Methods of Detection of HIV-specific CTL and Their Role in Protection Against HIV Infection

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INTRODUCTION

The observation that recovery from many virus infections is followed by the development of lifelong immunity is a fundamental principal of vaccinology. In contrast the perception that everyone who becomes infected with HIV remains persistently infected and will ultimately develop disabling immunodeficiency has been a serious discouragement to HIV vaccine development. In recent years a number of studies have suggested that a handful of people with documented HIV exposure who fail to seroconvert or develop detectable viraemia have a range of HIV-specific responses. These observations clearly raise the possibility that, under certain circumstances, HIV exposure can lead to potentially protective immune responses. In this article we will first review the methods now available for the detection and quantitation of HIV-specific cytotoxic T-lymphocytes (CTL) and then discuss the detection and potential role of HIV-specific CTL in highly-exposed persistently seronegative (HEPS) individuals.

MEASUREMENT OF HIV SPECIFIC CTL

One of the critical issues in interpreting the results of CTL studies in HEPS subjects is the method used to measure HIV specific CTL, for which a number of assay systems are employed. In general, the choice of assay technique will depend on the research question posed, the technical skill and experience of the operator, the type of sample under study, and the degree to which results need to approximate the in vivo milieu. These techniques may measure CTL qualitatively or quantitatively; in addition, it is possible to assess CTL responses directed against entire HIV gene products, against predetermined CTL peptide epitopes, or against individual MHC class I/peptide complexes.

1. Bulk CTL culture

The earliest description of HIV-specific CTL in HIV-infected people relied on the lysis by freshly isolated peripheral blood mononuclear cells (PBMC) of autologous B-cell lines (BCL) infected by recombinant vaccinia viruses expressing HIV-1 gene products [Walker (1987), Plata (1987)]. It is estimated that the detection of “fresh” CTL lysis requires a frequency of circulating effector CTL of the order of 1 in 100 [Gotch (1990)]. However, the use of fresh PBMC as effectors would be expected to fail to detect CTL that are present at low frequency, as well as resting or memory CTL. Somewhat surprisingly, two studies of uninfected infants born to infected mothers described the detection of HIV-specific CTL activity using unstimulated PBMCs in between 26% and 75% of children [Cheynier (1992), De Maria (1994)].

More usually, CTL activity is detected by expanding HIV-specific CTL in vitro, using co-culture of bulk PBMC with inactivated, autologous antigen presenting cells (APC) infected either by autologous virus [Nixon (1988)] or by recombinant vaccinia viruses expressing HIV-1 gene products [Lubaki (1994), van Baalen (1993)]. In this way, low frequency antigen-specific CTL present in the original sample are clonally expanded, and are detected by the lysis of Cr51-pulsed, class I MHC-matched targets, frequently autologous BCL. These methods have been the staple of HIV CTL studies in seropositive donors for over a decade. However, in HEPS donors who have no detectable viraemia, it is clearly not feasible to use autologous virus to restimulate CTL cultures. Some authors have employed stimulator cells infected with a laboratory strain of HIV [Fowke (1999)] or used the recombinant vaccinia method [Bernard (1999)] to elicit HIV-specific CTL from HEPS cohorts in vitro. In our experience, the most sensitive method for generating CTL cultures in HEPS donors is the use of specific CTL epitope peptides selected on the basis of the HLA class I type of that donor [Rowland-Jones (1995)]; the sensitivity of this method can be improved by the addition of IL-7 to the culture medium [Lalvani (1997b), Rowland-Jones (1998)].

Target cells may either be infected by recombinant vaccinia viruses expressing HIV-1 gene products [Nixon (1988), Walker (1987)], or pulsed with specific HIV CTL epitope peptides. Although there is some variation between laboratories, HIV-specific lysis of >10–20% after a 4–6 hour assay, over a range of effector/target ratios (often 50:1, 25:1 and 12.5:1) and with a >50% reduction of target cell lysis after CD8+ depletion, is generally used as the criterion for a positive CTL assay.

As described, the bulk CTL assay has two major disadvantages. The first is the requirement for prolonged in vitro stimulation, which necessarily results
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in the distortion of effector CTL frequency, and potentially their phenotype. This technique is therefore poorly able to approximate conditions under which HIV-specific lysis may occur in vivo. A second disadvantage lies in the fact that CTL bulk culture is a qualitative technique, useful only for HIV-specific CTL detection, rather than quantitation. This disadvantage can be overcome through the use of limiting dilution analysis (LDA) [Koup (1991), Carmichael (1993)]. Here, fresh PBMC are serially diluted, and these dilutions used to set up bulk CTL cultures. Mathematical techniques are then applied to calculate the CTL effector frequency in the original sample, based on the lowest dilution from which CTL can be detected after in vitro stimulation.

CTL bulk culture techniques provide the clearest measurement of functional CTL activity, since the assay end-point is the lysis by CTL of infected or peptide-pulsed targets. However, there are serious concerns that LDA may underestimate the actual effector CTL frequency [Gotch (1990), Moss (1995)], and recent work in human Epstein-Barr virus (EBV) infection has shown that LDA correlates only poorly with effector CD8+ T-cell frequencies estimated using ELISpot assays or HLA-peptide tetrameric complexes [Tan (1999a)]. The most likely explanation for this is that LDA measures only the subset of virus-specific cells which are able to grow and divide under limiting dilution conditions: this technique will therefore miss those CTL which are terminally differentiated or prone to apoptosis in tissue culture. This is likely to be a particular problem using cells from HIV-infected people. In addition, bulk CTL techniques are time-consuming, require high input numbers of effector cells, and involve the acquisition, handling and disposal of radioactive materials. Nevertheless, this method has been successfully used to demonstrate the presence of CTL precursors for a number of HIV gene products (particularly gag and env) in 13 out of 36 HIV-exposed uninfected partners of HIV+ donors [Goh (1999)].

2) Enzyme-linked immunospot (ELISpot) assay

The ELISpot is based on the same principles as an ELISA assay. However, the technique has been modified to permit the detection of cytokine release at the single cell level in response to a given stimulus [Czerkinsky (1988), Quiding (1991)], and has recently been applied to the enumeration of antigen-specific CD8+ T-cells [Di Fabio (1994), Miyahira (1995), Lalvani (1997a)]. In the ELISpot assay, nitrocellulose plates are coated with a first layer anti-cytokine antibody. Effector cells are then incubated in the presence or absence of a specific stimulus; in the HIV system, a suitable antigen can be a predefined HIV CTL epitope peptide [Hanke (1998), Rowland-Jones (1998), Herr (1998), Kaul (1999)], or a class I MHC-matched target infected by recombinant vaccinia expressing HIV-1 gene products [Larsson (1999)], or a laboratory strain of HIV [Zhang (1996)]. After incubation for 6–16 hours (overnight is convenient), cells are discarded, and the plate incubated with a second biotinylated anti-cytokine antibody. The use of an appropriate detector system allows each cell producing cytokine to be detected as a separate spot. These spots may either be counted by eye under a dissecting microscope, or by a variety of computer-assisted quantitation systems. HIV specific responses are calculated by the subtraction of background cytokine secretion (in response to media alone), and the results are reported as spot-forming units (sfu) per 10^6 cells. We have used the following criteria for a positive response: peptide-specific response at least twice that of background, an absolute number of specific sfu of > 20/10^6, and the demonstration of a titratable response with varying numbers of input cells [Kaul (1999)]. Other authors have proposed a cut-off of specific sfu of > 5 spots/well [Lalvani (1997a)], and 10 spots/well [Larsson (1999)], as long as this number is at least twice that observed in the negative control wells. Statistical methods can also be applied to calculate the probability that the response in the experimental wells is significantly greater than the background secretion of IFN-γ [Flanagan (1999)].

Although the ELISpot assay can be adapted to detect the release of any cytokine, the quantitation of PBMC secreting IFN-γ in response to CTL epitope peptides has been shown to correlate relatively well with CTL frequencies measured using lysis assays (either bulk culture or LDA) and more closely with HLA-peptide tetrameric complexes in a number of viral systems [Di Fabio (1994), Schmittel (1997), Lalvani (1997a), Tan (1999a), Larsson (1999)]. In general, IFN-γ ELISpot frequencies are around 2–5 fold higher than those measured using LDA bulk culture, and 2–5 fold lower than CTL frequencies measured using tetramers [Tan (1999a)].

Advantages of the ELISpot assay include its rapidity (an overnight assay, as opposed to 12–14 days for bulk CTL); the ability to screen for responses against multiple CTL epitopes and/or gene products in a single assay; and the need for relatively low cell input numbers (generally 2–4 × 10^5 per epitope/gene product tested). Of particular relevance in the study of HEPS individuals, where HIV specific CTL frequencies seem likely to be considerably lower than seen in HIV infected individuals [Goh (1999), Kaul (1999)]; the sensitivity of the IFN-γ ELISpot is greater than that of MHC class I/peptide tetramers. While ELISpot is able to detect antigen-specific responses to a frequency of 1/50,000 input PBMC, tetramers currently have a threshold of detection of 1/5,000 [Altsman (1996)]. Finally, ELISpot detects antigen-specific CD8+ T-cells ex vivo,
without the need for prolonged these cells in vivo more accurately than bulk CTL cultures. However there are also disadvantages of this assay system. Once the assay has been developed, the cells cannot be manipulated any further, and usually only one cytokine can be measured at a time. Moreover, although it is assumed that IFN-γ secretion and cytolysis are overlapping functions of antigen-specific CTL, this is not necessarily the case. Moreover, in a study of the functional phenotype of tetramer-positive cells specific for HIV and CMV antigens using intracytoplasmic staining for cytokines, although the great majority of tetramer-staining cells produced IFN-γ, a significant proportion (up to 25%) did not produce TNF-α (V. Appay, manuscript submitted). Thus the TNF-α Elispot assay would probably underestimate the number of antigen-specific cells detected using IFN-γ release.

The Elispot assay has been used in a number of studies of HEPS donors. We detected responding CD8+ T-cell frequencies in response to particular HIV peptides of up to 1 in 3000 PBMC using an IFN-γ ELISpot in a cohort of highly-exposed sex workers in Nairobi [Rowland-Jones (1998)]. In a survey of over 100 Nairobi prostitutes meeting the definition of HIV resistance, over 50% showed significant ELISpot responses to a range of known epitope peptides (R. Kaul, manuscript in preparation). An ELISpot assay for tumour necrosis factor (TNF-α) production was used to demonstrate responses to known HIV peptides presented by HLA-A2 and A3 in 5 of 11 seronegative people with documented HIV exposure: in this study, the frequencies of TNF-producing cells were in the order of 100–420 per 10⁶ PBMC [Herr (1998)].

3) HLA-peptide tetrameric complexes

HLA-peptide complexes allow the direct visualisation of antigen-specific CD8+ T-cells, independent of their functional characteristics. Four biotinylated, refolded HLA-peptide complexes are attached to a streptavidin molecule, creating a stable tetrameric structure which will specifically bind to T cells expressing an appropriate T-cell receptor (TCR) [Altman (1996), Ogg (1998)]. Although this technique does not rely on a functional endpoint, the antigen-specific CD8+ T-cell frequencies measured by flow cytometry have been shown to correlate with functional cell populations by a number of methods, including direct CTL lysis, CTL cloning, ELISpot and LDA [Ogg (1998), Dunbar (1998), Tan (1999a)]. One of the major advantages of the HLA-peptide tetramer technique is the ability to examine the co-expression of other surface antigens [Ogg & McMichael(1999)], such as markers of activation [Ogg (1998)], T-cell receptor Vβ usage [Wilson (1998)] or indicators of apoptosis [Tan (1999b)]. More recently, the use of intracellular staining has permitted more detailed phenotypic analysis of the virus-specific tetramer-positive population [Gillespie (1999)] (Victor Appay, manuscript submitted). Another advantage is the ability to sort specifically a population of antigen-specific cells, making growth and characterization of CTL clones relatively simple [Dunbar (1998), Dunbar (1999)]. The technique is time-effective, able to yield results within a few hours of blood being drawn, and works well with frozen cells. The fact that PBMC can be stained directly ex vivo means that, of the three techniques described, HLA-peptide tetramer studies are most likely to accurately reflect the phenotype of antigen-specific CD8+ T-cells in vivo.

The major disadvantage of tetramer studies in the characterization of HIV-specific CD8+ T-cells in HEPS populations is the decreased sensitivity of this technique compared to ELISpot or bulk CTL assays. The limit of detection for HLA-peptide complexes is approximately 1/5000 [Altman (1996)], as compared to 1/50,000 for ELISpot [Lalvani (1997a)] and 1/100,000 for LDA [Goh (1999)]. Since the CD8+ T-cell precursor frequency in the blood of HEPS populations is approximately 10-fold lower than in HIV-infected individuals [Kaul (1999)], in most cases these cells will be below the level of detection of the HLA-peptide tetramer assay. There are no reports to date of tetramer-positive CD8+ cells in HEPS cohorts.

HIV-SPECIFIC CTL IN HEPS POPULATIONS

It has become clear over the past decade that susceptibility to HIV infection is not uniform, and that some individuals can be identified who do not develop chronic HIV infection despite multiple exposures [Rowland-Jones & McMichael(1995), Fowke (1996)]. The first reports of such cases in exposed seronegative homosexual men raised the possibility that transient infection might have occurred which could lead to protective immunity [Imagawa (1989)]. Standard HIV-1 IgG ELISA assays and Western blots are negative in these subjects, as are sensitive HIV-1 PCR assays in the peripheral blood. There nevertheless remains a possibility that persistent infection has been established in such cases (Zhu et al., Sixth Conference on Retroviruses and Opportunistic Infections) but viraemia has been controlled to levels below the limits of detection: therefore, these individuals are more accurately referred to as HEPS than Exposed Uninfected (EU).

The association of HIV-specific CTL with non-progressive disease in HIV-infected persons [Pantaleo (1995), Harrer (1996), Betts (1999)], as well as the increased frequency of certain HLA class I alleles in HEPS populations [Plummer (1999)], has led several groups of researchers to look for HLA
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class I restricted, HIV-specific CTL in these groups. HIV-specific CTL have now been described in the peripheral blood of individuals exposed to HIV through a number of routes, including heterosexual/homosexual sex [Rowland-Jones (1995), Langlade-Demoyen (1994), Rowland-Jones (1998), Goh (1999), Bernard (1999)], percutaneous exposure to infected blood [Pinto (1995), Bernard (1999)], and exposure to maternal virus during pregnancy or delivery [Cheynier (1992), Rowland-Jones (1993), De Maria (1994), Aldous (1994)]. However, other investigators have not detected HIV-specific CTL in exposed uninfected infants [Luzuriaga (1991)]: this discrepancy between reports may be because the exposure is largely at a single time-point or because the neonatal immune system is less able to mount a cellular immune response than adults. In animal models, SIV-specific CTL have been associated with protection from SIV challenge in seronegative macaques exposed to either HIV-2 [Putkonen (1995)] or low doses of SIV [Murphey-Corb (1999)]. Where attempts have been made to quantitate CTL activity, the CTL in HEPS donors are usually present at a lower frequency than in people with persistent HIV infection [Goh (1999), Kaul (1999)]. CTL detection may also be intermittent over time in a given individual [Goh (1999)]. This could be due to the levels of CTL being close to the limits of detection of the currently available assays, or it may reflect a genuine relationship between CTL numbers and the timing and extent of HIV exposure. In studies of HEPS donors with a single HIV exposure (through a needlestick injury or at the time of birth) the detection of CTL has often been transient, with a maximum duration of around 12 months following exposure [Rowland-Jones (1993), Pinto (1995), although in one health care worker with a series of needlestick injuries CTL activity was still detectable 34 months after exposure [Bernard (1999)]. Resistance to HIV infection in Caucasian HEPS populations has been associated with inherited defects in coreceptors necessary for HIV cell entry [Liu (1996)], but these genetic defects do not account for the lack of infection in the great majority of HEPS individuals [Fowke (1998), Goh (1999), Bernard (1999)].

The hypothesis that HIV-specific CTL may be a crucial element in decreased susceptibility to HIV infection is suggested by the consistency with which they have been described in various HEPS populations, by a number of different investigators and using a variety of techniques. Despite the fact that the frequency of some HIV-specific CTL in these individuals may be close to the limits of detection, CTL can recognize a diverse array of HIV epitopes within a given HEPS individual [Rowland-Jones (1995), Rowland-Jones (1998)]. The HIV epitopes recognized do not appear to fall within any one region of the HIV genome, as responses have been described directed at env [Cheynier (1992), Pinto (1995), Rowland-Jones (1998), Goh (1999), Fowke (1999)]; gag [Cheynier (1992), Rowland-Jones (1993)] [Rowland-Jones (1995), Rowland-Jones (1998), Bernard (1999), Goh (1999)]; nef [Cheynier (1992), Langlade-Demoyen (1994), Rowland-Jones (1995), Rowland-Jones (1998), Bernard (1999), Goh (1999)]; and pol [Rowland-Jones (1995), Rowland-Jones (1998), Bernard (1999), Goh (1999)]. Interestingly, HIV-specific CTL have been recently been demonstrated in the genital tract of the Nairobi HEPS prostitutes [Kaul (1999)], at the likely site of viral exposure. It might be hypothesized that a primed CTL response at the site of exposure would be more relevant in protection against incident HIV infection than in blood, where HEPS CTL responses have been previously described. This is consistent with the finding that mucosal rather than blood CTL directed against an HIV envelope peptide were associated with protection against gp160-vaccinia viral challenge in a mouse model [Belyakov (1998)]. It has recently been reported that transient SIV infection of macaques following colonic exposure led to the generation of mucosal SIV-specific CTL which were strongly associated with protection against subsequent high dose SIV mucosal challenge [Murphey-Corb (1999)].

In most published studies, CTL are detected in the blood of approximately 30–60% of HEPS individuals examined. If the hypothesis that HIV-specific CTL play a role in the protection of exposed seronegative subjects is correct, why are CTL not detected in all HEPS donors? One key issue may be the sensitivity of the assay system. If, as our experience suggests, the most sensitive method to detect CTL is the use of the optimised specific epitope, either to stimulate CTL lines or in an ELISpot assay, then studies will be limited by the availability of immunodominant epitopes for the HLA haplotype of the donor. This is a particular problem for studies of non-Caucasian donors exposed to non-clade B strains of HIV-1, where little epitope mapping has been carried out [Dorrell (1999)]. Even for a well-studied HLA haplotype such as A*0201 there is a hierarchy of CTL epitopes recognised by different donors [Goulder (1997), Brander (1998)]. Moreover, this approach presupposes that the CTL responses of both resistant and infected donors will be directed at the same epitopes, which is not necessarily logical. In studies of CTL responses to peptides presented by the HLA molecule HLA-A*6802, which is associated with resistance to HIV infection in the Nairobi cohort [MacDonald (1999)], we have observed that the dominant response differs between infected and resistant donors (R. Kaul, T. Dong, manuscript in preparation). This would not be surprising if it is the case that some responses are more “protective” than others, but it does imply that the use of epitopes defined in seropositive donors as tools to detect CTL in HEPS donors may miss potentially important responses.
Another factor is that CTL are often only observed transiently, and this appears to be related to the duration and extent of recent exposure. Certainly, the highest rate of CTLs detected to date has been in a cohort of HEPS sex workers in Nairobi (R. Kaul, manuscript in preparation), whose predicted antecedent exposure to HIV is extraordinarily high [Fowke (1996)]. Other factors, such as intercurrent infection, may also influence the detection of CTL responses (R. Kaul, manuscript in preparation). Finally, it is equally possible that the nature of exposure has not been sufficient to elicit CTL responses and that HIV resistance is mediated by other mechanisms. A number of other HIV-specific immune responses have also been described in HEPS cohorts, including CD4+ T-cell responses associated with lymphoproliferation [Goh (1999)], production of IL-2 [Clerici (1992), Clerici & Shearer (1993), Clerici (1993), Clerici (1994), Beretta (1996a)] and β-chemokines [Furci (1997)] [Wasik (1999)], CD8+ T-cell associated suppression of viral replication [Levy (1998), Stranford (1999)], IgA in mucosa [Mazzoli (1997)] [Beyrer (1999), Kaul (1999)] and serum [Mazzoli (1999)], and antibodies to HLA molecules [Beretta (1996a), Beretta (1996b)] and CD4 [Burastero (1996)]. The precise relationship between these different immune responses and protection from subsequent infection remains unclear.

CONCLUSIONS AND FUTURE DIRECTIONS

A number of important questions remain to be answered about the role of HIV-specific CTL in HEPS donors. It is hard to explain what sort of immunological priming would stimulate the cellular arm of the immune response so specifically without leading to circulating antibodies: the mechanism of this priming needs to be addressed. It is also important to understand why only a small minority of very highly exposed people make these immune responses and resist HIV infection. It seems likely that it is a particular conjunction of the nature of the viral exposure (such as low-level mucosal exposure to a less efficiently replicating or defective virus) and the ability of the immune system to respond unusually effectively to this challenge. It has not so far been possible to link the immune responses of HEPS donors with compelling evidence of transient HIV infection [Frenkel (1998)].

Although there is strong circumstantial evidence that HIV-specific CTL responses in blood and mucosa are associated with resistance to HIV infection in people with extensive HIV exposure who remain apparently uninfected, it remains to be proven that these responses are actually mediating protection. Epidemiological studies looking at the correlation of HIV resistance in high-risk cohorts with the development of CTL responses during sustained HIV exposure should be helpful. Some support for the CTL protection hypothesis comes from the macaque studies described above. Further support has come from studies in which SCID mice reconstituted with T-cells from HEPS (but not low-risk) donors were resistant to HIV challenge: this protection was mediated by CD8+ cells [Zhang (1996)]. It should be possible to use the SCID-hu mouse model to test whether CTL clones derived from resistant donors are associated with protection from challenge: in that case, it would be valuable to compare the efficacy of clones from infected and HEPS donors in mediating protection. It will be important to determine whether there is a qualitative difference in the CTL from HEPS donors from those found in infected donors, such as an increased T-cell receptor affinity which improves recognition of the infected cell, or a pattern of cytokine and chemokine secretion which is better able to suppress viral replication. It is also of great importance to know whether protection is transient, in relation to the timing and extent of exposure, and would therefore require regular boosting to be maintained. However, the ultimate test of the role of HIV-specific CTL in protective immunity will come from studies of CTL-inducing vaccines in human subjects.

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REFERENCES


[Belyakov (1998)] I. M. Belyakov, J. D. Ahlers, B. Y. Brandwein, P. Earl, B. L. Kelsall, B. Moss, W. Strober, & J. A. Berzofsky. The importance of local mucosal HIV-specific CD8(+) cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by
HIV-specific CTL


HIV-specific CTL


