HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women

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A crucial requirement in the rational design of a prophylactic vaccine against the human immunodeficiency virus (HIV) is to establish whether or not protective immunity can occur following natural infection. The immune response to HIV infection is characterized by very vigorous HIV-specific cytotoxic T-lymphocyte (CTL) activity. We have identified four HIV-1 and HIV-2 cross-reactive peptide epitopes, presented to CTL from HIV-infected Gambians by HLA-B35 (the most common Gambian class I HLA molecule). These peptides were used to elicit HIV-specific CTLs from three out of six repeatedly exposed but HIV-seronegative female prostitutes with HLA-B35. These women remain seronegative with no evidence of HIV infection by polymerase chain reaction or viral culture. Their CTL activity may represent protective immunity against HIV infection.

The production of an effective prophylactic vaccine against human immunodeficiency virus (HIV) infection would be greatly assisted by an understanding of the requirements for protective immunity. One step towards this is to determine if such immunity can develop naturally following exposure to the virus. HIV can usually be isolated from seropositive subjects at any stage after infection[1], and cohort studies suggest that most people with HIV-antibodies will ultimately develop disease[2]. Thus there is little evidence that HIV-seropositive people can eliminate their virus. However, some individuals remain seronegative despite definite exposure to HIV and several studies suggest that cellular immune responses to HIV can occur in the absence of persistent antibody in such cases. These include T-helper (Th) cell proliferation and interleukin-2 (IL-2) secretion in response to envelope peptides[3–6], and inhibition of HIV replication by their $CD8^+$ T cells[7]. It remains unclear, however, whether these responses reflect exposure to replication-competent virus or merely to defective virus particles or protein antigens. The induction of major histocompatibility complex (MHC) class I-restricted CTL may be a more reliable indication of exposure to replicating virus[8,9], and the finding of HIV-specific CTL in a small number of apparently uninfected children born to HIV-infected mothers is believed to indicate exposure to live virus[10–12]. The most important subjects for study are those repeatedly exposed to HIV who remain uninfected and in whom persistent seronegativity might truly represent resistance to HIV infection. Preliminary studies in uninfected sexual partners of HIV-infected people suggest that they may have an increase in *nef*-specific CTL precursors[13]. We have extended these studies by looking for the presence of HIV-specific CTL in a group of repeatedly exposed but persistently seronegative female prostitutes in The Gambia, West Africa.

HIV-specific CTL activity can be readily detected in asymptomatic HIV-infected donors, often directly from unstimulated peripheral blood mononuclear cells (PBMCs)[14,15] suggesting that there is a high frequency of circulating CTL in response to continuing viral replication[16,17]. In contrast, CTL activity in the absence of persistent infection might be transient and temporally related to exposure, as seen in several of the babies born to infected mothers. We therefore selected for study a group of women who are likely to have been (and continue to be) repeatedly exposed to both HIV-1 and HIV-2. Approximately 35% of the female sex-workers in Gambian towns are HIV-infected and this has been increasing rapidly[18,19]. Whereas initially HIV-2 was predominant, most recent infections are with HIV-1. The seronegative women in this study have worked as prostitutes for more than five years, use condoms infrequently with clients and only rarely with their regular partners[20], and have a high incidence of other sexually transmitted diseases, making it less likely that they have escaped exposure to HIV simply by chance. Although the prevalence of HIV infection in their clients is not known, levels of infection in the general population are consistent with their exposure to at least one HIV-infected man every month.

HIV-specific CTL from infected donors are usually detected using their endogenous virus to restimulate the CTL *in vitro*[21], but this method would not be appropriate to elicit CTL from uninfected donors. One approach is to use exogenous HIV to stimulate CTL in culture, but this may damage the $CD4^+$ T cells needed to initiate the response and was not successful in these studies. Virus-specific CTL recognize viral antigens in the form of naturally processed peptides, eight to ten amino acids in length, which are bound in the cleft of class I MHC molecules on the surface of

Table 1. Identification of peptides presented by HLA-B35 to CTL from HIV-infected donors

Protein	Amino acid residues	HIV strain	Amino acid consensus sequence
Nef	75–82	HIV-1 & -2	VPLRPMTY
Pol	329–337	HIV-1	HPDIVIYQY
		HIV-2	NPDVILIQY
p24	260–269	HIV-1	PPIPVGDIY
		HIV-2	NPVPVGNIY
p17	130–138	HIV-1	NSSKVSQNY
		HIV-2	PPSGKGGNY

the infected cell and presented to the T-cell receptors of the CTL[22]. In order to detect CTL in the women in this study we first defined peptide epitopes from HIV-1 and -2, recognized by CTL from infected donors in association with the most common Gambian human leucocyte antigen (HLA) class I molecule, HLA-B*3501 (present in 32% of the major ethnic group[23]). These peptides showed evidence of cross-reactivity between HIV-1 and HIV-2, that is, CTL from donors infected with one strain of HIV frequently recognized the corresponding peptide from the other virus strain. The peptides were then used directly to stimulate CTL from the PBMCs of seronegative donors, a protocol which has previously been shown to be an efficient method of generating secondary specific (or memory) CTL responses *in vitro*[24–26]. Using this strategy, specific CTL activity against one or more HIV peptides was detected in three out of six highly exposed but apparently uninfected women with HLA-B35 but not in a panel of 19 volunteers with no history of HIV exposure.

Identification of peptides presented by HLA-B35 to CTL

The amino acid sequence motif of peptides binding to HLA-B35 has previously been determined and used to identify HLA-B35-restricted epitopes from *P. falciparum*[25]. In a similar way the consensus sequences of HIV-1 and-2 *gag*, *pol* and *nef* proteins were scanned for possible B35-binding epitopes, *i.e.* octamers or nonamers with proline or serine at position two of the peptide and tyrosine at the C terminus. Candidate peptides were synthesised and screened for binding to HLA-B35, using the previously described T2-B35 assembly assay[25]. Those which bound (15 out of 19) were tested for recognition by bulk CTL cultures from HIV (−1 or −2)-infected Gambians, identified as having HLA-B*3501 by serological typing and isoelectric focusing[27].

The majority of HIV-infected donors with HLA-B35 had B35-restricted CTL recognizing one or more peptide from *gag* (p17 and p24), *pol* and *nef* (Table 1). The *nef* peptide, which is conserved between HIV-1 and -2, has previously been identified as an epitope for CTL from HIV-1-infected donors[28] and was also recognized by CTL from HIV-2-infected donors (Fig. 1*a*). For the other peptides, CTL from most HIV-2-infected donors recognized both the HIV-2 peptide and the corresponding peptide from HIV-1 (Fig. 1*b*), suggesting that donors with a strong B35-restricted CTL response against HIV-2 may show cross-immunity to HIV-1. The HIV-1 *pol* and p24 peptides were subsequently confirmed as epitopes for B35-restricted CTL from HIV-1-infected donors (both European and Gambian), and also showed cross-reactivity between HIV-1 and -2 peptides (Fig. 1*c*). This is in contrast to the only previously described peptide epitope from HIV-2, where HLA-B53-restricted CTL from HIV-2-infected donors fail to recognize the corresponding HIV-1 *gag* sequence[29].

CTL studies in exposed seronegative donors

The study population consisted of 20 women — 14 had been prostitutes for more than five years and reported little condom usage with clients or regular partners[20] and six were long-term sexual partners of HIV-infected men. Cells from those donors clearly typed by serology as having HLA-B35 or B53 were stimulated *in vitro* with each of the candidate B35-restricted peptides from HIV-1 and -2 or the previously identified B53-restricted peptide from HIV-2 (ref. 29). Peptide-stimulated cultures were also set up from eight known seropositive donors with HLA-B35 or B53, and from a control group of volunteers at low-risk of HIV infection[19] with HLA-B35 (12 Gambian and seven European) and two Gambians with HLA-B53. For those donors without either HLA-B35 or B53, CTL cultures were established using autologous phytohaemagglutinin (PHA) blasts infected with HIV-1 (IIIB) and a Gambian strain of HIV-2 (ref. 30). Similar cultures were set

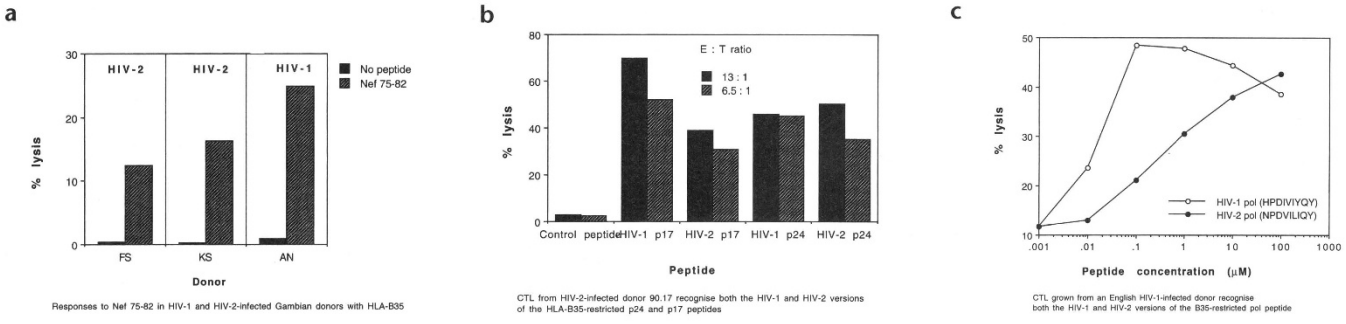

Fig. 1 Cross-reactivity between HIV-1 and HIV-2 in the recognition of peptides presented by HLA-B35 by CTL from HIV-infected donors. CTL assays were performed using 2-week-old bulk CTL cultures from donors with HLA-B35 known to be infected with either HIV-1 or HIV-2, using B35-matched targeted cell-lines pulsed with each of the peptides and a control peptide at a concentration of 10 mM. *a*, CTL bulk cultures from donors infected with either HIV-1 (AN) or HIV-2 (FS, KS) show specific recognition of the HIV nef peptide[75–82]. *b*, Cultures from HIV-2-infected donor 90.17 show specific recognition of HLA-B35 target cell-lines pulsed with either the HIV-1 or HIV-2 versions of the HLA-B35-restricted p17 and p24 peptides. *c*, CTL grown from a British HIV-1-infected haemophiliac donor are tested on HLA-B35-matched target cells pulsed with increasing concentrations of either the HIV-1 or HIV-2 versions of the peptide Pol 329–337.

up from four previously identified seropositive donors.

The results of these studies are shown in Table 2. Using the virus-stimulation protocol, CTL could only be generated in cultures from known HIV-infected donors and not in the exposed seronegatives. However, specific CTL activity against one or more peptides was repeatedly detected after 10–14 days in the peptide-stimulated cultures from three of the six high-risk seronegative women with HLA-B35 (OX11, 12 and 14, in Fig. 2), but not in their three counterparts with HLA-B53 nor in any of the low-risk volunteers (Table 2). These CTL grew in culture and continued to show specific activity without further restimulation for over four weeks. Of the responding seronegatives, donors OX11 and OX12 generated CTL to most of the HIV peptides tested and OX14 responded only to the HIV-1 *pol* peptide. The strongest responses were generated towards the HIV-1 *pol* peptide, which lies close to the active site of reverse trancriptase[31], and to the *nef* peptide, which is conserved between HIV-1 and -2 in a region thought to be important as a protein kinase phosphorylation site[32]. Three months later, further samples were taken from the six exposed seronegative women with HLA-B35, and persistent CTL activity was detected against the HIV-1 *pol* peptide in cultures from OX11 and OX12, but not against any of the other HIV peptides. These CTL killed target cells infected with recombinant vaccinia virus expressing the HIV-1 *pol* protein and recognized the peptide at 1×10^{-6} M (Fig. 3*a*,*b*).

CTL cultures were generated from both HIV-exposed and unexposed donors, using the same protocol, to an HLA-B35-restricted influenza matrix peptide (influenza A matrix protein 128–135; T. D. *et al.*, manuscript in preparation) and an HLA-B35-restricted Epstein-Barr virus (EBV) peptide (EBNA 3a 458–466, ref. 60). The kinetics of these secondary *in vitro* responses against EBV and influenza were virtually identical to those elicited by the HIV peptides in the exposed seronegative women (Fig. 3c).

Table 2. Detection of HIV-specific CTL

Method of stimulation	Risk group	CTL detected
Virus stimulation	High-risk seronegative	0/11
	Known seropositive	3/4
Peptide stimulation HLA-B53 donors	High-risk seronegative	0/3
	Low-risk seronegative	0/2
	Known seropositive (HIV-2)	2/2
Peptide stimulation HLA-B35 donors	High-risk seronegative	3/6
	Low-risk seronegative (Gambian)	0/12
	Low-risk seronegative (European	0/7
	Known seropositive (HIV-1)	2/3
	(HIV-2)	2/3

Occult HIV infection excluded

To exclude occult HIV infection in the women with detectable CTL, samples from each donor were tested for HIV-1 and -2 DNA by means of the polymerase chain reaction (PCR). Nested PCR was performed on two samples taken three months apart and was consistently negative. Attempts to isolate virus, by coculture of cells stored from each bleed with uninfected PHA blasts, monitoring for reverse transcriptase (RT) activity with a highly sensitive assay capable of detecting single virions[33], showed no evidence of HIV infection. HIV-specific CTL in seronegative subjects could potentially be a response to acute HIV infection, before the development of antibodies[34], but the women were still seronegative and virus-culture negative three months after the CTL were first detected, making recent infection extremely unlikely.

Discussion

The most probable explanation for the finding of HIV-specific CTL, able to kill virus-infected cells, in apparently uninfected but repeatedly HIV-exposed women is that they have been immunised by exposure to HIV. This could result from exposure to live or defective virus particles, but live virus would be the most efficient in inducing CTL. Other possible explanations are less likely. Occult HIV infection or delayed seroconversion is improbable in the absence of detectable viraemia using more sensitive methods available. Cross-reactivity from CTL primed by epi-

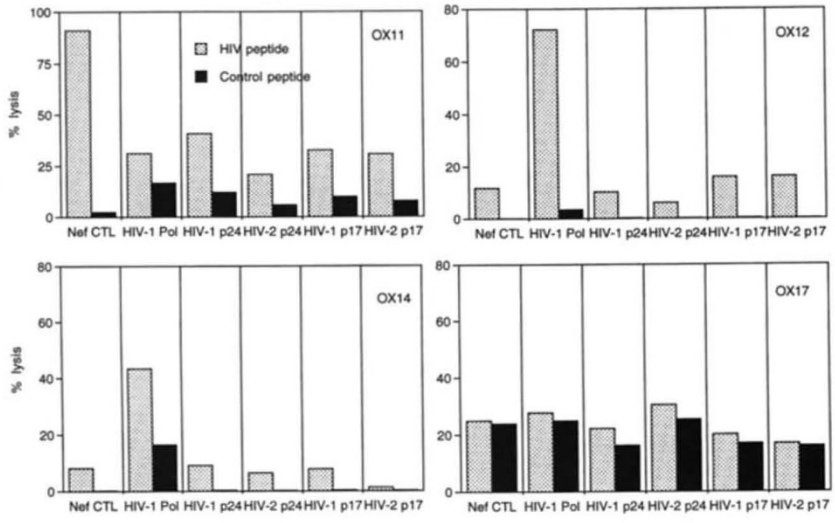

Fig. 2 HLA-B35-restricted CTL using peptide-stimulated cultures from high-risk seronegative donors OX11, 12, 14 and 17. The CTL assays shown were performed on day 12–14 in culture, using a target cell line matched only at HLA-B35, pulsed for one hour with each of the peptides and a control influenza peptide at 10 mM. The effector:target ratio (E:T) varied according to the number of cells that could be harvested from each culture, but was between 12–50:1.The lysis in the presence of a control influenza peptide is shown in the dark columns and that with the specific HIV peptide is shown in the dotted columns. Results were regarded as positive if recognition of the HIV peptide was more than 10% above that of a control peptide on at least two occasions. Donor OX17 was regarded as showing no specific CTL activity, and no other positive results were obtained in cultures from the remaining exposed seronegative donors with HLA-B35 or HLA-B53.

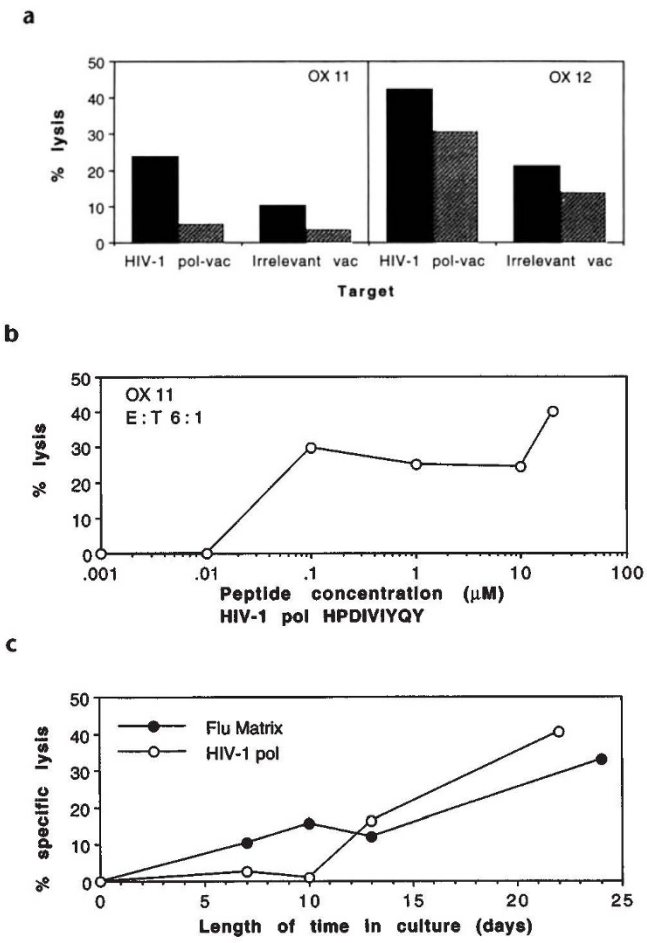

Fig. 3 Peptide-stimulated cultures from the second bleed of high-risk seronegative donors OX11 and OX12. *a*, Recognition of virus-infected cells. Peptide-stimulated cultures were established using the HIV-1 pol peptide as described, and used on day 15 in culture. Target cells were B-LCL from donor FS, matched with the CTL only at HLA-B35, infected with recombinant vaccinia virus expressing full-length HIV-1 Pol or an irrelevant protein, β-galactosidase. E:T ratios were 16:1 (dark columns) and 4:1 (striped columns). *b*, Peptide titration for the HIV-1 Pol peptide. Peptide-stimulated cultures from donor OX12 were used on day 22, at an E:T ratio of 6:1, in a CTL assay using HLA-B35 matched target cells with the HIV-1 Pol peptide diluted in the assay to the concentrations shown. *c*, Time course of CTL activity from the peptide-stimulated cultures. CTL assays using cultures stimulated with either the HIV-1 pol peptide in the case of donor OX12 or the influenza matrix peptide in the case of donor OX11 were performed using similar E:T ratios, over a 24-day period. The figures shown are specific lysis, *i.e.* with the lysis in the presence of a control peptide subtracted from that of the peptide of interest.

topes of similar sequence in another pathogen would be unlikely to lead to responses against more than one HIV peptide, and the recognition by the CTL of target cells pulsed with low concentrations of peptide or infected with virus is much more in keeping with a high-affinity specific response than a low-affinity cross-reaction. A related possibility is that the CTL responses represent cross-reactive alloreactive CTL[35] induced by pregnancies, but the donor with the strongest responses (OX11) is nulliparous whereas the Gambian control population included several multiparous women. Finally, these are unlikely to be primary *in vitro* CTL responses, given that no responses to HIV peptides could be elicited in any of the control donors in the absence of HIV exposure. The generation *in vitro* of primary CTL able to kill virus-infected cells requires special conditions that were not present here, such as the use of specialized antigen-presenting cells[36–38] or peptide modified to allow access to the class I MHC processing pathway[39]. The similarity of the kinetics of the HIV-specific responses and those raised against influenza and EBV peptides strongly suggests that these are secondary, or memory, CTL responses primed by exposure to live virus.

The lack of detectable CTL activity in the other exposed seronegative donors may simply mean that, against the background of relatively low HIV prevalence in the general population, those women have managed to avoid or escape exposure to infectious HIV. Alternatively, it may reflect the limitations of our restimulation protocol, which would be expected to detect only HLA-B35 or B53-restricted CTL primed by virus very similar in sequence to that of the peptides used, and would not therefore detect CTL with other specificities. In addition, the follow-up studies suggest that some HIV-specific CTL responses may be transient, perhaps in relation to the timing of exposure.

If the CTL have been primed by exposure to live HIV, it is surprising that these women remain persistently seronegative. These findings are consistent with the hypothesis that exposure to low doses of virus primes predominantly cellular (or Th-1) immune responses at the expense of humoral (or Th-2) responses[4,40]. In macaques, low-dose simian immunodeficiency virus (SIV) primes Th-1 responses without antibody production (CTL were not measured) and these animals are then protected against a high-dose challenge[41]. In murine leishmaniasis, protection results from cell-mediated immunity without antibody production induced by low-dose immunization[42]. The detection of responses which are cross-reactive between HIV-1 and -2 may be relevant, since Gambian prostitutes are likely to have been first exposed to HIV-2 which appears to be less pathogenic[43], and has a lower transmissibility and virus load than HIV-1 infection[44,45]. Initial exposure to HIV-2 may have led to protective immunity against HIV-1 in women who make a CTL response that is cross-reactive between the two viruses. Cross-reactive neutralizing antibody responses between HIV-1 and -2 are rare[46]; cross-reactive T-helper[47] and CTL[48] responses can occur but are not always seen[29,49–51], so cross-protection may be dependent on the extent of conservation between HIV-1 and -2 in the epitopes selected by particular class I molecules. The strongest responses observed in our seronegative donors were to epitopes which are probably conserved for functional reasons[31,32]. If an effective cross-reactive CTL response could clear infection with a second strain of HIV, this may explain why not all donors with dual seroreactivity for HIV-1 and -2 are actually infected with both viruses[52–54].

The question of whether these women are protected against HIV infection is crucial, and may depend on the persistence of their CTL. Although the CTL response to HIV can be extremely long-lasting at the clonal level in infected patients[17] (P. Moss *et al.* manuscript submitted), it is not known how long specific CTL persist in the absence of antigen. Some studies have demonstrated long-lasting CTL immunity without persisting antigen, although this remains controversial[55–57]. The observation of transient HIV-specific CTL in some uninfected infants of infected mothers[11, 12] and the diminution in detectable CTL after three months in these

women may indicate that CTL levels wane over time. However, studies in a comparable group of women in Nairobi show that a minority (5%) remain uninfected despite repeated exposure over long periods of time and appear to be genuinely resistant to infection (Plummer, F.A. *et al.* 1993, IXth International Conference on AIDS and manuscript submitted).

These findings of HIV-specific CTL in a group of repeatedly exposed but uninfected women suggest that CTL generation may be a central component of protective immunity against HIV and underline the importance of CTL induction in HIV vaccine design.

Methodology

HIV testing. Ethical approval was obtained from the The Gambia Government/MRC Ethical Committee and informed consent prior to HIV testing was given by each donor in the study. Donors were tested for HIV-1 and -2 infection with two type-specific competitive enzyme-linked immunosorbent assays (Murex Diagnostics, Dartford, Kent, U.K.) and with type-specific peptide-strips (Pepti-Lav, Diagnostics Pasteur, Marnes-la-Cougette, France).

Generation of HIV-specific CTL from infected donors. PBMCs were separated from whole blood, and CTL cultures were established by taking one eighth of cells, stimulating them for 24 hours with PHA, washing once and adding them back to the remaining cells[21]. Cells were cultured in RPMI 1640 (Gibco) with 10% fetal calf serum (Gibco) (R/10) and antibiotics for one week, then 10% Lymphocult T (Biotest) was added for the second week. Standard Chromium-51 release assays were performed after two weeks using HLA-B35-matched or control mismatched target B-lymphoblastoid cell lines (B-LCL's) labelled with Chromium-51 and pulsed with each of the HIV peptides or a control influenza peptide at a concentration of 10m M. Background Chromium release was less than 20%. Percent lysis was calculated from the formula 100 x (E-M/T-M), where E is the experimental release, M is the release in the presence of R/10 medium and T is release in the presence of 5% Triton X-100 detergent. Results were regarded as positive if recognition of the HIV peptide was greater than 10% above that of a control peptide on at least two occasions.

CTL studies in exposed seronegative donors. After testing serum for HIV antibodies, PBMCs were separated from 10–15 mls of heparinized-blood and 2 x 10^6 cells were stored at -80°C, for future virological studies. Class I HLA typing was performed by serology, and peptide-stimulated cultures were set up from donors with HLA-B35 or B53. Their cells were pulsed as a pellet for 1 hour with 100 mM of each B35-restricted peptides from HIV-1 and -2, or the previously identified B53-restricted peptide from HIV-2 (ref. 29), at a concentration of 100 mM, then diluted in R/10 to a final concentration of 10 mM and cultured at 2 x 10^6 cells per well in a 24-well Costar plate. IL-2, in the form of Lymphocult-T, was added to a dilution of 10% on day three. Assays for specific CTL activity were carried out after 7–14 days in culture, using HLA-B35 or B53-matched target cells pulsed with the appropriate peptides as above. Some CTL cultures were also tested against matched target cells infected with recombinant vaccinia virus expressing full-length HIV-1 *pol* or an irrelevant protein, b-galactosidase (kind gifts of B. Moss.)

Cells from the donors without HLA-B35 or B53 were stimulated with autologous PHA blasts infected with HIV-1 (IIIB) and a Gambian strain of HIV-2 (CBL-20) (ref. 30). These cultures were tested for HIV-specific CTL activity after 7–14 days using autologous or HLA-matched target cell-lines infected with recombinant vaccinia viruses expressing HIV *gag*, *pol* and *nef* genes.

Control studies. Peptide-stimulated cultures using HIV peptides were set up from known seropositive donors with HLA-B[35] or B and a control group of volunteers at low-risk of HIV infection. (Low-risk volunteers were European laboratory workers, seronegative donors from a rural area of The Gambia with low HIV prevalence and seronegative donors from The Gambian Blood Bank.) CTL cultures were generated from both HIV-exposed and unexposed donors, using the same protocol, to an HLA-B35-restricted influenza matrix peptide (Influenza A matrix protein 128–135) and an HLA-B35-restricted EBV peptide (EBNA 3a 458–466.)

Virological studies in seronegative donors with HIV-specific CTL. Nested PCR was carried out on DNA extracted from PHA blasts on two occasions three months apart for donors OX11 and OX12, and on one occasion for donor OX14, using *gag* primers for HIV-1 (ref. 58) and LTR primers for HIV-2 (ref. 59). Virus isolation was performed as described previously[30], on two samples from each donor taken three months apart. Briefly, PBMCs from the women were stimulated with PHA for 3 days, then cultured in the presence of IL-2 with fresh donor PBMCs added at weekly intervals over a 35-day period. Cultures were monitored for the appearance of syncitia or other cytopathic effects, and were cocultivated with indicator cell-lines at regular intervals. Supernatants were collected from the cultures every 3 to 4 days and assayed for RT activity using a highly sensitive PCR assay (at least 100 times more sensitive than conventional assays)[33].

RECEIVED 20 OCTOBER; ACCEPTED 16 NOVEMBER 1994

Acknowledgements

We thank the donors in this study for their participation and Ramou Jagne and Kenbougel Joof for their hard work in the collection of blood samples. We are grateful for the technical assistance of Fatim Cham, Robert Walton, Tony Brett and Philip Thornton-Evison, advice from Robin Weiss and Adrian Hill, and to Steve Lee and Alan Rickinson for sharing unpublished data. This study was supported by the MRC AIDS Directed Programme, and S.R-J. is an MRC Clinician Scientist Fellow. Follow-up studies were performed with a sample from donor OX12 one year after the original bleed, and persistent CTL activity against GAG, POL and NEF peptides was detected.

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