Co-evolution of human immunodeficiency virus and cytotoxic T-lymphocyte responses

Summary: After more than a decade of intensive research, the precise role of human immunodeficiency virus (HIV)-specific cytotoxic T lymphocytes (CTL) in determining the course of the infection remains open to argument. It is established that HIV-specific CTL appear early in the infection and are temporally associated with the clearance of culturable virus from the blood; that CTL are generally detectable at very high levels throughout the asymptomatic phase and decline at the time of progression to AIDS; and that CTL-mediated killing is sufficiently fast to prevent production of new virions by HIV-infected cells. However, viral turnover is high throughout the course of the infection, and infected individuals progress inexorably to disease in spite of the CTL response. In order to address the question of whether CTL play an active part in influencing the course of HIV infection, one approach has been to seek evidence for CTL-mediated selection pressure on the virus. Several clear examples of CTL epitope-specific mutations selected for fixation are described. We argue that CTL escape is a common event which occurs at all stages of the infection. Detailed longitudinal studies are required to detect CTL escape and to understand the complexities contributed by factors such as a polyvalent CTL response and the presence of epitope variants which antagonise the CTL response. In conclusion, there is strong evidence of a dynamic process in which CTL impose important selection constraints upon HIV from which the virus attempts to escape; ultimately, at the time of disease progression, the tenuous control of CTL over the virus is lost.

Introduction

The precise role played by cytotoxic T lymphocytes (CTL) in human immunodeficiency virus (HIV) infection remains open to some argument. It is established that HIV-specific CTL appear early in the course of infection, coinciding with the rapid fall in viraemia and clearance of culturable virus from plasma and peripheral blood mononuclear cells (PBMC) (1). Failure to generate an early CTL response is associated with a protracted seroconversion illness, a low CD4 count that fails to recover to basal levels, and an inability to clear culturable virus from the blood. In a second study (2), the level of HIV-specific CTL activity paralleled the efficiency of control of primary viraemia. Studies of the efficiency with which CTL can kill HIV-infected
targets have revealed that CTL-mediated lysis is sufficiently fast to eliminate infected cells before the production of new virions (3), and that even a relatively weak CTL response can eliminate a large fraction of productively infected cells (4).

It is also established that HIV-specific CTL are detectable at very high levels in many HIV-infected donors in the acute phase of infection (5, 6). Activated T cells with restricted clonality, probably HIV-specific CTL, in the blood may exceed 10% of CD8+ T cells in acute infection (5, 6), and may commonly reach 1% of CD8+ T cells (7–9) in chronically infected donors. Whilst these high levels of activated antigen-specific CTL may also be found in the blood of donors in the acute phase of other infections, such as Epstein-Barr virus (EBV) (10), these levels are not maintained in the asymptomatic phase.

The high levels of HIV-specific CTL in asymptomatic donors contrast, in most studies, with the decline and disappearance of CTL in individuals progressing to disease (11–14). This point is less clear, because there are many events taking place concurrently at this phase of disease, including the accelerated decline in CD4+ count (15), which may obscure the cause and effect of the CTL decline, and may affect the assays used to quantitate CTL numbers.

The particular importance of events occurring during the early phase of infection is that these are likely to determine the steady-state level of viral load which appears to persist stably through the asymptomatic phase (16, 17). This, in turn, is strongly predictive of outcome, and is the best single marker of prognosis (18). The strong dependency of outcome upon the age at time of infection, demonstrated by a study of haemophiliacs (19), highlights the role of host factors – including the CTL response – in determining outcome. Analysis of CD8+ T-cell receptor Vβ-specific expansions in seroconverters argues that it is the breadth of CTL response generated at this phase of the infection which is the critical factor determining rate of progression to disease (20).

Despite this large body of evidence to show that HIV-specific CTL strongly influence the course of disease in HIV infection, there is an opposing view which holds that the high-level CTL activity observed in most asymptomatic HIV-infected donors is merely reflective of, and not an important determinant of, the high turnover of virus in these individuals (21, 22). In addition, observations of progression to disease despite a strong CTL response (23–25) and the theoretical argument that clearance of HIV from the blood might occur in the absence of a documented CTL response (26) point to CTL-independent mechanisms responsible for determining the rate of disease progression in HIV infection.

Decline and fall of CTL?

The misconception that the asymptomatic phase of HIV infection might reflect a period of inactivity in virus replication was revealed in two studies which showed a high-level of viral replication in the lymphoid tissue of asymptomatic donors (27, 28). This finding is consistent with the earlier identification of high levels of activated HIV-specific CTL in the blood of many asymptomatic donors, and also with the observed genetic diversity of the virus within a single infected individual (29). Given a comparable error rate in RNA-dependent polymerases for HIV (30) and all other RNA viruses (31, 32), the greater genetic diversity observed in HIV must be explained by its remarkably high turnover.

A more precise quantification of the daily turnover of virus in HIV-infected individuals was provided by studies exploiting interruption of the viraemic steady state using potent antiviral drugs (33, 34). Both studies come to very similar conclusions that between 10⁶ and 10⁷ (35) virions are produced daily in HIV-infected individuals, a figure which was independent of viral load (range in patients studied: 12,000 to 643,000 RNA copies/ml plasma).

These data have been interpreted as inconsistent with CTL’s position as centrally important in determining disease progression (22). Can a daily viral turnover of 10⁶ or 10⁷ plasma virions be compatible with an effective CTL response? Kleinerman et al. (4) argue that the role of CTL is not in significantly altering the half-life of productively infected cells (which is stable in different individuals) in this cytopathic infection, but in limiting virus production and hence determining viral load. Similarly, Wain-Hobson suggests that the vast majority of infected CD4+ cells are killed by CTL (rather than the virus), leaving only a very small proportion able to produce virus (36, 37).

The question is therefore: do CTL play an active part in influencing the course of HIV infection, or simply follow ineffectually in the virus’s wake? An approach to answering this has been to look for evidence that the virus is under CTL-mediated selection pressure throughout infection. The demonstration of selection for viruses carrying epitope-specific mutations enabling the virus to escape CTL recognition would represent strong evidence for CTL-mediated killing as a dominant selection pressure on the virus (21, 38).

Epitope-specific selection for CTL escape: experimental virus infection

The earliest study demonstrating virus escape from a CTL response was in a lymphocytic choriomeningitis virus (LCMV)
model, in which mice transgenic for an LCMV-specific T-cell receptor (TcR) derived from a T-cell clone were infected with a high dose of LCMV, and mutant viruses were rapidly selected (39). Given the monoclonal T-cell response, this was a strong selection force, but one which might not be expected to occur naturally. The TcR was specific for an H-2D\textsuperscript{\textalpha}-restricted epitope in the LCMV glycoprotein-1 (GP-1), amino acid sequence KAVYNFATC (single letter amino acid code). The primary anchors for this restriction element are at position 5 (P5) and the carboxy terminus of the epitope (P6) and P2 and P3 are also buried (41). The observed mutations occurred at P2 (Ala to Thr), P3 (Val to Leu) and P4 (Tyr to Phe or Cys) in the epitope. Thus, virus escape in this instance is likely to have occurred either as a result of failure by the T cell to recognise a mutated epitope bound to H-2D\textsuperscript{\textalpha} or because of impaired binding to the class I molecule.

Selection of escape mutants in vitro was also demonstrated by culturing LCMV-infected targets cells with CTL clones specific for the GP-2 H-2D\textsuperscript{\textalpha}-restricted epitope SGVENPGGYCL (42). On this occasion, the majority of escape mutants carried a change (Asn to Asp) at the anchor position P5, which prevented binding to H-2D\textsuperscript{\textalpha}.

In order to address the question of whether mutation in a single epitope can be of significance in the context of a polyvalent CTL response, mice infected with LCMV containing between 0 to 3 mutated epitopes have been studied (43, 44). Despite the fact that LCMV-infected H-2\textsuperscript{\textbeta} mice generally generate CTL responses to three separate epitopes in a clear hierarchy of immunodominance, infection with a virus in which the immunodominant (GP-1) epitope alone had been mutated to prevent CTL recognition resulted in delayed clearance of the virus (43). Mutation in the subdominant (GP-2) epitope alone did not delay viral clearance, but when two epitopes were mutated elimination of virus was delayed (45).

Studies of mice infected with mouse hepatitis virus, strain JHM (MHV-JHM) have revealed a similar pattern (46). C57 B6 mice infected at the suckling stage are protected from developing acute, fatal encephalitis by being nursed with dams previously immunised to MHV-JHM, but a proportion develop hindlimb paralysis from demyelinating encephalomyelitis. B6 mice infected at a later stage do not develop hindlimb paralysis, and depend upon the virus-specific CTL response for virus control and clearance. The immunodominant epitope has been defined as the H-2D\textsuperscript{\textalpha}-restricted sequence CSLWNGPHL. All 9 suckling B6 mice infected with MHV-JHM who developed hindlimb paralysis carried infectious virus with mutations specifically within RNA encoding the immunodominant epitope, and no sequence changes elsewhere in PCR products 200 nucleotides in length. The sequence changes resulted in amino acid changes at positions 2 to 7 in the epitope, all of which resulted in inefficient CTL recognition of peptide-pulsed targets. In symptomatic mice, this pattern was not observed. The conclusion to be drawn from this study is that escape from a monospecific CTL response is obligatory for the development of disease in these MHV-JHM-infected mice.

An alternative mechanism of CTL escape is suggested by the study of murine leukaemia viruses (MuLV) (47), of which there are two major types: endogenous AKV/MCF-type MuLV and exogenous Friend/Moloney/Rauscher (FMR)-type MuLV. Disease resistance appears to be primarily determined by MuLV-specific CTL. The CTL response in H-2\textsuperscript{\textbeta} mice is type-specific. The immunodominant CTL epitope in H-2\textsuperscript{\textbeta} mice infected with AKV/MCF-type is the K\textsuperscript{\textbeta}-restricted sequence KSPWFTTL, a very conserved sequence in mouse type C retroviruses. FMR-type MuLV encode an alternative form of this epitope, with an Lys to Arg change at position 1. This change not only results in failure of recognition of peptide-pulsed targets by CTL specific for the KSPWFTTL-encoding MuLV, but also prevents adequate presentation of the RSPWFTTL variant on the cell surface, so that CTL specific for the Arg-variant cannot be generated (48). The mechanism for this failure of presentation of the Arg-variant is preferential proteasome-mediated cleavage C terminal to the arginine residue, so that the RSPWFTTL variant is generated at much lower levels than the KSPWFTTL epitope (49).

These studies illustrate the ground rules governing escape from CTL recognition as applied to RNA viruses. Most simply, mutation within an immunodominant CTL epitope may result in loss of recognition either through a change in the anchor residues, which affect binding to the MHC-presenting molecule, or, equally, in residues which interact principally with the TcR. In the latter case, the effects may be more profound if the CTL response is oligoclonal (see below). Changes within or proximate to the epitope may also significantly affect presentation of the epitope on the surface of infected cells.

The infidelity of reverse transcriptase limits the size of RNA viruses, and therefore the variety of mechanisms of CTL escape available to RNA viruses compared to DNA viruses. A growing list of strategies adopted by the herpes viruses, for example, are described which enable these viruses to avoid attack by CTL by a variety of mechanisms other than amino acid sequence variation (reviewed in (50)).

**Epitope-specific selection: HIV-specific CTL**

The first example of escape from a virus-specific CTL response identified in a natural infection was in HIV-infected donors.
Donors with HLA-B8 were described in whom epitope variants were observed which were not recognised by autologous CTL. Variation within HLA-B8-restricted epitopes was not seen in HLA-B8-negative donors. In contrast, little variation was observed within the immunodominant epitope recognised by HLA-B27-positive donors, and the few variants that were found were well recognised by autologous CTL. This could be relevant to the more rapid progression of HIV infection in patients with HLA-B8 compared to the average and the possible protective effect of HLA-B27.

Longitudinal study of these HLA-B8-positive donors appeared to show fluctuations in the frequency of the escape mutants which were paralleled by alterations in the immunodominance of the CTL response. Variants that were at one time point able to escape CTL recognition subsequently increased in frequency at the expense of the index sequence. A switch to a new CTL response specific for the variant peptide enabled the index sequence to increase in frequency once again.

Longitudinal data of this type are hard to obtain, but there is increasing evidence to support the theory that antigenic variation results in a shift in the immunodominant epitopes. In a study of the CTL responses of HLA-A*0201-positive donors, two HLA-identical haemophilic brothers who had been infected at the same time by transfusion with the same batch of contaminated Factor VIII were compared. The pattern of CTL response was quite dissimilar: donor 003 made strong responses to two epitopes in p17 Gag, the dominant response to SLYNTVATL (HLA-A2-restricted) and the subdominant response to RLRPGGKKK (HLA-A3-restricted); donor 023 responded to neither of these two epitopes, the CTL response being directed to two previously undescribed HLA-B7-restricted epitopes in p24 Gag and Nef. The p17 gag proviral sequences in the two brothers showed no variation over almost a decade of observation in donor 003, but fixation of mutations encoding CTL escape sequences (SLHNAWVL and RLRPGGKKK, respectively) in the non-responding donor 023. A third HLA-A*0201-positive haemophilic donor, 008, who was also infected by the same batch of Factor VIII, and who also made no response to the HLA-A*0201-restricted epitope in p17 Gag, had the sequence variant SLFNTVATL encoded by proviral DNA. Neither SLHNAWVL nor SLFNTVATL were recognised by CTL specific for the index peptide from the responders tested. The third residue in HLA-A*0201-binding peptides is a secondary anchor.

The conclusion that mutation, occurring early in the infection, in the immunodominant epitope results in a switch of the response to alternative epitopes was supported by the study of 24 HIV-infected donors with HLA-A*0201. The majority (71%) generated responses to the p17 Gag epitope in standard assays, using bulk cultured CTL which had been restimulated with autologous virus. In the minority who failed to respond to the epitope in standard assays, there was evidence of low-frequency memory CTL responses using peptide (SLYNTVATL) stimulation specific for the index peptide, and 5 out of 6 of these donors showed mutations in and around the p17 Gag epitope. In contrast, CTL escape mutation within the Gag epitope in the SLYNTVATL responders was not observed. The existence of a memory response for the index SLYNTVATL peptide implies that this proviral sequence must have been present at some stage.

In another cross-sectional study, two donors with HLA-A11 and three other donors with HLA-B18 were analysed in relation to the Nef-specific responses, concluding that autologous virus sequences did not show mutations that abrogated CTL recognition in the responders, but that these mutations were present in the non-responders. The longitudinal data from the study of HLA-A*0201 donors suggested that the mechanism for this observation is mutation in the epitope, driven by CTL, as opposed to the alternative possibility that non-responders are infected with viruses that already, by chance, carry mutations in the immunodominant epitopes.

A similar pattern of antigenic variation and fluctuating immunodominance to that described by Nowak was observed in one of the HLA-A*0201-positive donors studied. When first seen, the SLYNTVATL-specific response was one of three co-dominant responses in bulk cultured lymphocytes, restimulated by autologous virus. At this time, 50% of proviral sequences encoded the index sequence, and 50% the unrecognised variant SLHFNTVATL. Over the course of the next 6 months, the response to the index peptide SLYNTVATL became undetectable in bulk culture, although restimulation with the index peptide itself consistently permitted the generation of CTL lines specific for the index peptide; by this time, 100% of proviral sequences encoded an epitope variant, either SLYNTIAML or SLFNTVATL. A year later, 21% of proviral sequences encoded the index sequence, but no CTL response was detectable in bulk CTL.

Finally, 30 months after the original response was detected, proviral sequences encoding the index peptide SLYNTVATL were again in the majority (64%). Thus, the immunodominant CTL response shifted, in this case to the HLA-B62-restricted epitope GLNKIVRMY, from the SLYNTVATL epitope in this individual following mutation within the epitope that abrogated CTL recognition. After 2 years in which there was no CTL response to either the index
or the mutated epitope, there has been an increase in frequency of the wild-type sequence encoding what is now a subdominant epitope (R Goulder et al. unpublished data). Similar data of this type are also described in longitudinal studies of donors responding to epitopes within Nef (59).

**Objections to the CTL escape hypothesis**

These studies, which argue that HIV-specific CTL are mediating epitope-specific selection pressure on the virus, have also raised many further questions.

1) Why do selected escape mutants apparently fail to reach fixation?

2) Stable variation in immunodominant CTL epitopes is often not observed in HIV-infected donors even during the asymptomatic phase, and in the absence of fluctuations in the immunodominant CTL responses (51, 55, 60, 61). It might be expected that, during the asymptomatic phase of infection, at the time when the CTL response is reportedly at its strongest (11–13), evidence of CTL escape would be easily observed.

3) Variation arises so frequently in HIV infection that escape mutations would be selected very rapidly, as long as the mutant virus is able to replicate efficiently. Thus, CTL might select escape mutants, but would present no obstacle to the virus. For example, Nietfeld et al. (62) examined the immunodominant CTL response in HLA-B27-positive donors to the p24 Gag peptide KRWII.GLNK. It was argued that the Arg to Lys change at the anchor position P2 in the epitope, which would abrogate binding and therefore represent an escape mutation, would arise several hundred thousand times a day, and that viruses carrying this mutation would be selected very strongly, provided the epitope change did not affect viral replication. In vitro studies with mutant viruses demonstrated that viruses carrying this single Arg to Lys change at P2 in the epitope replicated at least as well as the wild-type virus.

4) The final principal objection concerns the difficulty in conceptualising the selection of mutant viruses that carry escape variation in a single epitope in the context of a polyvalent CTL response.

These points will be addressed in the following sections.

**CTL escape to fixation**

It was recognised early that the effect of antiretroviral therapy on HIV was to select drug-resistant mutants relatively rapidly (34, 63, 64). These drug-resistant mutations are entirely predictable and reach fixation. Should CTL-imposed selection pressure be expected to differ? The chief distinction between CTL and antiretroviral drugs as mediators of selection pressure is that CTL responses are generally polyvalent, directed at many different parts of the virus, and the immunodominance of the response may change over time as a result of the emergence of CTL escape variants (54, 55). Also, exposure to CTL of a particular specificity may not be even throughout the body. Antiretroviral drugs, in contrast, are directed at a single HIV protein, and the selection pressure is constant.

Recently there have been three reports of CTL escape to fixation (6, 14, 65) in which the selection pressure exerted by CTL in these instances was akin to that seen in individuals on antiretroviral therapy. In the first example, Borrow et al. (6) described a seroconverter “WEOU” who initially generated monospecific CTL response to HLA-B44-restricted Env epitope AENLIWVTVVY. Within 14 days of the response being detected, escape mutations appeared specifically within the region encoding the epitope, and within 3 months the Glu anchor residue at P2 had been completely replaced by Lys, Gly or Ala. Price et al. (65) also described a seroconverter, whose striking response to the HLA-B8-restricted Nef epitope FLKEKGGL was similarly followed rapidly by the emergence of mutations specifically within the region of the virus encoding the epitope. Once again, these mutations principally occurred at an anchor position, in this case P5, with the replacement of Lys by Asn, Gln or Glu, which abrogated binding to the presenting MHC molecule (66). Additional mutations were seen that deleted segments of the gene encoding the epitope. In the third example (14), CTL escape to fixation was observed in two haemophiliacs as they progressed to AIDS. Both had made strong CTL responses to the immunodominant HLA-B27-restricted epitope KRWII.GLNK, and in both donors the same escape variant was selected by an Arg to Lys change at the anchor position P2.

In each of these cases, the CTL response was essentially monospecific, and represented a particularly strong response; for example, patient WEOR carried CTL precursors specific for the immunodominant HLA-B44-restricted epitope AENLIWVTVVY at a frequency of 1/17 PBMC during acute infection with HIV-1 (6). In two reports, the examples of CTL escape to fixation were observed in seroconverters, and, in the third (14), in two donors progressing to AIDS. A further clear example of CTL escape driven by a strong, monospecific CTL response in association with progression to AIDS was revealed by the study of Koenig et al. (24), in which CTL escape by epitope deletion followed infusion of 5×10⁸ of a Nef-specific
CTL clone which had been expanded in vitro. Although the mutant did not reach more than 30% of the viruses, this is impressive for an attenuated (ADE-deleted) virus.

There is some evidence that, in each of these four instances, the monospecific CTL response may have been the strongest anti-HIV immune response present (1, 2, 67, 68). During the asymptomatic phase of infection, many anti-HIV immune forces are acting on the virus, including lytic and non-lytic T-cell responses (69, 70), and it is possible that CTL-mediated lysis of HIV-infected cells is not always the dominant selective force throughout the course of the infection. Even if CTL-mediated lysis were the sole selective force acting during asymptomatic phase, often the CTL response is polyclonal, and so epitope-specific selection for escape variants would in this case be inversely related to the frequency of escape variants present. As escape variants accumulate, the CTL precursor frequency decreases (for example (6)), and subdominant responses become dominant (54).

It is puzzling that the Arg to Lys change at P2 in the HLA-B27-restricted KRWITMLNK epitope was only selected in the two donors progressing to AIDS after 9 and 12 years of infection (14), when the mutation in all probability arises with very high frequency throughout infection (62). It may be that CTL-mediated lysis was not the dominant selective force throughout the asymptomatic phase in these donors. This would explain the apparent lack of epitope-specific variant selection by CTL in many non-progressors, in whom non-lytic inhibition of viral replication may be particularly potent (71, 72). Since inhibition of viral replication by the β chemokines RANTES, MIP-1α and MIP-1β is not predominantly MHC-restricted (69, 70), epitope-specific "escape" variation might carry less selective advantage. Although stimulation of β-chemokine release by CD8+ T cells should be epitope-specific, β-chemokine production by CD4+ T cells may be equally important in limiting HIV replication (72). Reduction in the levels of these β chemokines, or a switch in the phenotype of the virus from NS1 (which use the β-chemokine receptor CCR5 (73, 74)) to SI (which use the SDF-1 receptor CXCR4 (75)), as frequently occurs in association with disease progression, might enable CTL-mediated killing to become the dominant selection force at a late stage of infection. SI viruses would not be inhibited by β chemokines.

Alternative explanations for the long delay in selection for CTL escape viruses in these cases would be that the Arg->Lys change in the HLA-B27-presented Gag p24 epitope is disadvantageous in vivo to the virus, and can only be tolerated once the CTL response has weakened; or can only be tolerated when this mutation occurs in the context of several other mutations simultaneously. This latter explanation is favoured by the observation that no B clade virus isolates and only 10% of A clade viruses carry lysine at this position in the p24 Gag protein (76).

How common is CTL escape?

These clear examples of CTL escape described above are evidence that CTL are a selection force at different stages of HIV infection, but it is still not established how commonly these escape mutations occur. It might be supposed that, if it has taken 15 years of research to identify these recent unequivocal examples of CTL escape, then it is a rare and therefore unimportant phenomenon. It is argued below that CTL escape occurs very frequently, and is considerably underestimated for reasons summarised in Table 1.

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<th>Table 1. Sources of underestimation of CTL escape</th>
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<td>1. Undetected immunodominant CTL responses</td>
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<td>- bias in favour of conserved epitopes</td>
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<td>2. Cross-sectional studies</td>
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<td>- escape has already happened</td>
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<td>4. Limitations of CTL assays</td>
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<td>- miss processing escape mutants</td>
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<td>- miss rapid off-rate mutants</td>
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<td>5. CTL antagonism</td>
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Limitations of standard methods of identifying immunodominant CTL responses

The standard methods for identifying CTL responses (for example (77–80)) have been to infect autologous EBV-transformed B-cell lines, or tumour cell lines expressing particular MHC class I molecules, with a panel of vaccinia recombinants, each expressing one of the major immunogenic HIV proteins. Having identified a protein to which a CTL response is directed, a panel of overlapping peptides may then be used to pulse onto target cells and thereby identify the recognised epitope. The effect of using this method is that only sequences that are conserved between that used to make the vaccinia construct, and those present in the donor whose CTL are being tested are likely to be identified as epitopes. Conserved sequences are likely to be more resistant to epitope-specific selection pressure than sequences that occur in regions of the virus that can accommodate variation without detriment to the fitness of the virus. Thus, the standard method of identifying CTL responses may not only miss the immunodominant responses most likely to drive the selection of escape mutants, but also introduces bias.
Fig. 1. Amino acid sequence variation in Nef in donor SC2 at 5 time points spanning October 1995 to June 1996. Seroconversion date was early October 1995. A strong HLA-B8-restricted FLKEGGGL-specific CTL response was first detected at the second time point. The epitope (Nef_{154} residues 90–97) is shaded. Seventeen to nineteen clones from each time point were sequenced. The Kabat-Wu index (KWI), calculated at each amino acid position as the number of different residues/frequency of the commonest residue, provides a measure of variability: an index of 1 indicates that no variation was detected at the relevant position. At the initial time point, 100% of sequences encoded the index peptide FLKEGGGL; at the final time point, all but 2 of 17 clones encoded the CTL escape sequence FLKENGGGL. The bulk CTL response had disappeared by the third time point. Memory CTL were only detectable by peptide stimulation from this time onwards.

in favour of conserved epitopes, which are more resistant to escape mutation.

It is possible that this limitation may be relatively unimportant when studies of CTL responses to B clade viruses are undertaken. However, the shortcomings of the standard method are nowhere more apparent than when HIV-1 B clade-based vaccinia constructs are used to identify CTL responses in donors infected by non-B clade viruses. Recombinant vaccinia viruses expressing HIV-1 proteins from the same clade of virus as is present in the donor tested improve the identification of CTL responses present, as might be anticipated (for example (81)). Ideally, recombinants would be generated from autologous sequences, which would improve the identification of CTL responses (82), but this is not practicable in assessing every donor.

CTL escape is only easily identified if observed as it happens. Perhaps more importantly, the detection of CTL escape is only easily achieved if, by chance, it is observed as it happens. If CTL escape has already occurred, and the CTL response has consequently disappeared, the CTL that selected mutant viruses will only be identified by looking at sequences in donors who unusually fail to make a response to what is normally an immunodominant response. This method revealed strong evidence for CTL escape to fixation in several HLA-A*0201-positive donors who made no response to the immunodominant p17 Gag epitope SLYNTVATL (55). If CTL escape occurs rapidly, as in the unequivocal examples described above (6, 14, 24, 65), it is likely to be missed, other than by longitudinal studies. This feature is amply illustrated in Fig. 1. The majority of studies investigate viral sequence from a single time point, which makes interpretation difficult. A recent longitudinal study anal-
ysing sequence variation in env highlights the rapidity with which non-synonymous nucleotide mutations are selected in particular regions of the gene at particular time points, and the invisibility of these dynamics when viewed after the event (83).

Limitations of methods used to identify epitope-specific selection

This last point also illustrates the limitations of methods that are used to distinguish between variation occurring as a result of CTL-driven selection, and that which occurs as a result of an error-prone reverse transcriptase and a high viral turnover. The Kabat Wu index (KWI) (84) determines variation at each amino acid position by dividing the number of different residues at that position by the frequency of the commonest variant. If all regions of the virus could accommodate variability equally comfortably, without loss of replicative efficiency, this indeed would provide a measure of selection for amino acid change in different regions of the virus. However, perusal of the HIV-1 sequences in the Los Alamos database (76) underlines the fact that some regions of the virus are extraordinarily conserved, whilst others are relatively variable. For example, if an epitope is found in a very conserved region of the virus, such as the HLA-B27-restricted p24 Gag epitope KRWIILGLNK, it is likely that only a very restricted number of amino acid changes may be made without detriment to the fitness of the variant virus. Thus, it is probably not a coincidence that both donors who progressed to AIDS carried the identical escape mutation of Arg to Lys at P2 in the epitope, a position where there is no variation in 32 B clade sequences listed in the database (76). The maximum KWI value that could be obtained for escape mutation in this situation would be when, by chance, the donor's virus was sequenced at a time when 50% of clones encoded each variant; at the time of maximum selection, the value would be 4. This compares with a KWI value of over 8 in analysis of variation at P5 in the HLA-B8-restricted Nef epitope FLKEKGGL in the donor described by Price et al. (65). The explanation is less likely to be that there was stronger selection acting for change at P5 in the FLKEKGGL epitope, but that a wider variety of amino acid residues can be accommodated at this position without detriment to Nef function (7 different residues occur at this position in 65 sequences in the database (76)).

The calculation of rates of non-synonymous (dN) and synonymous (dS) nucleotide substitutions to assess selection intensity (85) similarly does not give sufficient weight to single amino acid changes occurring within a conserved epitope. Because dN/dS values for whole proteins will underrepresent the impact of epitope-specific selection occurring within only a small portion of that protein, dN/dS are more revealingly calculated in a sliding window analysis (as in, for example (65)). The size of the sliding window must not be too large, otherwise small regions where selection is acting strongly for amino acid change will be obscured by adjacent regions of the virus where selection is acting for amino acid conservation. Conversely, reducing the size of the sliding window increases sensitivity, but the window must not be too small otherwise random non-synonymous changes ("noise") will be over-represented. Thus, dN/dS analysis, using a sliding window analysis, will best demonstrate epitope-specific selection where there are non-synonymous nucleotide changes occurring at several positions in the epitope. For the reasons discussed in relation to the KWI above for variation in the HLA-B27 epitope KRWIILGLNK and the HLA-B8 epitope FLKEKGGL, amino acid changes at multiple positions in the epitope are more likely to be accommodated in the Nef epitope, and would be reflected in the dN/dS sliding window analysis. The equally significant change occurring only at a single position in the Gag epitope would not be demonstrated by the same method of analysis.

The other difficulty in using either of these methods, KWI or dN/dS analysis, is that neither will detect escape after it has occurred and the escape mutation has reached fixation (see Fig. 1). The recent longitudinal study on nucleotide sequence variation in env demonstrated the enormous disparity in dN/dS values for the same region sequenced at different time points (83).

Limitations of CTL assays in identifying CTL escape

One additional source of underestimation of CTL escape relates to the limitations of standard CTL assays. Normally, recognition of variant peptides is assessed by peptide pulsing of targets, using several concentrations of the peptide, in order to obtain titration curves. In the comparison of the index peptide KRWIIMGNLK (R2) and the variant KKWIIMGNLK (K2), it was initially surprising to observe little difference in the two peptide titration curves (Fig 2A) (14). However, when the assay was delayed for 2–24 h following pulsing of targets with the peptides, it was clear that the K2 variant failed to bind to targets for more than about an hour, whilst the index peptide could sensitise targets for lysis by CTL for over 24 h (Fig 2B). To resolve this question, minigenes encoding the two versions of the epitope were constructed, and cells transfected with the R2 minigene were well recognised by CTL, whilst cells transfected with the K2 minigene were not recognised (Fig 2C) (14). Most published analyses of CTL response to sequence variants have used synthetic peptides, either in the lysis assay throughout, or immediately after pulsing with peptide, often at high concentrations of peptide.
In the analysis of the R1 variant of the MuLV-specific K\(^r\)-restricted epitope KSPWFTTL, it was determined that this peptide does not reach the surface of target cells as a result of preferential cleavage by the proteasome C terminal to the arginine residue (49). Mutations within and adjacent to epitopes may thus have a major influence on whether the peptide in question reaches the cell surface, but this type of escape mutation would be invisible if the only criterion used was whether peptide-pulsed targets were recognised by CTL.

Clear immunodominance versus polyvalent CTL response
This discussion of CTL escape has to this point been mostly concerned with the situation in which there is a clear immunodominant CTL response, and the consequences of failure of CTL to recognise the mutated epitope, either through failure of the variant to bind the presenting molecule, failure of the epitope to be processed intracellularly or failure of the TcR to bind to the variant peptide. Whilst the immunodominance of the HIV-specific CTL response is often striking (for example, in donor 007, whose anti-HIV CTL response was directed at a single epitope for several years (14)), the situation is also observed where a number of responses appear to be acting simultaneously (for example (86)). This broad specificity of the anti-HIV response, which contrasts with the narrow responses seen to many other viruses, could actually result from epitope variation and consequent shifts in immunodominance.

Where there are comparably strong, co-dominant responses, mutation in one epitope might be enough to reduce the total antiviral response to give the variants even a small selective advantage. Data from the LCMV experiments described above (43) support this, in that escape in an immunodominant (GP-1) epitope delays clearance of virus even in the presence of other, subdominant CTL responses; and escape simultaneously in a subdominant (GP-2) epitope delays virus clearance further. However, there may be cases where mutation in a single epitope may only have minimal selective advantage, and this would be an alternative explanation for the lack of epitope variation sometimes observed in virus sequences from

Fig. 2. A. Recognition of index peptide KRWIIMGLNKN and variant peptide KKWIIIMGLNKN by CTL clone from donor 007. Targets (autologous EBV-transformed B-cell line: BCL) were incubated with the peptide at the concentrations shown for the duration of the assay. B. Peptide-HLA-B27:05 complexes are very unstable when exogenously added peptide is the KKWIIIMGLNKN variant compared to KRWIIMGLNKN index. Targets (autologous BCL) were pulsed with peptide for 1 h, then washed three times, before incubation at 37°C for the times shown prior to use in the assay. C. HLA-B*2705 molecules are not adequately loaded with the variant K2 peptide in the endoplasmic reticulum to sensitize target cells for lysis by CTL. pCEP4 targets transfected with plasmid expressing no epitope; KR-SR: targets transfected with minigene expressing the peptide: metKRWIIMGLNKNIVELSRYWYAIRTR; KK-SR: targets transfected with minigene encoding metKKWIIMGLNKNIVELSRYWYAIRTR; KR-SK: encoding metKRWIIMGLNKNIVELSRKWYAIRTR. Peptides (10 micromolar) used in assay: KR-pep – KRWIIMGLNKN; KK-pep – KKWIIIMGLNKN; SR-pep – SYFWAIRTR (influenza-specific HLA-B27-restricted epitope).
slow-progressing asymptomatic donors (55, 86). It should be noted however that some authors (87) have detected an increased level of epitope-specific sequence variation in slow progressors when compared to more rapid progressors. Whether this relates to the particular regions of the virus to which these CTL responses are directed, or the MHC class I molecules involved, is not clear.

**CTL antagonism and persistence of escape variants**

An added complication is that variation occurring at positions in the epitope which interact with the TcR (and also sometimes at anchor positions (88)) may allow antagonism of the CTL response towards the index peptide (89). This means that CTL that are unable to kill targets loaded with the variant peptide may also be unable to kill adjacent target cells loaded with the index peptide. In this case, frequency-dependent selection may operate, such that selection pressure favouring a variant peptide is inversely proportional to its frequency. Thus, although CTL escape is occurring, this is not reflected by an increase in the frequency of the antagonist variant to fixation. It is worth noting that all the examples in which fixation has been reported in escape from HIV-specific CTL responses involved mutations that affected anchor residues involved in binding to the HLA molecules rather than to the TcR (6, 14, 65).

Variant peptides that can still bind to the MHC-presenting molecules and fail to sensitise targets for killing by CTL may nonetheless stimulate the generation of CTL specific for the index peptide (89). This may provide an explanation for the apparent failure of CTL specific for the variant to be generated, and the persistence of CTL escape variants where variation in the epitope has occurred at a TcR contact residue. Alternatively, in many cases, TcR Vβ expansions are oligoclonal (5, 8, 10) (J. Wilson, M. Callan & A. McMichael, unpublished data) and the generation of new responses that are required for recognition of many variants may become increasingly difficult as the infection progresses and help from CD4+ T cells diminishes.

**Conclusion**

The enormous variation observed in HIV is the consequence of an error-prone reverse transcriptase and the very high viral turnover in infected individuals. CTL are very active in HIV infection. Since CTL can kill virus-infected cells before the production of viral progeny, there must be selection pressure for the virus to escape the CTL response. The strength of this selection pressure depends on the dominance of the CTL response over the other antiviral immune responses, including the effect of β chemokines produced by CTL and other cells.

CTL escape is most clearly seen when CTL-mediated killing of virus-infected cells is the dominant antiviral immune response and the CTL response itself is monospecific or there is clear immunodominance. This may occur at seroconversion or later including the progression to AIDS. The most likely escape mutations affect MHC binding.

Detailed longitudinal studies are required to detect the occurrence of CTL escape, with frequent monitoring of the CTL response and the composition of viral quasispecies present. In the commonly observed situation in mid-infection, where many anti-HIV CTL responses are present, epitope-specific mutations affecting residues binding the TcR may be present and CTL escape to fixation may not be observed. Interpretation of such "snapshots" is very difficult, although the absence of responses to normally immunodominant epitopes and sequence changes in these epitopes are highly suggestive of previous escape. The accumulation of longitudinal data is extremely labour-intensive, but more would determine whether escape is occurring continuously throughout the course of infection, as suggested by Nowak et al. (54), or whether it is limited to the beginning and end of the infection.

The role of CTL escape in hastening disease progression is also yet to be unequivocally established. Drug-resistant viruses may or may not replicate less efficiently in vitro than the wild-type virus, but there is no evidence that individuals harbouring these resistant viruses progress to disease and death more slowly (90, 91). Does shift to less immunodominant responses lead to weaker control of virus replication? If so, this would lead to higher viral loads, more rapid decline in CD4+ T-cell count, reduction in CD4+ T-cell "help", and eventual failure to maintain existing CTL and inability to respond to new variants. Detailed longitudinal studies of HIV infection, in which the dynamics of CTL responses are followed through the course of the infection, using peptide-MHC complexes specific for particular CTL (9), should provide answers to these questions.

HIV is unusual among viruses in not being controlled adequately by the immune response and in particular by the CTL response. The ability of the virus to infect critical immunocytes, CD4+ T cells, dendritic cells and macrophages must be relevant to this. Yet initially the immune response does quite well. We have argued here that the exceptional accumulation of virus variants and their ability to evade immune responses must slowly undermine this control. Thus, the virus evolves in response to the selection force of the immune response and the CTL response in turn evolves, attempting to regain control, but ultimately fails.
References


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