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Induction of feline immunodeficiency virus-specific cell-mediated and humoral immune responses following immunization with a multiple antigenic peptide from the envelope V3 domain

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SUMMARY

Cytotoxic T-cell determinants should be an important component of a vaccine against feline immunodeficiency virus (FIV). Epitope mapping studies have revealed an immunodominant neutralization epitope within the third variable (V3) domain of the viral envelope glycoprotein comprizing 17 amino acids (residues 390–406: RAISSWKQRNRWEWRPD). We have investigated the induction of FIV-specific cytotoxicity and anti-peptide antibody in cats immunized with a multiple antigenic peptide (MAP) containing this epitope. Virus-specific lymphocytotoxicity was determined using autologous or allogeneic skin fibroblasts as target cells labelled with chromium-51 and pulsed with overlapping 10 amino acid peptides. Cytotoxic effector cells derived from fresh peripheral blood were detected in five out of 10 immunized cats. The cell-mediated immune response appeared to be directed to envelope peptide 1 (RAISSWKQRN) and peptide 2 (SWKQRNRWEW), with recognition of peptide 3 (QRNRWEWRPD) in only one cat. An antibody response to the 17 amino acid peptide immunogen was detected in seven immunized cats, which was directed to envelope peptides 2 and 3. These results suggest that different epitopes may be recognized by the cell-mediated and humoral immune responses. None of the cats was protected from challenge with the $Glasgow_8$ isolate of FIV (FIV/GL-8). This study has implications for vaccine strategies using synthetic peptides to induce virus-specific cell-mediated immune responses.

Determination of the appropriate immune responses and the fine recognition specificity of such responses are an important prerequisite to the rational design of an effective vaccine against lentiviral infection. There is an increasing volume of indirect evidence to suggest that $CD8^+$ cytotoxic T lymphocytes (CTL) represent an important component of the protective immune response following infection with human immunodeficiency virus (HIV-1). During the early stages of HIV a major expansion of $CD8^+$ T cells with a predominant $V\beta$ usage mediating HIV-specific cytotoxicity has been described which is concomitant with a dramatic down-regulation in viraemia.[1] Vigorous HIV-specific CTL activity is observed at various sites throughout the body following infection, with recognition of both major viral structural and regulatory gene products.[2,3] This activity decreases with disease progression,[4] indicating a possible role of CTL in maintaining the asymptomatic stage of HIV-1 infection.[5] Further support for a protective role for CTL comes from reports of HIV-1-specific CTL activity in highly exposed yet seronegative individuals,[6,7] and in virus-free children born to HIV-1-infected mothers, suggesting that a CTL response may have eliminated the infection.[8,9]

Feline immunodeficiency virus (FIV), a recently discovered lentivirus occurring as a persistent infection in feline populations worldwide,[10] represents an increasingly important model for HIV in humans. Although the immune responses to FIV and the viral antigens recognized are as yet incompletely characterized, the envelope glycoprotein is likely to be involved in virus neutralization. The third variable region (V3) of FIV and HIV-1 have a number of analogous structures[11] and this region of the HIV-1 external glycoprotein contains important immunoreactive domains involved in virus neutralization.[12–14] The identification of specific epitopes is an important contribution to the design of a vaccine against lentiviral infection that stimulates a Major Histocompatibility Complex (MHC)-restricted cytotoxic response. Epitope mapping studies using synthetic peptides[15] or overlapping fragments of the envelope protein[16] of FIV have revealed immunodominant neutralization sites within this region. In the present study, cats were immunized with a synthetic peptide of the FIV envelope V3 domain as a multiple antigenic peptide (MAP) complex with Quil A and aluminium hydroxide as adjuvants. The capacity of this immunization regime to induce virus-specific cell-mediated

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Table 1. Amino acid sequences of FIV envelope peptides.

Peptide	Residue span	Sequence*
17-residue	390–406	RAISSWKQRNRWEWRPD
env peptide 1	390–399	RAISSWKQRN
env peptide 2	394–403	SWKQRNRWEW
env peptide 3	397–406	QRNRWEWRPD

* One-letter code is used for amino acids. The amino acid sequence shown for FIV is based on the Glasgow 8 and Petaluma isolates of the virus.

and humoral immune responses was evaluated, and the fine recognition specificities of these responses examined.

The 15 12-week-old, outbred, specific-pathogen free, domestic cats selected for this study were serologically negative for FIV. Ten cats were immunized subcutaneously on three occasions 3 weeks apart with 100 μg of a multiple antigenic peptide (MAP; MW = 20·6 K) comprizing a 17-residue peptide from the *env* V3 region of FIV (Table 1) on an eight-branched poly-lysine core with 50 μg Quil A (Superfos Biosector a/s, Vedbaek, Denmark) and 1% aluminium hydroxide as adjuvants in 1 ml of sterile phosphate buffered saline (PBS). Five control cats were immunized three times with 50 μg Quil A in 1% aluminium hydroxide alone at 3-week intervals. The cats were challenged intraperitoneally 3 weeks after the final immunization with 10 cat-infectious doses 50% (cid 50) of the $Glasgow_8$ isolate of FIV (FIV/GL-8). Following challenge peripheral blood mononuclear cells (PBMC) were collected at 3-week intervals for virus isolation.

Two weeks following the third immunization 5 ml peripheral venous blood was collected into an equal volume of Alsevers' solution (Scottish Antibody Production Unit, Carluke, UK), and PBMC were prepared by centrifugation over Ficoll–Paque (Pharmacia LKB, Biotechnology Inc., Piscataway, NJ) for determination of virus-specific lymphocytotoxicity. At the same time 2 ml of blood was collected into a heparinized container for determination of peptide-specific antibodies in the plasma. Fibroblast cell lines were derived from skin biopsy samples (4 mm in diameter) obtained from all cats under general anaesthesia prior to immunization, and maintained in minimal essential medium (MEM) ALPHA medium with ribonucleosides and deoxyribonucleosides (Gibco Biocult, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, and 100 IU of penicillin, 100 μg streptomycin, 10 ng of human recombinant epidermal growth factor (Sigma, Poole, UK) per ml.

Virus-specific CTL present in fresh PBMC were detected using autologous or allogeneic skin fibroblast target cells which had been pulsed with 1 μM FIV *env* peptides (see Table 1) in complete MEM ALPHA medium for 1·5 hr at 37°, washed once in this medium, and then labelled with 50 μCi of sodium ^{51}chromate (Amersham International PLC, Amersham, UK) per 10^6 cells for 1 hr at 37° and washed three times prior to use in the assays. Microcytotoxicity assays were performed in triplicate by adding appropriate numbers of effector cells to 2×10^4 target cells to give effector:target (E:T) ratios of 50, 25 and 12·5:1, in a total volume of 200 μl of RPMI-1640 medium (Gibco Biocult) supplemented with 10% FBS, 2 mM

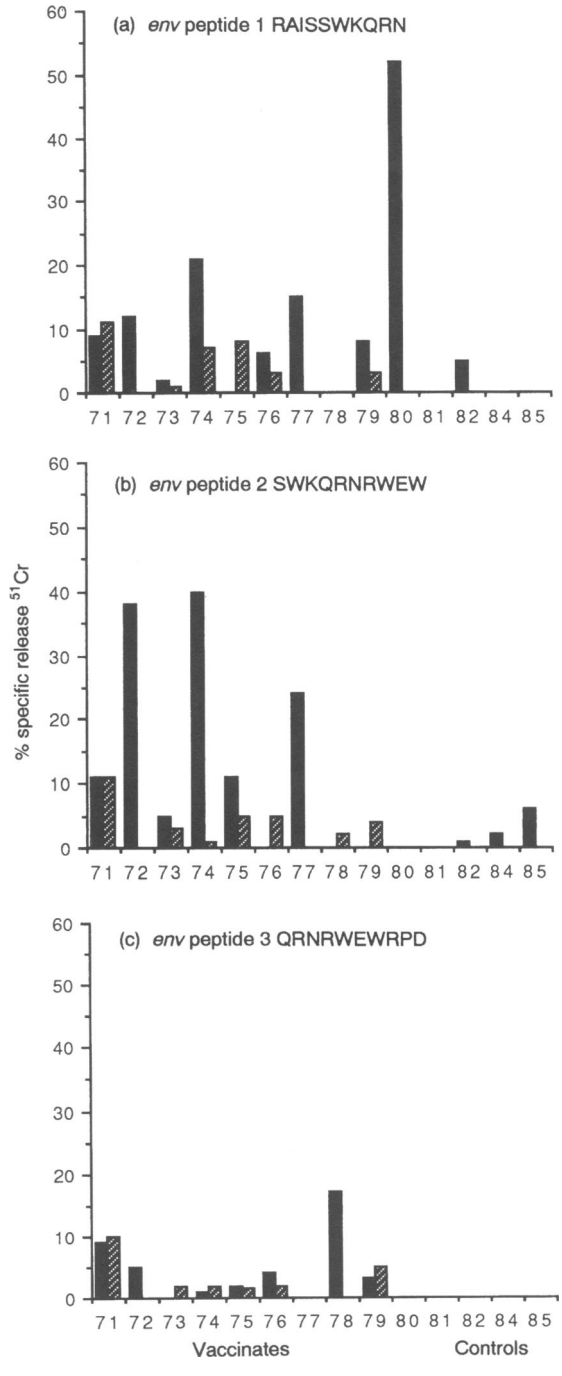

Figure 1. Detection of FIV envelope-specific CTL responses. The FIV *env* peptide-specific CTL activity of PBMC isolated from 10 immunized cats (71 to 80 inclusive) and four control cats (81 to 85 inclusive) immunized with adjuvant alone was determined. Autologous (■) or allogeneic (▨) skin fibroblasts were labelled with ^{51}Cr and pulsed with overlapping 10 amino acid peptides from the *env* V3 loop of FIV corresponding to residues RAISSWKQRN (a), SWKQRNRWEW (b), or QRNRWEWRPD (c). The release of ^{51}Cr was detected in the culture supernatant after 4-hr incubation at 37°. The results shown are from triplicate cultures at an E:T ratio of 50:1. The values for control target cells pulsed with medium alone have been subtracted from the values shown.

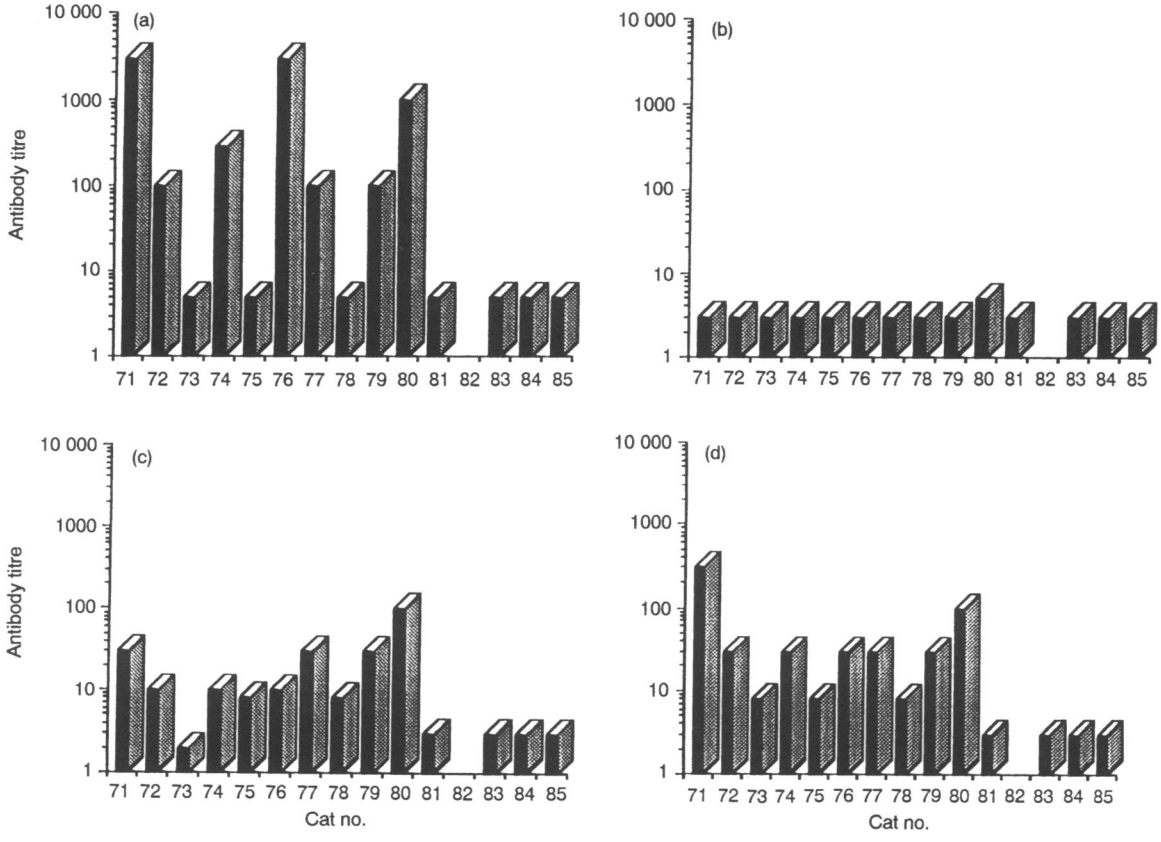

Figure 2. Induction of FIV envelope-specific antibody responses. Plasma samples were collected from immunized (71 to 80 inclusive) and control (81 to 85 inclusive) cats 2 weeks following the third immunization with the *env* V3 MAP, and examined by ELISA for the presence of antibodies to the a linear 17 amino acid peptide with the same sequence as the immunogen (A, RAISSWKQRNRWEWRPD), *env* peptide 1 (B, RAISSWKQRN), *env* peptide 2 (C, SWKQRNRWEW), and to *env* peptide 3 (D, QRNRWEWRPD).

L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 IU penicillin and 100 μg streptomycin per ml in V-bottomed microtitre plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). After 4 hr incubation at 37° in a humidified atmosphere containing 5% CO_2, 50 μl of supernatant was removed from each well and added to the wells of a 96-well 'Luma Plate' (Canberra Packard, Pangbourne, Berkshire, UK), allowed to dry and then the ^{51}Cr-specific activity measured in a 'Top Count' Microplate Scintillation Counter (Canberra Packard, UK). Maximum release was obtained by treating the labelled target cells with 1% Triton X-100 (BDH, Poole, Dorset, UK) and spontaneous release was determined using uninfected target cells. Percentage cytotoxicity was calculated follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

Peptide-specific CTL activity was detected in the fresh peripheral blood of five out of 10 immunized cats (Fig. 1). Of these cats, three recognized both *env* peptide 1 (RAISSWKQRN) and *env* peptide 2 (SWKQRNRWEW), one recognized *env* peptide 1 only, and one recognized *env* peptide 3 (QRNRWEWRPD) only. The response was only observed when autologous target cells were used in the assay, suggesting that the response was MHC restricted. There was negligible recognition of target cells pulsed with medium alone (data subtracted from results shown), nor was any FIV peptide-specific CTL activity observed in control cats immunized with adjuvant alone. The ability of viral synthetic peptide immunogens to elicit virus-specific CTL is well recognized and has been described for influenza virus,[17] Sendai virus,[18] lymphocytic choriomeningitis virus,[19,20] and HIV.[21] However, all of these earlier studies were performed with mice and the majority used adjuvants that are not currently commercially acceptable. There are no reports currently available on the use of MAPs to elicit virus-specific CTL responses, although protein antigens administered with ISCOMS do induce antigen-specific CTL activity,[22,23] and linear peptide administered with Quil A has been shown to induce virus-specific CTL in cats.[24] In the present study, peptide-specific CTL were detected in only half of the immunized cats, suggesting that such peptide complexes enter into the endogenous antigen processing pathway for subsequent association with MHC Class I molecules with a relatively low level of efficiency, or that the overlapping 10-residue peptides used in the detection assay may have been inappropriate for the epitopes generated following immunization with the 17-residue peptide from the *env* V3 region. However, in the five cats that did exhibit a CTL response, high levels of lymphocytotoxicity were observed (up to 52% at an

E:T of 50:1). Furthermore, this CTL activity was demonstrable in freshly isolated PBMC, without any requiremnet for prior *in vitro* restimulation of the effector cells.

Anti-peptide antibodies were detected as described previously.[24] Seven out of 10 immunized cats had an antibody response to the linear 17-residue peptide that had the same sequence as the peptide used in the MAP immunogen (Fig. 2a). No response was observed in control cats immunized with adjuvant alone. The elicitation of peptide-specific antibodies as a result of this immunization regime would suggest that the MAP and the adjuvants used were capable of stimulating $CD4^+$ helper T cells in an MHC Class II-restricted manner, thus resulting in the production of anti-peptide antibodies. In an attempt to define the residues involved in antibody binding the assays were repeated using smaller constituent 10-residue peptides. There was no detectable recognition of *env* peptide 1 (Fig. 2b), but cats that had an antibody response to the 17-residue immunogen also had a response to *env* peptides 2 and 3 (Fig. 2c and d), although the antibody titre was higher to *env* peptide 3 than peptide 2. These peptides share the common sequence QRNRWEW, suggesting that this region may comprise the B-cell epitope present in the 17-residue peptide of the *env* V3 region. The epitope involved in CTL recognition would appear to be different from that involved in antibody binding, since the CTL responses were directed against peptides 1 and 2 with the recognition of peptide 3 observed in only one cat. Following challenge with FIV/GL-8, none of the cats was protected. In a similar study, cats immunized with a linear peptide of the V3 domain of FIV developed neutralizing antibodies, but these cats also were not protected from challenge.[25] Taken together these studies suggest that neither FIV *env*-specific CTL, nor virus-neutralizing antibodies would appear be sufficient on their own to protect cats from challenge. Nevertheless, HIV V3 loop synthetic peptides have also recently been shown to be highly immunogenic *in vivo* and may prove an important component in an effective vaccine against HIV.[26]

In summary, immunization of cats with a 17-residue synthetic peptide of the third variable domain of the FIV envelope glycoprotein induced high levels of FIV-specific cell-mediated and humoral immune responses in a proportion of immunized cats. Epitope mapping studies revealed differences in the fine recognition specificity of the MHC Class I- and Class II-restricted immune responses observed. This study has important implications for the design of a vaccine which is required to stimulate both cell-mediated and humoral immune responses; however, larger numbers of animals with defined MHC haplotypes should be examined before any firm conclusions on the epitopes recognized can be drawn.

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REFERENCES

1. Panteleo G., Demarest J.F., Soudeyns H. *et al.* (1994) Major expansion of $CD8^+$ T cells with a predominant Vβ usage during the primary immune response to HIV. *Nature* **370**, 463.
2. Nixon D.F., Townsend A.R.M., Elvin J.G., Rizza C.R., Gallwey J. & Mcmichael A.J. (1988) HIV-1 *gag*-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. *Nature* **336**, 484.
3. Nixon D.F., Broliden K., Ogg G. & Proliden P.-A. (1992) Cellular and humoral antigenic epitopes in HIV and SIV. *Immunology* **76**, 515.
4. Nixon D.F. (1992) The cytotoxic T cell response to HIV. In: *Immunology of HIV* (ed. G. Bird), p. 59. Kluwer Academic Publishers, Liverpool.
5. Baltimore D. & Feinberg M.B. (1989) HIV revealed: Towards a natural history of the infection. *N Engl J Med* **321**, 1673.
6. Levy J.A. (1993) HIV pathogenesis and long-term survival. *AIDS* **7**, 1401.
7. Salk J., Bretscher P.A., Salk P.L., Clerici M. & Shearer G.M. (1993) A strategy for prophylactic vaccination against HIV. *Science* **260**, 1270.
8. Cheynier R., Langlade-Demoyen P., Marescot M.-R. *et al.* (1992) Cytotoxic T lymphocyte responses in the peripheral blood of children born to human immunodeficiency virus-1-infected mothers. *Eur J Immunol* **22**, 2211.
9. Rowland-Jones S.L., Nixon D.F., Aldhous M.C. *et al.* (1993) HIV-specific cytotoxic T-cell activity in an HIV-exposed but uninfected infant. *Lancet* **341**, 860.
10. Pedersen N.C., Ho E.W., Brown M.L. & Yamamoto J.K. (1987) Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science* **235**, 790.
11. Pancino G., Fossati I., Chappey C. *et al.* (1993) Structure and variation of feline immunodeficiency virus envelope glycoproteins. *Virology* **192**, 659.
12. Javaherian K., Langlois A.J., McDanal C. *et al.* (1989) Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *Proc Natl Acad Sci USA* **86**, 6768.
13. Ohno T., Terada M., Yoneda K. *et al.* (1990) A broadly neutralizing monoclonal antibody that recognizes the V3 region of human immunodeficiency virus type 1 glycoprotein gp120. *Proc Natl Acad Sci USA* **64**, 5701.
14. Gorny M.K., Conley S., Karwowska S., Buchbinder A., Xu J.-Y., Emini E.A., Koenig S. & Zolla-pazner S. (1992) Neutralization of diverse human immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. *J Virol* **66**, 7538.
15. Lombardi S., Garzelli C., La Rosa C. *et al.* (1993) Identification of a linear neatralization site within the third variable region of the feline immunodeficiency virus envelope. *J Virol* **67**, 4742.
16. Deronde A., Stam J.G., Boers P. *et al.* (1994) Antibody response in cats to the envelope proteins of feline immunodeficiency virus: Identification of an immunodominant neutralization domain. *Virology* **198**, 257.
17. Gao X.-M., Zheng B., Liew F.Y., Brett S. & Tite J. (1991) Priming of influenza virus-specific cytotoxic T lymphocytes *in vivo* by short synthetic peptides. *J Immunol* **147**, 3268.
18. Kast W.M., Roux L., Curren J. *et al.* (1991) Protection against lethal Sendai virus infection by *in vivo* priming of virus-specific cytotoxic T lymphocytes with a free synthetic peptide. *Proc Natl Acad Sci USA* **88**, 2283.
19. Aichele P., Hengartner H., Zinkernagel R.M. & Schulz M. (1990) Antiviral T cell response induced by *in vivo* priming with a free synthetic peptide. *J Exp Med* **171**, 1815.
20. Schulz M., Zinkernagel R.M. & Hengartner H. (1991) Peptide-induced antiviral protection by cytotoxic T cells. *Proc Natl Acad Sci USA* **88**, 991.
21. Hart M.K., Weinhold K.J., Scearce R.M. *et al.* (1991) Priming of anti-human immunodeficiency virus (HIV) $CD8^+$ cytotoxic T cells *in vivo* by carrier-free HIV synthetic peptides. *Proc Natl Acad Sci USA* **88**, 9448.
22. Takahashi H., Takeshita T., Morein B., Putney S., Germain

R.N. & BERZOFSKY J.A. (1990) Induction of CD8+ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMS. *Nature* **344**, 873.

23. MOWAT A.M., DONACHIE A.M., REID G. & JARRETT O. (1991) Immune stimulating complexes containing Quil A and protein antigen prime class I MHC-restricted T lymphocytes *in vivo* and are immunogenic by the oral route. *Immunology* **72**, 317.
24. FLYNN J.N., CANNON C.A., BEATTY J.A. *et al.* (1994) Induction of feline immunodeficiency virus-specific T cells *in vivo* with carrier-free synthetic peptide. *J Virol* **68**, 5835.
25. LOMBARDI S., GARZELLI C., PISTELLO M. *et al.* (1994) A neutralizing antibody-inducing peptide of the V3 domain of feline immunodeficency virus envelope glycoprotein does not induce protective immunity. *J Virol* **68**, 8374.
26. RUBINSTEIN A., GOLDSTEIN H., PETTOELLO-MANTOVANI M. *et al.* (1995) Safety and immunogenicity of a V3 loop synthetic peptide conjugated to purified protein derivative in HIV-seronegative volunteers. *AIDS* **9**, 243.