

Cellular immune responses induced by *in vivo* priming with a lipid-conjugated multimeric antigen peptide

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SUMMARY

This report investigates the generation of cytotoxic T lymphocytes (CTL) by *in vivo* administration of a synthetic antigen linked to a lipid moiety, tripalmitoyl-S-glyceryl cysteine (P3C). The antigen consisted of a 17-mer peptide, derived from the gp120 envelope protein of the human immunodeficiency virus type-1 (HIV-1), in a tetravalent multiple antigenic peptide (MAP) configuration. A single injection of MAP-P3C elicited a long-lasting CTL response in mice. The route of administration was not a determining factor, since intravenous (i.v.) and intraperitoneal (i.p.) priming were both effective. The HIV strain-specific cytotoxic lymphocytes were of the CD8⁺ subset and class I restricted. A broad cytolytic activity could be achieved by priming with a mixture of homologous peptides from gp120 IIIB and MN strains. Following the administration of the monoclonal antibody GK1.5, resulting in the depletion of the CD4⁺ T-lymphocyte subpopulation, mice were able to mount a strong CTL response. This finding demonstrates that in priming with a peptide antigen covalently linked to a lipid, such as MAP-P3C, CD4⁺ cells are not required for the generation of CD8⁺ cytotoxicity. In contrast, the elimination of macrophages by the carrageenan pretreatment caused suppression of the T-cell lytic activity, suggesting a substantial contribution of the phagocytic cells in mounting CTL response. Taken together, these results may lead to new strategies in designing a human immunodeficiency virus type-1 (HIV-1) vaccine based on synthetic peptides.

INTRODUCTION

Much attention has been devoted to the importance of eliciting neutralizing antibodies in the design of subunit vaccines against human immunodeficiency virus type 1 (HIV-1); however, cell-mediated immunity in addition to the humoral response may be needed for an effective prevention of the disease.¹ In fact, there is a general consensus regarding the contribution of cytotoxic T lymphocytes to the limitation and clearance of viral diseases, through the killing of the host-infected cells.²⁻⁵ Cell-mediated responses are sufficient to protect against viral antigens which do not elicit neutralizing antibodies and they represent the body's first reaction to the viral exposure. The primary mediators are identified as major histocompatibility complex (MHC) class I-restricted CD8⁺ lymphocytes, which recognize processed

peptides from viral external glycoproteins or internal non-structural proteins.

Since intracellular *de novo* protein synthesis and the consequent cytosolic antigen localization are a strict requirement for the activation of the class I presentation pathway, an optimal mean to achieve *in vivo* induction of specific CD8⁺ cytotoxic T lymphocytes (CTL) is the immunization with replicating viruses.⁶ Nevertheless, several other methods for *in vivo* CTL priming with proteins or synthetic peptides have been described. Immunostimulating complexes (ISCOM)⁷ or free peptides administered in Freund's adjuvant have been used to induce specific cellular immunity.⁸⁻¹⁰ In addition, class I-restricted cytotoxicity has been obtained with soluble proteins formulated in squalene and Tween-80¹¹ or by liposome encapsulation.¹² A common feature of these different methods is the presence of lipophilic substances in the antigenic formulations. In our approach to develop a peptide-based vaccine for the HIV-1 infection, we extended the observation of Deres *et al.*¹³ that a synthetic CTL epitope could elicit virus-specific cytotoxicity when covalently linked to a lipophilic molecule. In prior work,¹⁴ a peptide, containing a sequence from the principal neutralizing domain of gp120, was coupled to the lipid moiety P3C, a synthetic analogue of the N-terminus of bacterial lipoprotein. We utilized this hydrophobic moiety for the following advantages. It has adjuvant properties¹⁵ and, at the same time, allows

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Abbreviations: CTL, cytotoxic T lymphocyte(s); HIV-1, human immunodeficiency virus type-1; i.p., intraperitoneal; i.v., intravenous; MHC, major histocompatibility complex; MAP, multiple antigenic peptide; PFC, plaque-forming cells; SRBC, sheep erythrocytes; P3C, tripalmitoyl-S-glyceryl cysteine.

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the peptide to be inserted into liposomes or aggregated in micelles. The resulting immunogen elicited neutralizing antibodies as well as T-cell response to the HIV-1 antigen in mice.

The present study was conducted to examine the characteristics and mechanism of the cellular responses generated by a tetravalent multiple antigenic peptide conjugate to P3C (MAP-P3C). Induction of long-term CD8⁺ cytotoxic cells is effectively achieved by a single immunization. Using *in vivo* depletion, we have analysed the contribution of CD4⁺ cells to the priming. Our data demonstrate that they are not involved in the generation of the cytotoxic response to a synthetic peptide, when it is covalently linked to a lipophilic moiety. The results also suggest that macrophages are important antigen-presenting cells in the initiation of class I-restricted immunity.

MATERIALS AND METHODS

Mice

BALB/c, 8–12 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME) and used three to four per group for each immunization.

Peptides

B2M (KSIRIQRGPGRAFVTIGK) from gp120 IIB strain and B8M (KRKRIHIGPGRAFYTTKN) from gp120 MN strain were synthesized in a MAP format consisting of four identical repeats. They were prepared manually by a stepwise solid-phase procedure on a 9-fluorenylmethoxycarbonyl (Fmoc)-Ala-OCH₂-resin as described.¹⁶ MAP-P3C conjugates were synthesized as described in detail elsewhere.¹⁴

Vaccinia viruses

The recombinant vaccinia virus v.env5, containing HIV-1 gp160 gene, was obtained from S. L. Hu (Bristol-Myers Squibb, Seattle, WA). The wild-type vaccinia virus WR was obtained through the AIDS Research and Reference Program (NIH, Bethesda, MD).

In vivo treatment with anti-CD4 monoclonal antibody

BALB/c mice were depleted of CD4⁺ T cells by i.p. inoculation of 300 µg of GK1.5 antibody on days -6, -3, -1, +1, +3, +5. The antibody was prepared by precipitation with ammonium sulphate from ascitic fluid of *nude* mice injected with the rat anti-mouse hybridoma [American Type Culture Collection (ATCC), Rockville, MD]. The protein concentration was assessed by measurement of the OD at 280 nm.

In vivo treatment with carrageenan

Carrageenan Iota was purchased from Sigma (St Louis, MO). It was dissolved (10 mg/ml) in physiological saline by heating in boiling water. It was then dialysed against saline and stored at 4°. Aliquots were rewarmed to the liquid form prior to injecting. Animals were injected i.p. with 1 mg carrageenan at days 5 and 2 prior to the *in vivo* priming.

Generation of cytotoxic T cells

CTL effectors were generated from the spleen cells of mice primed following different protocols of immunization. Lymphocytes (8×10^6) were cultured for 5 days in the presence of 0.4 µM B2M or 1 µM B8M peptide in 2 ml of culture medium [RPMI-1640 supplemented with 10% foetal bovine serum (FBS),

□ gp120 peptide, aa 312–329

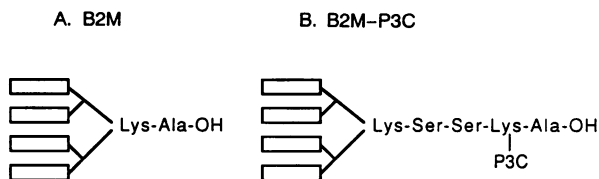


Figure 1. Schematic representations of B2M and B2M-P3C. (A) The gp120 peptide (aa 312–329) was synthesized in a MAP format consisting of four identical repeats. (B) B2M was covalently linked at the carboxyl terminus to the lipid tail P3C through a hydrophilic spacer (Ser, Ser).

antibiotics, L-glutamine and 5×10^{-2} M 2-mercaptoethanol (2-ME)]. MAP peptides or the corresponding linear peptides were used at equimolar concentrations in the *in vitro* assays with comparable results.

Cytotoxicity assay

Target P815 cells (2×10^5 /ml) were incubated for 2 hr or overnight at 37° in the presence or absence of the specified peptides. After washing, cells were labelled with 100 µCi ⁵¹Cr for 40 min at 37°. They were washed three times and added (5×10^4) to serial dilutions of effector cells for 4 hr in a volume of 0.2 ml culture medium. The percentage of the specific ⁵¹Cr release was calculated by the formula:

% specific release =

$$\frac{\text{c.p.m. experimental release} - \text{c.p.m. background release}}{\text{c.p.m. 5\% T} \times 100 \text{ release} - \text{c.p.m. background release}} \times 100.$$

The background release was always <10% of the maximal release. Standard errors of the means of triplicate cultures were always <5% of the mean. Vaccinia virus-infected target cells were prepared by the incubation of P815 cells in serum-free medium for 90 min with 50 plaque-forming units (PFU) per cells. After overnight incubation in culture medium, the cells were labelled with ⁵¹Cr and used as above. The background release of the infected cells was ≤30% of the maximal release.

In vitro lymphocyte depletion

Effector cells were incubated with purified anti-CD4 monoclonal antibody (obtained by Becton Dickinson, San Jose, CA) or anti-CD8 (purchased from Accurate, Westbury, NY) for 1 hr at 4°. After washing, cells were incubated with 1/10 dilution of low tox rabbit complement (Accurate) for 1 hr at 37°. Cells were washed and used for the cytotoxicity assay as described above.

Antibody-forming cell assay

The antibody response to sheep erythrocytes (SRBC) was measured in terms of direct plaque-forming cells (PFC) by the method of Jerne *et al.*¹⁷ Mice were immunized i.p. with 4×10^8 SRBC. PFC response was evaluated with the haemolytic plaque assay 4 days later.

RESULTS

In vivo priming with B2M-P3C

We have shown previously that a series of peptides from the third hypervariable region (V3) of gp120, assembled in a MAP

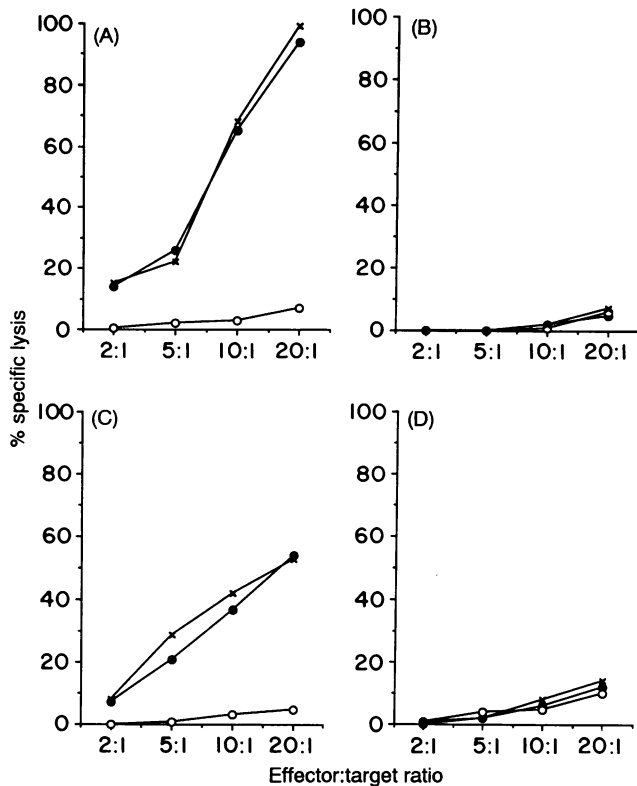


Figure 2. Priming specificity. CTL activity after i.p. priming of mice with B2M-P3C (●) or with 10^7 PFU of the recombinant vaccinia virus v.3env5 (×) expressing HIV-1 gp160 glycoprotein. Recipient spleen cells were stimulated *in vitro* for 5 days against $0.4 \mu\text{M}$ of the relevant B2M peptide; B2M-P3C-primed cells were restimulated also against an equimolar amount of the unrelated T peptide (○). ^{51}Cr -specific release was determined of syngeneic P815 target cells preincubated with B2 peptide (A) or with T peptide (B), infected with v.3env5 (C) or with the wild type vaccinia virus (D).

format, elicited humoral immunity in various animal species.¹⁶ In this study we used one of these antigens, B2M, consisting of the amino acid sequence 312–329 from the IIIB isolate (KSIR-IQRGPGRAFVTIGK), synthesized in a tetravalent configuration (Fig. 1A). This peptide contains a gp120 immunodominant epitope recognized by CTL.¹⁸ In order to elicit cellular immunity *in vivo*, B2M was covalently linked at the carboxyl terminus to the P3C molecule through a hydrophilic spacer consisting of two serines; this conjugate is referred to as B2M-P3C (Fig. 1B). BALB/c mice were injected once i.p. with B2M-P3C in phosphate-buffered saline and the splenic cytotoxic activity was tested on syngeneic P815 target cells. As shown in Fig. 2, the priming was antigen specific, with B2M-P3C-primed spleen cells lysing P815 cells pulsed with B2M peptide or infected with the recombinant vaccinia virus v.3env5 (expressing gp160). No cytotoxicity was observed when the target cells were preincubated with equimolar concentrations of a control peptide T, corresponding to a T-helper sequence in gp120 IIIB (amino acid 429–443), or when they were infected with the control wild-type vaccinia virus WR (not presenting the HIV-1 envelope antigen). Lysis of the v.3env5-infected targets demonstrated that the response was specific for endogenously processed gp160 epitopes as well as exogenously added peptide containing the CTL epitope. *In vitro* restimulation with the

Table 1. Induction of peptide-specific CTL memory by B2M-P3C intraperitoneal or intravenous injection*

	Days after priming			
	3	7	30	200
<i>Intraperitoneal immunization: % lysis†</i>				
E:T				
20:1	56 (8)	94 (6)	95 (2)	58 (2)
10:1	41 (2)	92 (5)	81 (1)	45 (0)
5:1	27 (1)	85 (3)	62 (1)	30 (0)
<i>Intravenous immunization: % lysis</i>				
E:T				
20:1	94 (9)	92 (4)	75 (3)	ND‡
10:1	86 (6)	85 (2)	52 (2)	
5:1	71 (3)	75 (1)	33 (2)	

* BALB/c mice were injected i.p. or i.v. with $100 \mu\text{g}$ of B2M-P3C. The spleen cells were restimulated *in vitro* with B2M peptide ($0.4 \mu\text{M}$) at different times after the priming.

† CTL activity was determined on P815 target cells preincubated with B2M peptide using the effector to target cell ratios (E:T) indicated. The values obtained on untreated P815 cells are in parentheses.

‡ Not done.

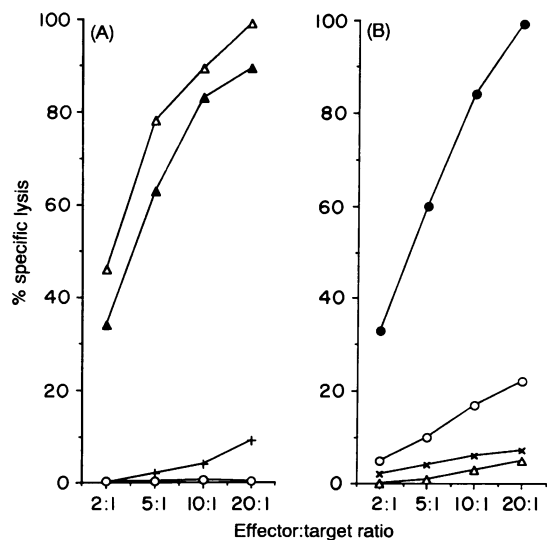


Figure 3. CTL phenotype and MHC class I restriction. Spleen cells from BALB/c mice, primed *in vivo* with $20 \mu\text{g}$ of B2M-P3C, were stimulated *in vitro* against the B2M peptide. (A) Effector populations were depleted of CD8^+ (+) or CD4^+ (Δ) cells by the treatment with specific antibodies plus complement, or treated with complement alone (\blacktriangle). CTL activity was determined on P815 target cells preincubated with the relevant peptide or untreated (○). (B) Lysis of P815 (H-2^d) (●), EL-4 (H-2^b) (×), or RDM4 (H-2^k) (Δ) targets preincubated with B2M peptide, and untreated P815 cells (○).

Table 2. CTL activity induced by priming with a single peptide or with a mixture

<i>In vivo</i> priming	HIV-1 isolates	% specific lysis of peptide-pulsed targets (E:T)			
		P815 + IIIB peptide		P815 + MN peptide	
		20:1	10:1	20:1	10:1
B2M-P3C	IIIB	84	60	30	18
B8M-P3C	MN	10	5	62	38
B2M-P3C + B8M-P3C	IIIB + MN	90	84	57	38

BALB/c mice were immunized i.p. with B2M-P3C (20 µg) and B8M-P3C (80 µg) alone or B2M-P3C plus B8M-P3C (20 + 80 µg). After 5-day culture, the cytotoxic activity of the IIIB- and MN-specific CTL was tested against ⁵¹Cr-labelled P815 targets either pulsed with 1 µM of B2M or 4 µM of B8M at the effector:target cell ratios (E:T) indicated.

Table 3. Effect of anti-CD4 monoclonal antibody treatment on CTL generation and antibody production

<i>In vivo</i> treatment*	Days -6 -3 -1 +1 +3	Day 0	Cytotoxic response†		
			E:T	% lysis	PFC/spleen‡
—		B2M-P3C	20:1	94 (3)	
			10:1	79 (0)	
			5:1	58 (0)	
			2:1	39 (0)	
Anti-CD4		B2M-P3C	20:1	98 (5)	
			10:1	87 (1)	
			5:1	61 (0)	
			2:1	43 (0)	
—		SRBC		68,750	
		Anti-CD4 SRBC		< 1000	

* Mice were injected i.p. with the monoclonal antibody GK1.5 (300 µg/mouse) or left untreated (—). The mice used for the CTL assay received an additional injection at day +5. On day 0, all mice received i.p. injection of B2M-P3C (20 µg/mouse) or SRBC (4 × 10⁸/mouse).

† On day +7, spleen cells were restimulated with B2M peptide (0.4 µM/ml). After 5 days, the cultures were assayed for CTL activity on ⁵¹Cr-labelled P815 target cells, presensitized with the peptide, at various effector to target cell ratios, E:T. The values obtained on untreated P815 cells are in parentheses. Results are expressed as the mean of three individual observations.

‡ The spleens were removed at day +4. The values are means for three mice in each group.

irrelevant peptide T failed to generate CTL response. B2M-P3C immunization led to a level of cytotoxic activity similar to that elicited by the infection with live recombinant vaccinia-gp160 vector.

In order to determine the priming conditions, mice were immunized once i.p. with increasing concentrations (1–100 µg) of B2M-P3C and the level of CTL induction was assayed 3 weeks later. The concentration of 4 µg produced a low cytotoxicity and the maximal response was reached with a concentration of about 20 µg (data not shown). The effect of increasing the time interval between priming and *in vitro* restimulation was determined by analysing CTL activity at 3, 7,

30 and 200 days post-immunization (Table 1). High levels of lysis were observed for up to 6 months after the priming. An identical experimental paradigm was conducted with i.p. and i.v. immunization: high cytotoxic activity was obtained with both routes of administration (Table 1).

CTL phenotype

The cytotoxic effector cells generated by B2M-P3C priming are conventional CD8⁺ CTL, since the activity was abrogated by the treatment with anti-CD8 plus complement, but not by anti-CD4 plus complement (Fig. 3). Allogeneic (H-2^b and H-2^k) MHC class I target cells preincubated with B2M were not lysed, indicating a restriction specificity for class I H-2^d. It should be mentioned that the tumour cell lines used as targets in these experiments do not express class II MHC gene products and that a V3 sequence, aa 308-322, was recognized by CD8⁺ CTL in association with the D^d molecule.¹⁸

In vivo priming with peptide mixture

The observation that the priming was peptide specific was further substantiated when we used for the immunization two homologous peptides, derived from the V3 sequences of HIV-1 IIIB and MN isolates (Table 2). A significantly reduced cytotoxicity was observed when splenocytes from mice immunized with B2M-P3C (IIIB) were tested against target cells pulsed with the homologous MN sequence (KRKRIHIGP-GRAFYTCKN), designated as B8M. Conversely, MN-specific CTL did not recognize the IIIB peptide. Therefore, it was of interest to determine the feasibility of eliciting a broader cytotoxicity by injecting a mixture of the two peptides. Mice were injected with 20 µg of B2M-P3C together with 80 µg of B8M-P3C. Positive killing of the target cells presenting IIIB or MN sequences was then obtained. The levels of lysis were comparable to those obtained with IIIB- or MN-specific CTL.

Priming does not require CD4⁺ T cells

The role of CD4⁺ lymphocytes in priming was investigated by producing *in vivo* deficiency of CD4⁺ T cells.¹⁹ BALB/c mice were injected with 300 µg of monoclonal antibody GK1.5 before (day -6, -3, -1) and after (day +1, +3, +5) the priming. Flow cytometry analysis of the splenocytes from the treated

Table 4. Inhibition of CTL activity by carrageenan pretreatment

In vivo treatment		% specific lysis of targets (E:T)					
		P815+B2M-P3C			RDM4		
		10:1	5:1	2:1	10:1	5:1	2:1
—	B2M-P3C	62	32	20	48	34	21
Carrageenan	B2M-P3C	13	7	3	43	27	12

BALB/c mice (H-2^d) were immunized with B2M-P3C or injected with carrageenan on days 5 and 2 prior to the priming. Responder cells were stimulated *in vitro* against B2M peptide or irradiated allogeneic RDM4 cells (H-2^k). CTL activity was determined against P815 pulsed with B2M peptide and RDM4 cells at the effector to target cell ratios (E:T) indicated. The results are means of two separate experiments.

mice demonstrated a greater than 90% depletion of the splenic T-cell subset (data not shown). However, removal of the CD4⁺ T cells did not affect the development of the anti-peptide CTL response (Table 3). The elimination of functional helper cells in GK1.5-treated animals was clearly demonstrated by the inhibition of the mouse antibody response to a T-dependent antigen, such as SRBC, as measured by the plaque-forming assay.

Possible role of macrophages as antigen-presenting cells

We also investigated whether macrophages are able to function as antigen-presenting cells for the generation of B2M-P3C-specific CTL. We used an immunopharmacological manipulation known to deplete macrophage function selectively without inhibiting the reactivity of B and T lymphocytes.²⁰ Animals were given two i.p. injections of 1 mg carrageenan 5 and 2 days before immunization. Following this treatment, we observed a marked reduction in the number of splenic PFC to sheep erythrocytes in all the mice (data not shown), in agreement with the finding that carrageenan suppresses humoral responses to T-dependent antigens by interfering with the macrophage function.²¹ One week after priming, spleen cells of carrageenan-treated mice were used to generate peptide-specific CTL responses. As shown in Table 4, the exposure to carrageenan inhibited the generation of the cytotoxic activity against target cells presensitized with the HIV peptide. However, carrageenan-treated mice were still able to mount CTL response against alloantigens, demonstrating that they were not impaired in their T-lymphocyte repertoire.

DISCUSSION

The use of lipo-peptide immunogens, such as MAP-P3C, is advantageous for the generation of CD8⁺ CTL. We have demonstrated that high levels of cytotoxicity can be induced with a single injection. The response, detectable by 3 days after immunization, was associated with the induction of a long-lasting (>6 months) CTL memory. Previous studies have indicated that the route of immunization with a free peptide was crucial for mounting CTL response.⁹ In contrast, the route used for MAP-P3C immunization is not critical. Intraperitoneal priming was as efficient as intravenous immunization. Furthermore, we have found that other routes of administration are also effective. Experiments performed giving B1M-P3C (a leng-

thened B2M sequence) subcutaneously²² or intragastrically (B. Nardelli and J. P. Tam, manuscript in preparation) resulted in the induction of specific CTL activity in murine splenocytes.

The priming efficiency of the MAP-P3C conjugate could potentially be explained by the stimulation of CD4⁺ T-helper cells, achieved through the activation of macrophages by P3C and the subsequent release of interleukin-1.²³ Alternatively, the peptide *per se* could directly activate T-helper cells, since the *in vivo* induction of CTL by a free peptide depends on its binding to CD4⁺ T cells.²⁴ The latter explanation is unlikely because, while the B2 sequence is able to activate both CD8⁺ and CD4⁺ lymphocytes²⁵ our previous data suggest that the MN sequence does not contain a murine T-helper epitope.¹⁶ Nevertheless, we achieved specific CTL priming with either sequence. However, the experiments conducted on CD4-depleted mice clearly demonstrate that the CTL response can be generated in the absence of functional CD4⁺ cells. This conclusion is not surprising since it has been demonstrated that CD8⁺ T cells are sufficient to control viral infections in the absence of CD4⁺ help.¹⁹ It is possible that in our experimental system lymphokines are produced by T-helper cells of the CD8⁺ phenotype,²⁶ by the CTL themselves,²⁷ or that the CTL precursors are totally independent of helper factors for their maturation.²⁸ Our results contrast with those of other groups with use free peptides in Freund's adjuvant for the immunization. In those experiments, successful priming required CD4⁺ T-helper cells. Taken together, these findings indicate that the cellular events triggered by a peptide covalently linked to a lipid moiety are distinct from those elicited by the immunization with a peptide presented in a lipid environment, such as Freund's adjuvant, where the adjuvant's role is to activate T-helper cell populations.

Specialized cells phagocyte, process, and present degraded products of exogenous antigens in association with class I glycoproteins to the T-cell receptors.^{29,30} Debrick *et al.*³¹ have recently proposed that macrophages play the leading role in the *in vivo* class I-restricted cytotoxicity, through the cognate interaction with CTL precursors. On the basis of this finding, we evaluated the effect of macrophage depletion on CTL induction by the administration of carrageenan, a cytotoxin for phagocytic cells. Our results demonstrate that macrophages are indeed important for antigen presentation in priming with the MAP-P3C construct. Our hypothesis is that macrophages process lipid-peptide conjugates and present them in association with class I molecules directly to the resting T lymphocytes without a

significant contribution of CD4⁺ T-helper cells. However, we cannot rule out that dendritic cells are involved in the antigen processing or in its presentation to the CTL precursors, subsequent to macrophage degradation. Dendritic cells have been reported as potent stimulators of T-cell responses *in vitro* and are able to support the generation of CD8⁺ CTL in absence of CD4⁺ T help.³²

The mechanism by which the exogenous MAP-P3C antigen gains access to the processing pathway required for class I-restricted CTL response remains to be elucidated. The lipophilic portion of the antigen may allow its entry into the cytosolic compartment of the cell and the subsequent association with class I molecules. Kabanov *et al.* have suggested lipid modification as a general step for protein translocation across biological membranes.³³ Indeed, using electron energy loss spectroscopy (EELS), it was found that P3C penetrates the macrophage plasma membrane and could be located within the cytoplasm.³⁴ Alternatively, the hydrophobic P3C moiety may generate a strong interaction with the plasma membrane of accessory cells. In this regard, it has been suggested that a stable association of the antigen with the macrophage cytoplasmic membrane could provide access of the peptide to unoccupied MHC class I molecules at the cell surface.³⁵

In conclusion, we believe that lipo-peptide conjugates, such as MAP-P3C, provide a valuable means of inducing a general immune response and should be considered as a useful vaccine model for HIV-1. The conjugate overcomes a major limitation in developing successful subunit vaccines. Since the induction of B and CD4⁺ T lymphocytes is usually not paralleled by an equally efficient CD8⁺ T-cell response, which may be important in controlling the infection. Moreover, it is particularly encouraging that the administration of a mixture of homologous P3C-peptides can raise cross-specific CTL. This is a required step in the production of a multivalent vaccine that might overcome the sequence variation in the CTL epitopes of different HIV-1 strains and, potentially, genetic restriction.

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