$) lymphoma [American Type Culture Collection (ATCC)], EL4 ($H\text{-}2^b$) lymphoma (ATTC), or P815 mastocytoma cells (from J. Yewdell, National Institutes of Health) precoated with 200 μg of peptide per ml overnight. Similar results were obtained using target cells sensitized with peptide for 1 hr before labeling. In some experiments, DM-1 cells transfected with the $H\text{-}2D^d$ molecule or LM-1 cells transfected with the $H\text{-}2L^d$ molecule (from J. Seidman, Harvard University, Boston) (26) either were precoated with peptide or were infected with a vaccinia construct containing HIVIIIB env gp160 (VPE16) (27) or with a vaccinia construct (PSCR2502) with the DNA sequence encoding amino acids 278–468 of HIVMN (containing the V3 loop region of HIVMN) substituted for homologous IIIB sequences (VacMN) (28). Target cells were labeled with 0.12 mCi of ^{51}Cr (1 Ci = 37 GBq; New England Nuclear) for 2 hr at 37°C and washed three times before use. Percent specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100, where spontaneous release was determined from target cells incubated in medium alone and maximum release was determined from target cells incubated in 0.5% Triton X-100.

RESULTS

T1-SP10MN(A) and F-T1-SP10MN(A) Peptides Prime $CD8^+$ Anti-HIV CTLs *in Vivo*. Following immunization of BALB/c ($H2^d$) and C57BL/6 ($H2^b$) mice three to five times with HIV env synthetic peptides, mouse splenocytes were cultured *in vitro* for 7 days and tested for cytolytic activity against peptide-coated target cells (Table 2). Surprisingly, spleen cells from BALB/c mice immunized with either free T1-SP10MN(A) or free F-T1-SP10MN(A) synthetic peptide lysed *H-2*-compatible target cells (L5178Y) precoated with T1-SP10MN(A) or F-T1-SP10MN(A) but did not lyse *H-2*-incompatible target cells (EL4) coated with the same peptides (Table 2). L5178Y target cells precoated with T1-SP10MN or F-T1-SP10MN peptide lacking the (A) region were not lysed by the BALB/c effector cells, indicating that the additional five residues comprising the (A) region were critical for CTL recognition. Pretreatment of effector cells with anti-Thy 1.2 plus complement or anti-CD8 plus complement markedly reduced the lytic activity, whereas pretreatment with anti-

Table 2. Free T1-SP10MN(A) and F-T1-SP10MN(A) synthetic peptide constructs prime CTLs when given to BALB/c mice *in vivo*

Mouse	Peptide *in vivo*	Cytolytic activity against peptide-coated target cells, % specific lysis					
		T1-SP10MN(A)		T1-SP10MN	F-T1-SP10MN(A)		F-T1-SP10MN
		L5178Y	EL4	L5178Y	L5178Y	EL4	L5178Y
BALB/c	T1-SP10MN	10	0	0	17	0	6
	T1-SP10MN(A)	81	0	1	87	0	7
	F-T1-SP10MN	6	0	2	11	0	5
	F-T1-SP10MN(A)	50	0	1	57	0	5
C57BL6	T1-SP10MN	3	2	1	4	0	4
	T1-SP10MN(A)	0	2	ND	ND	0	9
	F-T1-SP10MN	1	0	1	0	0	4
	F-T1-SP10MN(A)	0	0	1	0	0	1

The background lysis observed against untreated target cells has been subtracted from the presented data and did not exceed 16%. Spontaneous release of the target cells generally did not exceed 20% of the maximum release. Whereas the specific lysis observed by spleen cells from mice immunized with T1-SP10MN or F-T1-SP10MN in this experiment ranged from 6% to 17% on peptide-coated target cells, the mean percent specific lysis observed in groups of 4 mice immunized with T1-SP10MN or F-T1-SP10MN was 9.3% ± 1.4% and 4.6% ± 1.4%, respectively, using T1-SP10MN(A)-coated target cells. Naive BALB/c splenocytes stimulated *in vitro* with T1-SP10MN(A) or F-T1-SP10MN(A) peptide did not lyse peptide-coated target cells (data not shown). Results similar to those presented here were also obtained when the splenocytes were cultured with 25 μg of T1-SP10MN(A) per ml *in vitro* (data not shown). Data shown are representative of those obtained testing 4–14 BALB/c mice and 3–12 C57BL6 mice for each peptide immunogen. ND, not determined.

CD4 plus complement did not, indicating that the *in vivo* primed anti-HIV cytotoxic cells were $CD8^+$ T cells (Fig. 1*A*). Thus, addition of either the T1 sequence or the T1 and F sequences N-terminal to the SP10MN(A) sequence created a peptide capable of inducing $CD8^+$ anti-HIV CTLs *in vivo*.

Either F or T1 Sequence Can Confer the Ability to Prime $CD8^+$ CTLs *in Vivo*. To determine the relative contributions of the T1 and F sequences to the ability of a peptide to induce

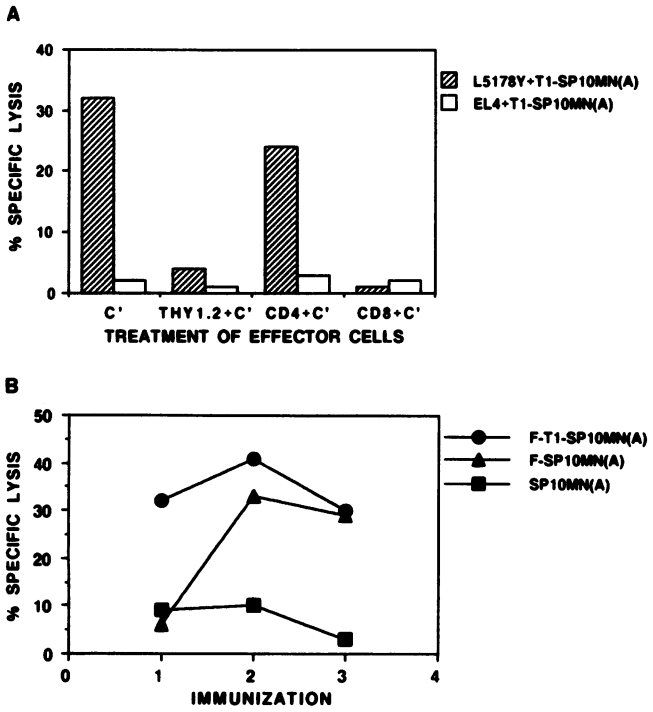

FIG. 1. CTLs induced by HIV env synthetic peptides are $CD8^+$ T cells (*A*) and can be induced by the F-SP10MN(A) peptide *in vivo* (*B*). (*A*) Complement-mediated lysis of effector cells pooled from three BALB/c mice immunized with F-T1-SP10MN(A) peptide was performed by incubating the effector cells (5×10^6 per ml of RPMI medium/5% human albumin/10 mM Hepes) with antibody (1 hr, 4°C), washing, resuspending cells in medium or a 1:15 dilution of Low-Tox M rabbit complement (Accurate Chemicals, Westbury, NY), and incubating cells (1 hr, 37°C) with periodic mixing. Cells were washed and resuspended at 2.5×10^6 per ml based on the original number of cells in the tube. Antibodies used were Thy 1.2 (1:20, Accurate Chemicals), Lyt2.2 (anti-CD8, 1:200, New England Nuclear), and 172.4 (anti-CD4, 1:6 final dilution of supernatant, from R. Ceredig, Lausanne, Switzerland). A 50:1 effector:target ratio was used in a 6-hr ^{51}Cr-release assay, using L5178Y target cells precoated with T1-SP10MN(A) peptide. Data are representative of three experiments performed. (*B*) Percent specific lysis observed using spleen cells from BALB/c mice immunized with F-T1-SP10MN(A), F-SP10MN(A), or SP10MN(A) peptide. Mice were given one, two, or three immunizations as indicated at 2-week intervals and sacrificed 2 weeks after their final immunization. Animal care was carried out in accordance with institutional guidelines. Spleen cells were cultured for 7 days with the F-T1-SP10MN(A) peptide and then tested for their ability to lyse L5178Y target cells precoated with F-T1SP10MN(A) or T1-SP10MN(A) peptide. Results shown were obtained using a 50:1 effector:target ratio. Background lysis observed against untreated L5178Y cells has been subtracted. The mean percent specific ^{51}Cr release observed using splenic effector cells from BALB/c mice immunized with SP10MN(A) *in vivo* was 3.3% ± 1.5% ($n = 9$), whereas a mean of 24.3% ± 2.7% specific lysis ($n = 22$) was observed using splenic effector cells from mice given F-T1-SP10MN(A) *in vivo* ($P < 0.001$). Complement-mediated lysis of effector cells with anti-CD4, CD8, and Thy-1 mAbs as in *A* demonstrated effector cells induced by F-SP10MN(A) peptide to be $Thy\text{-}1^+$ and $CD8^+$ (not shown). Data represent results from individual mice for immunizations 1 and 2 and are representative of three animals studied after immunization 3.

$CD8^+$ CTLs, two additional peptides, F-SP10MN(A) and SP10MN(A) (Table 1), were compared with F-T1-SP10MN(A) for their ability to induce $CD8^+$ CTLs in BALB/c mice (Fig. 1*B*). As expected, free SP10MN(A) did not prime CTLs *in vivo*, whereas F-SP10MN(A) did prime anti-HIV CTLs. Moreover, in time course experiments, priming of CTLs capable of killing peptide-coated L5178Y target cells was seen after one immunization with the F-T1-SP10MN(A) peptide *in vivo* (Fig. 1*B*). Thus, the presence of either T1 *or* F sequence N-terminal to CTL epitope sequences conferred on the SP10MN(A) peptide the ability to induce $CD8^+$ CTLs *in vivo*. The induction of anti-HIV $CD8^+$ CTLs by the F-SP10MN(A) peptide does not necessarily mean that the T-helper epitope was not required for CTL priming, since we have previously shown that a T-helper epitope is present between amino acids 310 and 322 of the SP10 peptide (14). Moreover, the presence of this T-helper epitope in the SP10 region also suggests that the inability of the SP10MN(A) peptide to generate CTLs *in vivo* was not due solely to lack of a T-helper cell epitope.

Minimal lysis of peptide-coated target cells was observed when BALB/c mice were immunized *in vivo* with the T1-SP10MN or F-T1-SP10MN peptides and the splenocytes were restimulated *in vitro* with F-T1-SP10MN(A) (Table 2), indicating that CTL responses in mice immunized with F-T1-SP10MN(A), T1-SP10MN(A), or F-SP10MN(A) peptide were the result of *in vivo* priming with synthetic peptides containing the complete D^d-restricted CTL epitope and were not the result of *in vitro* priming with the F-T1-SP10MN(A) or T1-SP10MN(A) peptide. Similarly, naive BALB/c mouse splenocytes primed *in vitro* with F-T1-SP10MN(A) peptide did not kill F-T1-SP10MN(A)-coated $H\text{-}2^d$ targets (not shown). Whereas F-T1-SP10MN(A) and T1-SP10MN(A) peptides primed CTLs *in vivo*, in five of eight experiments, the level of target cell lysis was greater when the F-T1-SP10MN(A) peptide was used for *in vitro* stimulation as compared to the T1-SP10MN(A) peptide (data not shown).

Peptide-Induced CTLs Are MHC Class I-Restricted. The genetic restriction of CTL induced by carrier-free F-T1-SP10MN(A) and T1-SP10MN(A) peptides was mapped to the $H\text{-}2D^d$ molecule by testing the ability of the CTLs to lyse murine L cells transfected with the $H\text{-}2D^d$ molecule (DM-1) or the $H\text{-}2L^d$ class I molecule (LM-1) (26). T1-SP10MN(A)-coated DM-1 cells expressing the D^d molecule were killed by BALB/c effector cells, whereas no lysis of peptide-coated L^d target cells was observed (Fig. 2*A*). No lysis of peptide-coated D^d (DM-1) or L^d (LM-1) target cells was mediated by C57BL/6 effector cells (data not shown). Similarly, C57BL/6 effectors did not kill peptide-coated L5178Y target cells (Table 2).

HIV Carrier-Free Peptides Prime Anti-HIV $CD8^+$ CTLs that Kill gp160-Expressing Targets. DM-1 target cells were infected with vaccinia constructs containing the gp160 molecule from HIVIIIB (VPE16) (27) or a construct designated VacMN, in which DNA sequences encoding the HIVMN env amino acid 278–468 region containing the HIVMN V3 loop were substituted for homologous IIIB sequences (28). Effector cells primed *in vivo* with the F-T1-SP10MN(A) peptide from HIVMN lysed DM-1 cells infected with the VacMN construct (Fig. 2*A*), indicating that the peptide-primed CTLs were able to kill target cells producing HIV gp160. CTLs primed *in vivo* with the F-T1-SP10IIIB(A) peptide also lysed DM-1 cells infected with VPE16 or coated with T1-SP10IIIB(A) peptide and did not lyse DM-1 cells infected with the VacMN construct (Fig. 2*B*) or LM-1 cells infected with either construct, indicating that lysis was $H\text{-}2D^d$-restricted and type-specific for the HIV isolate sequence used as immunogen. Similarly, CTLs primed *in vivo* with carrier-free HIV env synthetic peptides lysed peptide-coated target cells in a type-specific manner (Fig. 2*C*). Similar data were

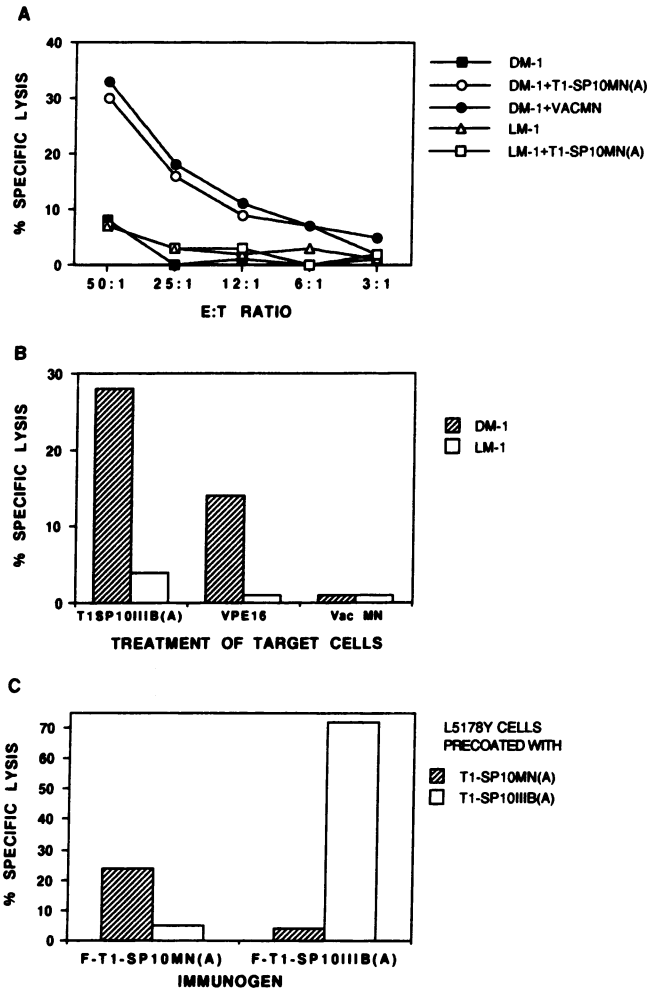

FIG. 2. Peptide-induced BALB/c CTLs are restricted to the *H-2D*^{d} class I molecule and recognize native HIV gp160. (*A*) Spleen cells from a BALB/c mouse immunized with F-T1-SP10MN(A) and restimulated *in vitro* with F-T1-SP10MN(A) tested in a ^{51}Cr-release assay at various effector:target (E:T) ratios for lysis of T1-SP10MN(A)-coated L cells transfected with the *H-2D*^{d} (DM-1) or *H-2L*^{d} (LM-1) class I molecules (26). The target cells were incubated with 200 μg of peptide per ml overnight, mechanically dislodged from wells, and tested in a ^{51}Cr-release assay. No lysis of VacMN-infected LM-1 cells was observed above the background lysis of untreated LM-1 cells (2% and 7%, respectively) at a 50:1 E:T ratio. Similarly, no specific lysis of treated DM-1 or LM-1 cells was observed using C57BL6 effector cells (not shown). Data are representative of five experiments performed. (*B*) Lysis of gp160-expressing target cells by F-T1-SP10IIIB(A)-primed effector cells. DM-1 and LM-1 cells (26) were infected with a vaccinia construct containing HIVIIIB env gp160 (VPE16) (27) or with a vaccinia construct (PSCR2502) with amino acids 278–468 containing the V3 loop region of the MN isolate substituted for homologous IIIB sequences (VacMN) (28). Target cells were incubated overnight with the vaccinia constructs at a 5:1 multiplicity of infection ratio or with 200 μg of T1-SP10IIIB(A) peptide per ml, labeled with ^{51}Cr, and used in a 6-hr ^{51}Cr-release assay (50:1 E:T ratio). Effector cells were obtained from BALB/c mice immunized with F-T1-SP10IIIB(A) peptide and restimulated with the same peptide *in vitro*. Data shown are representative of three experiments performed. (*C*) Peptide-primed CTLs lysed peptide-coated L5178Y target cells in a type-specific manner. Effector cells obtained from BALB/c mice immunized with either F-T1-SP10MN(A) or F-T1-SP10IIIB(A) and restimulated with the same peptide *in vitro* were tested in a 6-hr ^{51}Cr-release assay for the ability to lyse L5178Y target cells (50:1 E:T ratio) precoated with T1-SP10MN(A) peptide or T1-SP10IIIB(A) peptide. No specific lysis was observed on peptide-coated *H-2*-incompatible EL4 cells (data not shown). F-T1-SP10IIIB(A) peptide also primed CTLs *in vivo* when only incomplete Freund's adjuvant was used for *in vivo* immunizations (not shown). Data are representative of four experiments performed.

obtained using P815 cells infected with VacMN or VPE16 (not shown).

DISCUSSION

One of the major issues in AIDS vaccine development is whether antibody responses alone can protect an individual from cell-free HIV and HIV-infected cells or whether antigen-specific cytotoxic T cells are required as well (3, 29). Viral infection *in vivo* (as opposed to immunization with peptide fragments) is usually required to prime MHC class I-restricted CD8$^+$ CTLs (2, 4–6), although rare examples of induction *in vivo* of CD8$^+$, MHC class I-restricted CTLs by free peptides or protein fragments have been reported (7–11). Conjugation of synthetic peptides to lipid tails (5) or to Quil A immunostimulatory complexes (6) facilitates *in vivo* CD8$^+$ CTL induction, but the use of these types of derivatized synthetic peptides in humans is complicated by the pharmacology of the derivatizing material. In contrast, a carrier-free nonderivatized synthetic peptide vaccine for HIV would have many advantages over live or killed HIV or over larger subunit derivatized HIV immunogens. Carrier-free synthetic peptides carry no risk of inducing HIV infection, can be designed to delete HIV protein components capable of enhancing HIV infection (30), and carry no toxicities associated with a derivatizing molecule. Therefore, it would be desirable to design carrier-free synthetic peptide immunogens capable of inducing class I-restricted CTLs as well as T-helper cell and neutralizing antibody responses to HIV.

We have demonstrated in this study that hybrid synthetic peptides containing either the T1 or F HIV env region N-terminal to a HIV CTL epitope have the ability to induce CD8$^+$ MHC class I-restricted CTLs *in vivo* in mice. The basis for the ability of these hybrid HIV env peptides to prime CD8$^+$ CTLs *in vivo* is not yet known. Clues to the mechanism of this effect may come from the proposed structures of the T1 and F env sequences. The T1 T-cell epitope is thought to form an amphipathic α-helix (12), whereas the F peptide likely forms a highly hydrophobic helical structure (23–25). Synthesis of amphipathic T-cell epitopes of immunogens adjacent to B-cell epitopes markedly augments B-cell epitope immunogenicity (31–35). The hydrophobic F helical sequence promotes the formation of high molecular weight protein micelles by F-derivatized SP10(A) peptides in aqueous solutions (D. L. Cotsamire, R.M.S., P. Cresswell, and B.F.H., unpublished). In some circumstances, there may be a rationale for using the combination of F and T1 sequences in hybrid peptides, as addition of F and T1 regions to the SP10MN(A) peptide augmented the ability of the resulting peptide to induce anti-HIV neutralizing antibodies in mice (M.K.H. and B.F.H., unpublished).

Protein antigens are capable of being processed by antigen-presenting cells by two routes, the exogenous and endogenous antigen-presenting pathways (reviewed in refs. 1–5). Exogenous soluble peptides and protein antigens are generally taken into antigen-presenting cells via endocytosis and cleaved into small fragments, some of which associate with MHC class II molecules in endocytic vesicles (reviewed in ref. 36). Peptide fragments presented on the surface of antigen-presenting cells in the context of MHC class II molecules are recognized by CD4$^+$ T cells (1–5). In the endogenous pathway, peptide fragments of protein antigens presented in MHC class I molecules to CD8$^+$ T cells are generally derived from intracellular host proteins or proteins of intracellular infectious agents (1–5).

The observation that hybrid peptides containing multiple regions of HIV envelope sequences interact with antigen-presenting cells in a manner that leads to priming of CD8$^+$ CTLs *in vivo* suggests that peptide structure may, in part, determine the antigen-processing pathway a protein enters

upon contact with an antigen-presenting cell. Given the recent observations that small peptide fragments of nine amino acids in length preferentially fit into the MHC molecule groove (reviewed in ref. 36), it is likely that T1-SP10 hybrid peptides require antigen processing prior to presentation rather than directly binding to MHC molecules on antigen-presenting cells. However, the molecules to which T1-SP10 peptides bind and the mechanisms of peptide internalization and processing in antigen-presenting cells are not known. Nonetheless, recent reports of free peptides (9–11), proteins in immunostimulatory complexes (6), and peptides with a lipid tail (5) priming $CD8^+$ CTLs *in vivo*, together with the data in our study, suggest that exogenous protein priming of $CD8^+$ CTLs may be more common than originally appreciated.

T1-SP10 and F-T1-SP10 peptides are potent stimulators of proliferation of T-helper cells *in vivo* and *in vitro* (ref. 14; B.F.H. and M.K.H., unpublished). The ability of T1-SP10MN(A) and F-T1-SP10MN(A) peptide constructs to prime anti-HIV CTLs *in vivo* may relate to the capacity of T1-SP10 peptides to stimulate T-helper cell proliferation for CTL induction. It is also possible that the use of T1-SP10(A) and F-T1-SP10(A) peptides *in vitro* to expand primed $CD8^+$ CTLs facilitated the successful measurement of $CD8^+$ CTLs *in vitro*. Studies of tracking the route of entry of hybrid peptides into antigen-presenting cells are necessary to ultimately determine the mechanisms of hybrid peptide-induced generation of $CD8^+$ CTLs.

We (14, 37) and others (18, 38, 39) have proposed a strategy for the development of a polyvalent mixture of HIV env gp120 V3 region synthetic peptides capable of inducing antibody responses that neutralize HIV field isolates. However, major problems remain for the development of an effective HIV vaccine, including the need to induce mucosal as well as systemic anti-HIV immunity, the ability of HIV to mutate viral genome sequences, and the need for an effective anti-HIV immune response to eliminate allogeneic HIV-infected cells. Similarly, immunotherapy with HIV antigens carries the theoretical risk of accelerating destruction of the immune system by facilitating the cytolysis of infected or gp120-coated immune cells (40–43). Nonetheless, for the use of synthetic peptides to remain a viable strategy for HIV immunotherapeutic or vaccine development, it is important to be able to construct multiple carrier-free nonderivatized synthetic peptides that are capable of inducing $CD8^+$ CTLs to native HIV proteins.

We thank P. Cresswell, F. Ward, and D. Bolognesi for discussions and review of this manuscript, J. Yewdell, B. Moss, C. Scott, and J. Seidman for reagents, Ms. Dawn Jones for technical assistance, and Ms. Kim R. McClammy and Ms. Joyce Lowery for expert secretarial assistance. This work was supported by Grants CA28936, CA43447, A128662, and AI29852 from the National Institutes of Health. B.F.H. is a recipient of the Carter-Wallace Fellowship Award in Retroviral Research.

1. Morrison, L. A., Braciale, V. L. & Braciale, T. J. (1986) *Immunol. Res.* **3**, 294–304.
2. Bevan, M. J. (1989) *Nature (London)* **342**, 478–479.
3. Bangham, C. R. M. & McMichael, A. J. (1990) *Nature (London)* **348**, 388.
4. Townsend, A. R. M., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D. & McMichael, A. J. (1986) *Cell* **44**, 959–968.
5. Deres, K., Schild, H., Weismuller, K. H., Jung, G. & Rammensee, H. G. (1989) *Nature (London)* **342**, 561–564.
6. Takahashi, H., Takeshita, T., Morein, B., Putney, S., Germain, R. N. & Berzofsky, J. A. (1990) *Nature (London)* **344**, 873–875.
7. Staerz, U. D., Karasuyama, H. & Garner, A. M. (1987) *Nature (London)* **329**, 449–451.
8. Ishioka, G. Y., Colon, S., Miles, C., Grey, H. M. & Chestnut, R. W. (1989) *J. Immunol.* **143**, 1094–1100.
9. Aichele, P., Hengartner, H., Zinkernagel, R. M. & Schultz, M. (1990) *J. Exp. Med.* **171**, 1815–1820.
10. Schulz, M., Zinkernagel, R. M. & Hengartner, H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 991–993.
11. Kast, W. M., Roux, L., Curren, J., Blom, H. J. J., Voordouw, A. C., Meloen, R. H., Kolakofsky, D. & Melief, C. J. M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2283–2287.
12. Cease, K. B., Margalit, H., Cornette, J. L., Putney, S. D., Robey, W. G., Ouyang, C., Streicher, H. Z., Fischinger, P. J., Gallo, R. C., Delisi, C. & Berzofsky, J. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4249–4253.
13. Palker, T. J., Clark, M. E., Langlois, A. J., Matthews, T. J., Weinhold, K. J., Randall, R. R., Bolognesi, D. P. & Haynes, B. F. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1932–1936.
14. Palker, T. J., Matthews, T. J., Langlois, A. J., Tanner, M. E., Martin, M. E., Scearce, R. M., Kim, J. E., Berzofsky, J. A., Bolognesi, D. P. & Haynes, B. F. (1989) *J. Immunol.* **142**, 3612–3619.
15. Hart, M. K., Palker, T. J., Matthews, T. J., Langlois, A. J., Lerche, N. W., Martin, M. E., Scearce, R. M., McDanal, C., Bolognesi, D. P. & Haynes, B. F. (1990) *J. Immunol.* **145**, 2677–2685.
16. Rusche, J. R., Javaherian, K., McDanal, C., Petro, J., Lynn, D. L., Grimaila, R., Langlois, A., Gallo, R. C., Arthur, L. O., Fischinger, P. J., Bolognesi, D. P., Putney, S. D. & Matthews, T. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3198–3202.
17. Goudsmit, J., Debouck, C., Meloen, R., Smit, H. L., Bakker, M., Asher, D. M., Wolff, A. V., Gibbs, C. J. & Gajdusek, D. C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4478–4482.
18. Javaherian, K., Langlois, A. J., McDanal, C., Ross, K. L., Eckler, L. I., Jellis, C. L., Profy, A. T., Rusche, J. R., Bolognesi, D. P., Putney, S. D. & Matthews, T. J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6768–6772.
19. Takahashi, H., Cohen, J., Hosmalin, A., Cease, K. B., Houghton, R., Cornette, J. L., Delisi, C., Moss, B., Germain, R. N. & Berzofsky, J. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3105–3109.
20. Takahashi, H., Houghten, R., Putney, S. D., Margulies, D. H., Moss, B., Germain, R. N. & Berzofsky, J. A. (1989) *J. Exp. Med.* **170**, 2023–2035.
21. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C. & Wong-Staal, F. (1985) *Nature (London)* **313**, 277–284.
22. Myers, G., Josephs, S. F., Rabison, A. B., Smith, T. F. & Wong-Staal, F., eds. (1988) *Human Retroviruses and AIDS* (Los Alamos National Laboratory, Los Alamos, NM), Vol. 2, pp. 68–92.
23. Gallaher, W. R. (1987) *Cell* **50**, 327–328.
24. Bosch, M. L., Earl, P. L., Fargnoli, K., Picciafuoco, S., Giombini, F., Wong-Staal, F. & Franchini, G. (1989) *Science* **244**, 694–697.
25. Brasseur, R., Cornet, B., Burny, A., Vandenbranden, M. & Ruysschaert, J. M. (1988) *AIDS Res. Hum. Retrovir.* **4**, 83–90.
26. Weiss, J. H. & Seidman, J. G. (1985) *J. Immunol.* **134**, 1999–2003.
27. Earl, P. L., Hugin, A. W. & Moss, B. (1990) *J. Virol.* **64**, 2448–2451.
28. Scott, C. F., Silver, S., Profy, A. T., Putney, S. D., Langlois, A. J., Weinhold, K. & Robinson, J. E. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8597–8601.
29. Levy, J. A. (1988) *J. Am. Med. Assoc.* **259**, 3037–3038.
30. Robinson, W. E. J., Montefiori, D. C. & Mitchell, W. M. (1988) *Lancet* **i**, 790–794.
31. LeClerc, C., Przewlocki, G., Schutze, M. P. & Chedid, L. (1987) *Eur. J. Immunol.* **17**, 269–273.
32. Celada, F. & Sercarz, E. E. (1988) *Vaccine* **6**, 94–98.
33. Milich, D. R., Hughes, J. L., McLachlan, A., Thornton, G. B. & Moriarty, A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1610–1614.
34. Tam, J. P. & Lu, Y.-A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9084–9088.
35. Golvano, J., Lasarte, J. J., Sarobe, P., Gullon, A., Prieto, J. & Borras-Cuesta, F. (1990) *Eur. J. Immunol.* **20**, 2363–2366.
36. Parham, P. (1991) *Nature (London)* **351**, 523–524.
37. Haynes, B. F., Hart, M. K., Matthews, T. J., Langlois, A., Scearce, R. M., Martin, M. E., Lerche, N., Berzofsky, J. A., Bolognesi, D. P. & Palker, T. J. (1990) *AIDS Res. Hum. Retrovir.* **6**, 38–39.
38. LaRosa, G. J., Davide, J. P., Weinhold, K., Waterbury, J. A., Profy, A. T., Lewis, J. A., Langlois, A. J., Dreesman, G. R., Boswell, R. N., Shadduck, P., Holley, L. H., Karplus, M., Bolognesi, D. P., Matthews, T. J., Emini, E. A. & Putney, S. D. (1990) *Science* **249**, 932–935.
39. Javaherian, K., Langlois, A. J., LaRosa, G. J., Profy, A. T., Bolognesi, D. P., Herlihy, W. C., Putney, S. D. & Matthews, T. J. (1990) *Science* **250**, 1590–1593.
40. Salk, J. (1987) *Nature (London)* **327**, 473–476.
41. Lanzavecchia, A., Roosnek, E., Gregory, T., Berman, P. & Abrignani, S. (1988) *Nature (London)* **334**, 530–532.
42. Siliciano, R. F., Lawton, T., Knall, C., Karr, R. W., Berman, P., Gregory, T. & Reinherz, E. L. (1988) *Cell* **54**, 561–575.
43. Oehen, S., Hengartner, H. & Zinkernagel, R. M. (1991) *Science* **251**, 195–198.