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Cytotoxic T-lymphocyte escape viral variants: how important are they in viral evasion of immune clearance in vivo?

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Summary: Although viral variants which are not recognized by epitope-specific cytotoxic T lymphocytes (CTL) have been shown to arise during a number of persistent virus infections, in many cases their significance remains controversial: it has been argued that the immune response is sufficiently plastic to contain their replication. In this review, we describe the mechanisms by which amino acid changes in viral proteins may affect epitope recognition by virus-specific CTL, and discuss the viral and immunological basis for the emergence of viral variants bearing such amino acid changes during infection. We then consider the impact that viral variation may have on the host CTL response and its ability to contain virus replication. We argue that the emergence of a viral variant demonstrates that it must have an *in vivo* replicative advantage, and that as such, the variant must tip the balance between virus replication and immune control somewhat in favor of the virus. Further, we suggest that although the immune response can evolve to recognize new viral epitopes, the CTL generated following such evolution frequently have a reduced ability to contain virus replication. We conclude that this escape mechanism likely does make a significant contribution to persistence/pathogenesis during a number of different virus infections.

Introduction

Following virus infection, a series of complex interactions occur between the virus and the host immune system. The host aims to eliminate the infection and minimize associated pathological consequences, whilst the virus tries to avoid clearance by the host immune response so that it can persist and be disseminated to other hosts over a long time period. Viruses have evolved a variety of different strategies for avoiding clearance by the host immune response; a single virus often employs multiple strategies simultaneously to increase its chances of persisting in the face of an adaptive host defense system with an array of different effector mechanisms (1). As cell-mediated immune responses, in particular the virus-specific CD8^{+} cytotoxic T-lymphocyte (CTL) response, frequently play a key role in the elimination of established virus infections, many viral evasion strategies are targeted to this arm of the immune response.

Viral immune evasion strategies may broadly be divided into two categories: those that enable the virus to avoid detec-

tion by the host immune response, and those that impair the functioning of the host immune system. Viral impairment of the functioning of the host immune system may be non-antigen-specific, as exemplified by the production of homologs of host cytokines or their receptors that mimic (in the case of immune downregulatory) or block (in the case of immune-stimulatory or antiviral effector) the actions of host cytokines (2), or by infection of cells of the immune system, causing either impairment of their functions or resulting in their destruction; the latter may occur either directly or by the cells being rendered targets for immune lysis (3). However, as too severe a generalized impairment of host immune functions can lead to the death of the host, thus reducing the opportunity for virus spread, a preferable strategy is impairment of the virus-specific immune response. Examples of how this may be achieved are by viruses infecting and targeting the destruction of antigen-specific B cells (4) or inducing exhaustion of high-affinity clones of virus-specific T cells (5).

Strategies viruses may employ to avoid detection by the host immune response include i) the establishment of latent infections during which viral protein production is minimized, a strategy commonly employed by herpesviruses and perfected by herpes simplex virus (6); ii) infection of immune-privileged sites (to which access of the immune system is restricted and where levels of major histocompatibility complex (MHC) antigen expression are low and immune downregulatory mediators may be present), the best example being the brain where a surprisingly large number of different viruses persist (7); iii) virus-induced downregulation of levels of MHC antigens or adhesion molecules on the surface of infected cells so that they do not trigger host T-cell recognition (8); and iv) evolution of antigenic variants whose recognition by the specific immune response is impaired. Antigenic variation was initially documented as a means for viral escape from antibody neutralization (9), but more recently has also been shown to confer viral escape from CTL control (10).

Although the above immune evasion mechanisms all have the potential to promote viral persistence, the contribution that some of these mechanisms in fact make to persistence *in vivo*, particularly during human virus infections, is not completely clear. For example, although viral variants which are not recognized by epitope-specific CTL have been found during a number of persistent virus infections (11), in many cases their significance remains controversial: it has been argued that the immune response is sufficiently plastic to contain their replication (12). In this review, the mechanisms by which amino acid changes in viral proteins may affect epitope recognition by virus-specific CTL are described, and the viral and immunolog-

ical basis for the emergence of viral variants bearing such amino acid changes during infection is discussed. The impact that viral variation may have on the antiviral CTL response during the course of virus infections is then considered, and the contribution that this escape mechanism may make to persistence/pathogenesis in different virus infections is discussed.

Mechanisms by which amino acid changes may affect epitope recognition by virus-specific CTL

For a viral epitope to be recognized by CD8^{+} T cells, it must be processed and presented on the cell surface in association with MHC class I molecules. Amino acid changes may thus affect epitope recognition by specific CD8^{+} T cells by altering any of the steps involved in this pathway, from the initial generation of the epitopic peptide to its formation of a stable complex with MHC. They may of course also affect the interaction of the peptide-MHC complex with the T-cell receptor (TCR), either ablating TCR binding altogether, or altering the signaling which occurs via the TCR. As described below, examples have been found of amino acid changes in viral proteins that act via each of these mechanisms.

Alteration of antigen processing/peptide transport

The efficiency of processing and presentation of CTL epitopes is determined not only by the sequence of the epitope itself, but also by the residues which surround it in the protein. Amino acid mutations in flanking sequences may thus affect the recognition of viral CTL epitopes, as was originally demonstrated in experiments using cytomegalovirus and influenza virus-specific CTL (13, 14). The precise mechanism by which antigen processing was affected was not defined in these studies or a subsequent paper describing mutations in the Nef protein of human immunodeficiency virus type 1 (HIV-1) which also affected presentation of a nearby CTL epitope (15). However, in a more recent report, alteration of proteasome-mediated degradation due to a single amino acid difference within a CTL epitope was shown to form the basis of lack of presentation of this epitope by infected cells (16). In mice infected with AKV/MCF type murine leukemia virus, an epitope in the p15E transmembrane viral envelope protein constitutes the immunodominant sequence recognized by virus-specific CTL. These CTL do not recognize cells infected with Friend/Moloney/Rauscher type murine leukemia virus, where the epitope sequence differs by a single residue (17); this is because the amino acid difference causes epitope destruction by specific proteasomal cleavage. Mice infected with the latter virus thus do not mount a CTL response to this epitope. Amino acid

changes that alter the processing of CTL epitopes can therefore have a dramatic effect on the *in vivo* antiviral CTL response.

It is unclear how commonly such mutations are selected for during persistent virus infections. That relatively few examples of amino acid changes in viral proteins which affect CTL recognition via this mechanism have been described could be a reflection of the fact that such mutations are not readily picked up by the methods most frequently used to analyze antiviral CTL responses. Alternatively, the requirements for antigen processing/peptide transport may be so much less stringent than those for peptide interactions with MHC and the T-cell receptor that amino acid changes which affect the former arise less often.

Alteration of peptide binding to MHC

Amino acid mutations within epitopic peptides may ablate peptide binding to MHC altogether, or reduce the affinity of peptide-MHC interaction so that the peptide-MHC complex has an extremely short half-life and is unlikely to trigger T-cell activation. Viral mutations which alter peptide interactions with MHC class I molecules in each of these ways have been described; examples in HIV were recently reviewed (19). As mutations which prevent the stable association of peptides with MHC confer escape from recognition by all epitope-specific T cells regardless of their TCR usage, this is an efficient mechanism for viruses to avoid CTL recognition. It is of interest that the human leukocyte antigen (HLA)-A11 epitope loss from Epstein-Barr virus (EBV) isolates derived from populations where the frequency of HLA-A11 is extremely high is conferred via mutations in anchor residues of the epitope that are important for binding to HLA-A11(19, 20). EBV is a genetically stable DNA virus which generates mutants at a relatively low frequency; mutants will thus only come to prevail in the population if they confer advantages for the spread of virus to new hosts, in which very different TCRs may be used to recognize the same peptide-MHC complex. Mutations that ablate binding of the immunodominant HLA-A11-restricted EBV CTL epitope to MHC will allow escape from recognition from epitope-specific T cells in any host.

Alteration of peptide interaction with the TCR

Amino acid changes in a peptide which do not prevent it from being presented in association with MHC may result in alterations to the surface recognized by the TCR. This surface may be altered by changes in the TCR contact residues, or by changes in other residues in the epitope that cause the peptide to bind to MHC in a distorted conformation (21). Epitopes bearing mutations that cause alterations in the TCR contact surface have been termed altered peptide ligands (APL) (22). Epitope-specific T cells bearing distinct TCRs may be affected by APL in different ways. The altered peptide-MHC complex may fail to interact with a particular TCR altogether; or it may be recognized, but the T cell may receive a reduced or even a different type of signal when it recognizes an APL-bearing cell (23–26). The responding T cell may thus be only partly activated (e.g. to proliferate but not induce cell lysis (27)), or even anergized (28). Presentation of certain APL to T cells can therefore inhibit the response to the index peptide not just by competing with it for binding to MHC, but by negatively signaling the responding T-cell population: this phenomenon is known as T-cell antagonism. The ability of APL to act as CTL antagonists can be tested by measuring the capacity of cells presenting the APL to inhibit lysis of labeled target cells presenting the index peptide (29). Using this technique, mutations which confer antagonistic properties on epitopic sequences have been shown to exist in the persisting virus population in individuals chronically infected with both hepatitis B virus (HBV) (30) and HIV-1 (31).

Although viral mutations which result in the generation of APL may not provide as efficient a means of simple escape from immune recognition as mutations which abrogate epitope presentation altogether (because the APL may still be immunogenic to a proportion of host T cells which could be expanded following emergence of the mutant), they could potentially have powerful effects on the overall control of virus replication via other mechanisms. If they are able to act as T-cell antagonists, they may not only inhibit the lysis of cells on which the mutant epitope is presented, but also that of cells in which non-mutant virus is replicating, hence conferring protection from immune control on the entire viral quasispecies. Further, as some APL are able to stimulate and sustain the growth of CTL despite the fact that they do not induce CTL lysis (27), they could drive an ineffectual CTL response and hence modulate the CTL repertoire in a detrimental fashion, reducing the overall efficiency of CTL control of virus replication.

Viral variability: the basis for evolution of escape variants

Genetic variation is a strategy viruses exploit to promote their survival not just in the face of the host immune response, but under any environmental conditions they may encounter. They can achieve variation by a number of different mechanisms, including mutation, homologous and non-homologous recombination and (for viruses with a segmented genome) reassortment: different virus families utilize these to different extents. RNA viruses (including retroviruses) and DNA viruses such as

hepadnaviruses, whose genome replication involves an RNA intermediate, have extremely high mutation rates. This is due in large part to the absence or very low efficiency of proof-reading-repair activities associated with RNA replicases and transcriptases (32), and the lack of post-replicative error correction mechanisms such as those that normally operate during replication of cellular DNA. Misinsertion errors during RNA replication and reverse transcription have been estimated to be in the range of 10^{-3} to 10^{-5} substitutions per nucleotide per round of copying; for a 10 kb genome, this would result in each progeny RNA strand including an average of 0.1 to 10 mutations (33). RNA viruses thus exist not as homogeneous populations, but as complex, dynamic mixtures of heterogeneous sequences termed quasispecies (34, 35). If a virus is replicating under a constant set of environmental conditions to which it is optimally adapted, although the precise composition of the quasispecies will continually be fluctuating, the average sequence of the viral population will remain unchanged. However if conditions alter, mutations which confer an increase in fitness (the relative ability of the virus to produce infectious progeny) will be selected for and come to predominate in the viral population.

The quasispecies nature of RNA viruses, coupled with the short replication times and high viral yields they frequently exhibit, favors rapid adaptation to environmental changes. Dramatic examples of viral evolution in response to environmental pressure have been provided by the emergence of drug-resistant viral variants in HIV-1-infected patients treated with antiretroviral agents (e.g. (36–38)). HIV is a retrovirus with an *in vivo* mutation rate of approximately 3×10^{-5} nucleotides per replication cycle (39). Human infection with this virus is characterized by high levels of persisting virus, much of which is actively replicating and turning over at remarkable rates (40–42). As many as 10^{10} virions may be produced per day (42), allowing great potential for variation (43), although not all of these will be infectious, and the effective population size may be much smaller (44). Where a single nucleotide change is sufficient to confer a high level of resistance to antiretroviral treatment, resistance may be selected for very rapidly. For example, a single nucleotide change can reduce HIV's susceptibility to the non-nucleoside reverse transcriptase inhibitor nevirapine by 100–1,000-fold. Viral variants bearing this mutation have been shown to pre-exist in the plasma HIV RNA in untreated patients (45), and in some patients high level resistance to this drug may be acquired within weeks of starting therapy (37). In cases where multiple mutations are required to achieve drug resistance, either because a series of mutations needs to occur before a high level of resistance to one antiretroviral agent is achieved (38, 46) or because the patient is being treated simultaneously with a combination of several antiretroviral agents (47, 48), it takes longer for resistance to evolve. Indeed, in some patients, particularly where virus replication has been reduced to very low levels, it may not do so (49, 50). However, these observations illustrate the tremendous potential for RNA viruses to overcome controlling forces during the course of an infection via selection for resistance-conferring mutations.

Under what conditions are CTL escape viral variants selected *in vivo*?

The above outline of how mutations are selected for in a virus population allows a number of predictions to be made about the *in vivo* conditions under which CTL escape virus variants are likely to emerge. Firstly, as a mutation will only be selected for if it confers a fitness advantage on the virus bearing it, CTL escape virus variants will only grow out in infections where CTL pressure exerts significant control over virus replication. It is thus perhaps not surprising that the first report of the *in vivo* emergence of a CTL escape virus variant came from a study performed using lymphocytic choriomeningitis virus (LCMV) (10), a murine virus: during infection with LCMV the CD8+ CTL response is well known to be the critical determinant of control of virus replication (reviewed in (51)). Conversely, demonstration that viral variants bearing mutations which confer escape from CD8+ T cells come to dominate the viral quasispecies during a particular virus infection can be used to provide evidence for the importance of this arm of the immune response in controlling the infection, which in human virus infections may otherwise be difficult to demonstrate convincingly (52–54). Secondly, as the stronger the control over virus replication exerted by the CTL response directed against a particular epitope, the greater the selective pressure it will exert for escape mutations in this epitope to emerge in the viral population, the frequency of CTL directed against a particular epitope and the avidity of their target cell interaction will clearly influence the selection of escape mutations in this epitope. The original demonstration of CTL escape virus selection in the LCMV system was actually made in TCR transgenic mice where CD8+ T cells specific for the epitope in which escape mutations were observed made up 75–90% of the peripheral T-cell population prior to infection (10). This was an artificial system; however, due to advances in methods for quantitating epitope-specific T cells, it has recently become apparent that epitope-specific CD8+ T cells can reach extremely high frequencies in natural virus infections in both mice and humans, particularly during the primary immune response (55–57). Primary infection is

thus clearly a setting under which escape-conferring mutations may potentially emerge very rapidly. Although lower frequencies of epitope-specific CTL will also have the capacity to drive the selection of escape viral variants, this selection will occur more gradually over the course of a greater number of rounds of viral replication.

The greater the selective advantage conferred by a particular viral mutation, the faster this mutation will be selected for in the viral quasispecies. Thus, not only the strength of the CTL response to a particular epitope but also the immunodominance of this response will have a great impact on the likelihood that escape mutations will be selected for within the epitope. Escape mutations in subdominant or even co-dominant CTL epitopes may confer such a slight selective advantage on the viral variants bearing them (whose replication will still be controlled by the more dominant or co-dominant CTL responses in the host (58, 59)) that they may never emerge *in vivo*. Virus replication must clearly be ongoing for viral variants to be generated and selected. Particularly where the selective advantage conferred by a certain amino acid mutation is not very great, many cycles of virus replication may need to occur before viruses bearing it become predominant in the viral quasispecies. How dominant the CTL response to a certain viral epitope is in the overall control of virus replication is thus also an important determinant of the emergence of escape mutations in this epitopic sequence from this perspective. If other arms of the immune response or CD8+ T-cell responses to other epitopes in the virus which are not affected by the mutation concerned are able to reduce virus replication to very low levels or mediate viral clearance, the escape mutation will not have time to become fixed in the viral population during the course of infection. CTL escape virus variants will thus be most likely to emerge in the face of a host CTL response which is highly dominated by T cells of a single epitope specificity; even a very strong response to a particular epitope is unlikely to have the chance to select for escape viral variants in the context of strong responses to other epitopes.

This is illustrated by observations made in the murine LCMV infection model. The CTL response mounted to LCMV by infected C57BL/6 mice has a broad epitope specificity: in the viral glycoprotein (GP) and nucleoprotein (NP) alone, at least five different CTL epitopes have been identified (60, 61). Although the response to some of these epitopes is extremely strong (T cells recognizing the epitope at NP 396–404 constitute up to 40% of the CD8+ T-cell population, and those recognizing the epitope at GP 33–41 up to 29% at the peak of the antiviral immune response (56)), the infection is rapidly cleared, and CTL escape viral variants do not emerge. If mice

are immunized with one of these epitopes in a carrier protein prior to infection, the CTL response mounted upon LCMV infection is then biased in favor of this epitope. Viral clearance still occurs; however, if virus is isolated from the mice just prior to clearance and grown up in vitro, it is found to contain escape mutations in the epitope to which the CTL response was biased (62). Escape viral variants thus arise in the context of this more immunodominant CTL response, but they do not grow out *in vivo* as the infection is cleared too rapidly. The LCMV TCR transgenic mice discussed above represent a situation of epitope immunodominance at the extreme of the spectrum: their T-cell repertoire is so dominated by transgene-expressing cells recognizing the LCMV GP 33–41 epitope that CTL responses cannot be effectively mounted to other viral epitopes. Thus, when they are infected with LCMV and viral mutants arise which cannot be recognized by the GP 33–41-specific CTL, their replication is not contained by CTL of other specificities and they emerge in the context of viral persistence (10).

The above predictions that CTL escape viral variants are most likely to emerge in the context of a strong host CTL response which is highly focused on a single viral epitope are supported by the observation that one of the clearest examples of the emergence of CTL escape virus variants during a human virus infection occurred under just these conditions. Both we and others have shown that strong CD8+ CTL responses are mounted very early following infection with HIV-1, prior to seroconversion, and have hypothesized that they play an important role in containing viral replication (63, 64). In one patient we studied (53), the early CTL response (16–20 days following the onset of symptoms indicative of acute HIV-1 infection, which occurred 20 days after the homosexual encounter during which he initially contracted the virus) appeared to be strongly directed against a single viral protein, Gp160. Epitope mapping performed using the Gp160 sequence of the patient's autologous early HIV-1 population indicated that this response was in fact extremely focused on a single epitope encompassing Gp160 amino acids 30–38(9), recognized in association with HLA-B44. The frequency of epitope-specific CTL was extremely high: at the earliest timepoint available for study, which may have been slightly after the peak of the primary immune response, 1 in 17 peripheral blood mononuclear cells (PBMCs) were found to score as virus-specific CTL precursors by limiting dilution analysis, a technique which has recently been shown to greatly underestimate the total number of epitope-specific T cells (55, 56). As shown in *Fig. 1*, viral variants bearing mutations in the epitopic sequence which conferred escape from recognition by epitope-specific CTL rapidly appeared in this patient, and then increased in frequency until
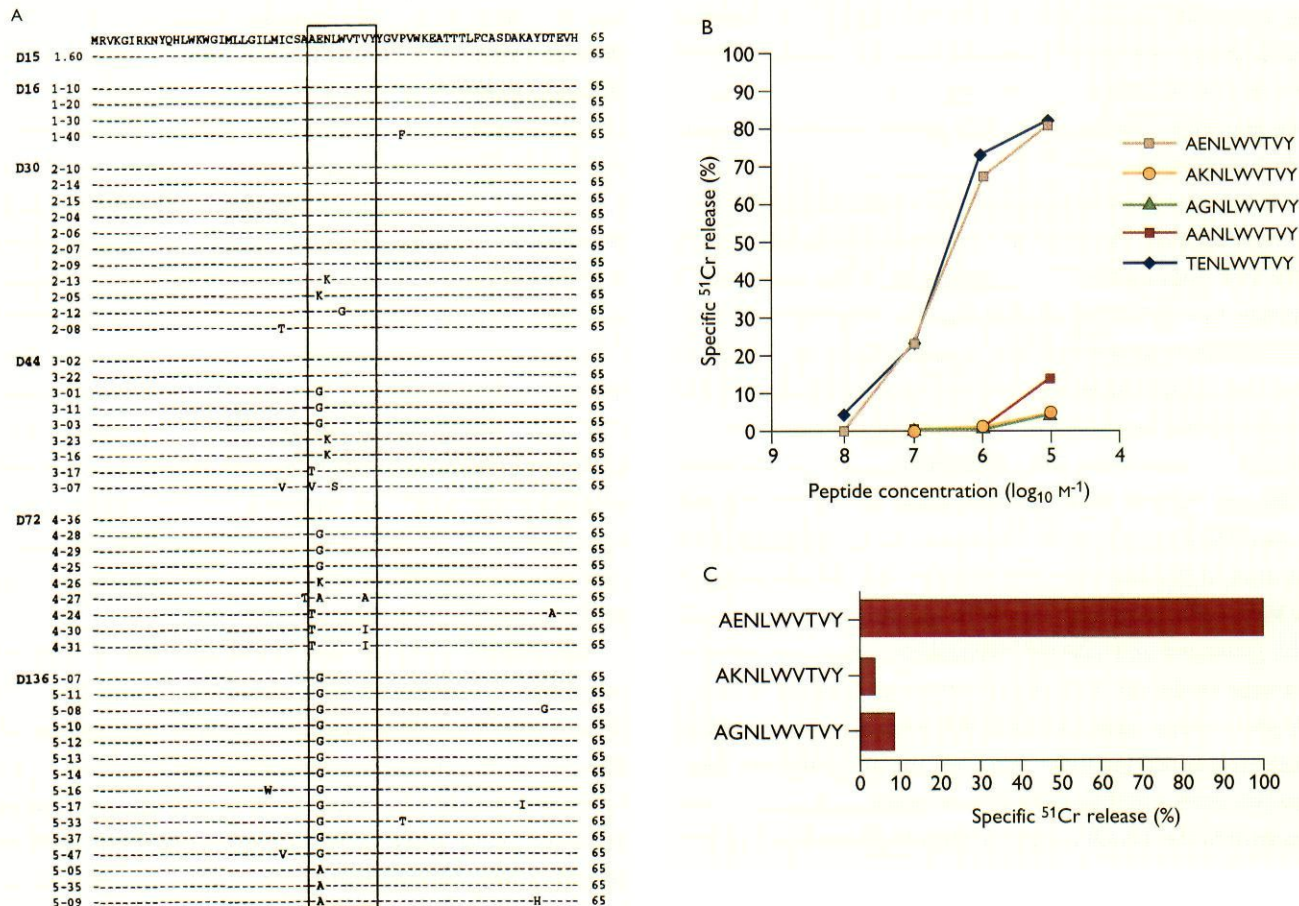
Fig. 1. Selection for mutations conferring escape from recognition by the immunodominant early CTL response in an HIV-1-infected patient. The patient mounted a primary CTL response that was highly focused on an immunodominant viral epitope in Gp160 at amino acids 30–38. **A** shows the deduced amino acid sequence of the N-terminal 65 residues of Gp160 of individual plasma viral RNA (cDNA) clones derived at the indicated number of days (D) following onset of the acute retroviral syndrome. The CTL epitope at position 30–38 is boxed. **B** and **C** illustrate the relative abilities of synthetic peptides corresponding to Gp160 amino acids 30–38 of the virus population in the patient at the earliest timepoint available for study, as well as the predominant mutant populations that emerged later, to sensitize autologous target cells for lysis by (B) a CTL clone or (C) bulk *in vitro* restimulated patient PBMC derived from the patient very early in the infection.

they had completely replaced the transmitted viral strain. Interestingly, the variants which came to predominate in the viral quasispecies all possessed changes at Gp160 amino acid 31, the position which constituted the major anchor residue for HLA-B44. As discussed above, mutations which ablate peptide binding to MHC confer escape from recognition by all epitope-specific T cells, and indeed, not only individual CTL clones but also bulk CTL derived from this patient were unable to recognize peptides corresponding to the mutant virus sequences in *in vitro* assays (Fig. 1).

Thus in both human and murine systems, there is evidence that mutations which confer resistance to control by epitope-specific CTL are most likely to be selected for in highly immunodominant epitopes. Under conditions of epitope immunodominance, a single mutation which provides escape from recognition by CTL of one specificity will confer a greater replicative advantage on the mutant virus, thus the selection for it will be stronger; plus there will be a greater chance that virus replication will be able to continue for long enough for the mutation to completely replace the index residue in the viral quasispecies.

Evolution of the CTL profile in response to the emergence of escape viral variants

The viral quasispecies can clearly evolve in response to host immune pressure: as the host immune response is also adaptive, can it in turn evolve in response to the emergence of

escape viral variants? The specific immune response is ultimately driven by antigen; the emergence of a CTL escape mutation in a virus population during the course of an infection could potentially affect the antigen to which the immune system is exposed, and hence the CTL profile, in several ways. Firstly, unless the mutation selectively affects CTL stimulation so that only effector functions are diminished, the level of CTL specific for the index epitope will decline as the level of antigen available to stimulate them decreases. Secondly, if the mutant sequence is presented to and can stimulate novel populations of host CTL, these will increase in frequency. Thirdly, if the mutation reduces the efficiency with which the epitope is presented, this may allow the level of presentation of other viral epitopes to increase, and hence CTL of different specificities to increase in frequency, or novel populations of host CTL to be stimulated. Finally, as the mutant virus must have a replicative advantage over the original virus in order to be selected, the antigen load will increase, which will drive the overall expansion of virus-specific CTL. As discussed earlier, CTL escape mutations are most likely to be selected for in immunodominant CTL epitopes. The end result of the above effects will be that the breadth of the CTL response will increase, with the previously dominant T-cell clones declining in frequency, and subdominant or novel responses being stimulated. Such broadening of CTL specificity in response to the emergence of viral variants able to escape recognition by CTL directed against a highly immunodominant epitope was apparent in the HIV patient described in the previous section (53). As illustrated in Fig. 2, whereas PBMC derived from the patient very early during the infection mediated detectable lysis after in vitro restimulation of only target cells expressing the immunodominant Gp160 30–38(9) epitope, as viral variants bearing mutations in this epitope emerged, responses to epitopes elsewhere in Gp160 and in other viral proteins became apparent. A decline in the frequency of CTL directed against the Gp160 30–38(9) epitope occurred simultaneously (53).

This patient clearly had the capacity to make CTL responses to a broad range of epitopes in HIV; why then was his initial response so highly focused on a single epitope? How epitope immunodominance is dictated during an antiviral immune response is not clear: both the level of presentation of different viral epitopes and the available T-cell repertoire impact on this. The former can be affected by epitope processing (65), peptide transport into the endoplasmic reticulum (66, 67), the binding affinities of peptides to class I molecules (68) and the stability of peptide-MHC complexes on the cell surface (69). If CTL epitopes overlap, different MHC molecules may compete for their presentation (70). The T-cell repertoire is initially deter-

mined by positive and negative selection in the thymus, and subsequently reshaped in the periphery during the course of successive immune responses. The influence that the previous infection history may have on the subsequent immune response to a virus infection has been illustrated in murine arenavirus infections (71). In the HIV patient discussed above, both properties of the epitopic sequence and the T-cell repertoire at the time of infection may have contributed to the extreme immunodominance of the early CTL response. The Gp160 30–38(9) peptide may have had advantages over other potential epitopic sequences in its processing and transport: there appear to be differences in the processing of transmembrane and cytoplasmic proteins for presentation with class I which may give the former a presentation advantage under certain circumstances (72); further, it is of interest that during the natural processing of Gp160, signal peptide cleavage occurs between amino acids 29 and 30, thus generating the same N terminus as the 30–38(9) epitope. In addition, this epitope is predicted to have a very high binding affinity to HLA-B44. T-cell repertoire effects may also have played a role: it is possible that prior unrelated infections in this patient left him with a population of memory T cells that cross-recognized this epitope, and that these cells, being present at higher frequency and more readily activated than naïve T cells, dominated the initial HIV-1-specific immune response. This cannot be demonstrated conclusively, as no samples are available from this patient prior to infection; however, the fact that epitope-specific CTL were contained in an oligoclonal population of Vβ19+ T cells which underwent a massive expansion very early after infection in this patient (73) would be consistent with this.

Another study in the HIV system which emphasized the effect that escape mutations in CTL epitopes may have on CTL specificity focused on the CTL responses of two HLA-identical hemophiliac brothers who were exposed to the same batch of contaminated Factor VIII and became seropositive within 10 weeks of one another (74). Their CTL responses were very dissimilar: one made strong responses to two HLA-A2 and HLA-A3-restricted epitopes in Gag, whilst the other instead responded to two HLA-B7-restricted CTL epitopes. Mutations in both immunodominant epitopes of the Gag responder were seen in proviral sequences from the non-responder; it was thus very likely that he had initially made a CTL response to these epitopes, and that following the emergence of escape mutant viruses, this response had been downregulated and the B7-restricted responses stimulated.

In this individual, the escape mutant viruses did not revert to the index sequence when the CTL response against them diminished, implying that the mutations they bore were not
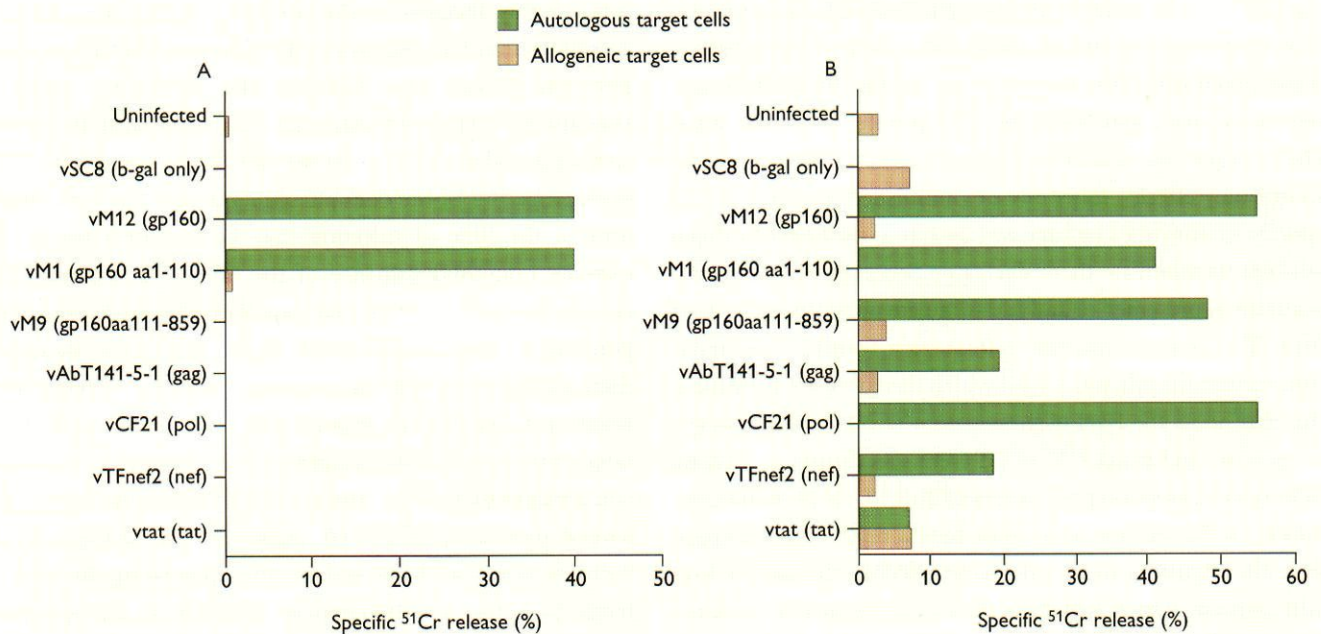
Fig. 2. Increase in breadth of the virus-specific CTL response during the early stages of infection in an HIV-1-infected patient where viral variants able to escape recognition by the initial immunodominant CTL response rapidly emerged. PBMC cryopreserved from the patient 20 (Panel A) or 30 days (Panel B) following onset of the acute retroviral syndrome were restimulated *in vitro* using an anti-CD3 antibody, and the ability of the effector cells generated to lyse autologous and allogeneic EBV-lymphoblastoid cell line target cells infected with recombinant vaccinia viruses encoding β-galactosidase (b-gal) only, full length Gp160 or sections thereof derived from the autologous virus in this patient 15 days following onset of symptoms, or other HIV proteins as indicated was tested in an *in vitro* [51]Cr release assay.

detrimental to viral replication. In other cases, however, CTL escape mutations may reduce viral fitness (75). If they impair virus replication too severely, they will not be selected for in the first place; this may contribute to the fact that in some studies escape variants have not been seen to arise in particular CTL epitopes despite the fact that CTL pressure is exerted on them over long periods of time (76, 77). If they have a smaller impact on viral fitness, they will emerge when epitope-specific CTL are exerting a strong pressure on viral replication, as overall they will confer an advantage on the virus, but as the frequency of epitope-specific CTL declines, they will tend to be replaced in the viral quasispecies by the index sequence. This in fact occurred in the HIV patient we described, where a strong selection occurred in the viral quasispecies for viral variants bearing mutations at the residue which constituted the anchor motif for the HLA-B44-restricted CTL epitope to which a dominant response was made in the early phase of the infection. Later in the infection, when the frequency of epitope-specific CTL had declined significantly, clones bearing the index sequence again began to emerge in the viral quasispecies (X. Wei, P. Borrow,

H. Lewicki, B. H. Hahn, M. B. A. Oldstone, G. M. Shaw, unpublished data). Epitope immunodominance and mutant virus frequency thus drive one another, which can lead to complex fluctuations in the makeup of the viral quasispecies and CTL specificity over the course of a persistent infection. Such fluctuations have been observed in patients chronically infected with HIV-1, and mathematical models developed to describe them (78–80).

These models predict that antigenic variation in immunodominant epitopes can shift responses to weaker epitopes and thereby reduce immunological control of the virus. That this certainly can occur has been illustrated in experiments performed in the LCMV model system. CTL clones directed against the three most dominant epitopes recognized during the antiviral CTL response in C57BL/6 mice were used to select *in vitro* for LCMV variants bearing escape-conferring mutations in one, two or all three of these epitopes (81–83). When C57BL/6 mice were infected with the mutant viruses, they mounted CTL responses to subdominant epitopes including epitope(s) in the viral polymerase which are not readily apparent during the response to wild-type virus, but the responses driven by the in

vitro-generated escape viral variants controlled virus replication less efficiently than the response to wild-type virus (58, 59). These experiments indicate that although the immune response is extremely plastic, its capacity to evolve to efficiently contain the replication of escape mutant viruses may be limited, particularly in inbred mice where the diversity of MHC alleles is more restricted than in the outbred human population. The evolution of escape viral variants may thus make a significant contribution to viral persistence during some infections by driving a suboptimal immune response.

What contribution do escape variants make to viral persistence and pathogenicity *in vivo*?

For a CTL escape viral variant to emerge, it must tip the balance between virus replication and the host antiviral immune response at least somewhat in favor of the virus. This replicative advantage can in itself affect the course of a virus infection, as was seen in the experiments originally demonstrating the *in vivo* selection of CTL escape viral variants in LCMV epitope-specific TCR transgenic mice: these animals developed a persistent infection following inoculation with a dose of virus which non-transgenic mice were rapidly able to clear (10). However, in this system, the TCR repertoire was artificially restricted, leaving open the question of whether CTL escape viral variants may also have biologically significant effects on the course of infection under more natural circumstances, where the immune system has the capacity to respond to a much broader range of viral epitopes and, as discussed above, the CTL response may co-evolve with the viral quasispecies. Mutations which affect epitope-specific CTL lysis *in vitro* have been described in a number of different viruses; here, their likely significance in some of these virus infections is discussed.

Mouse hepatitis virus

Mouse hepatitis virus-strain JHM (MHV-JHM) is a coronavirus which produces an acute fatal encephalitis in most inbred strains of mice. If C57BL/6 mice are infected whilst suckling on dams previously immunized to the virus, they are protected from the acute encephalitis; a proportion of mice then control the infection, but the majority develop a persistent infection associated with chronic demyelinating encephalomyelitis (84). The virus-specific CD8+ T-cell response is important in controlling this infection; in C57BL/6 mice this is directed against two epitopes in the viral surface GP (S), an immunodominant epitope at S 510–518, and a subdominant epitope at S 598–605 (85). A recent study showed that mutations which cause a loss of recognition are present in the immunodominant epitope sequence in

almost all virus sampled from symptomatic mice, but not in other T-cell epitopes (86). Mutations in this epitope were not detected in mice with acute encephalitis (which die very soon after the antiviral T-cell response is mounted) and were found at only low frequency in the residual viral RNA in the central nervous system of animals which had cleared infectious virus and remained asymptomatic at late times after infection. Further, when MHV-JHM variants bearing mutations in epitope S 510–518 isolated from infected mice were used to infect naïve mice, they produced an increased morbidity and mortality (87). This is thus a convincing example of an infection in which CTL escape variant selection does appear to be a key factor influencing virus pathogenesis. This infection may represent a situation where the host's ability to control virus replication is tenuous (CD8+ T-cell control of virus replication in the central nervous system may not be that efficient), and the replicative advantage conferred on the virus by escape mutations may tip the balance firmly in favor of the virus.

Epstein-Barr virus (EBV)

EBV is a human gamma herpesvirus that is carried by the majority of individuals as a lifelong asymptomatic infection, but has oncogenic potential and is implicated in the pathogenesis of a range of malignancies. The increased risk of development of EBV-positive malignancies associated with immune suppression illustrates the importance of immune control in maintaining a non-pathogenic equilibrium between this potentially oncogenic virus and its host; the potent antiviral CTL response is thought to play an important role in controlling virus replication and spread and the development of disease (20, 88, 89). The ability of this virus to evade clearance from the infected host has been attributed to a number of different mechanisms (reviewed in (20)), but CTL escape variant selection is not thought to play a major role. Unlike the other viruses discussed in this section, EBV is a genetically stable DNA virus; mutations which may confer escape from the prevailing host CTL response are thus likely to arise too infrequently for escape variant generation and selection to constitute an efficient means of immune evasion within a given host.

Mutations have been documented in the virus population prevailing in certain areas which confer escape from recognition by CTL directed against epitopes which are presented by HLA alleles possessed by a high proportion of the local populace. The best known example involves HLA-A11-restricted CTL epitopes in the EBNA3B protein which are conserved in the majority of type 1 EBV isolates from Caucasian and African populations, where the HLA-A11 allele frequency is low, but are mutated in virtually all isolates from highly A11-positive

populations in China and coastal New Guinea (19, 90). The majority of these mutations are located within anchor residues and abrogate epitope binding to the HLA-A11 molecule; as discussed earlier, such mutations would provide escape from epitope-specific CTL regardless of their TCR usage, suggesting that they may have conferred a selective advantage on the virus in the A11-positive populations from which they were isolated. However, EBV isolates from South-East Asia differ from Caucasian and African isolates at a number of loci, and other polymorphisms have been shown to affect the antigenicity of epitopes that are not present at high frequency in South-East Asian populations (91), raising the possibility that the changes observed in the A11-restricted epitopes may be coincidental. Support for the hypothesis of specific epitope loss was not obtained when EBV isolates from a highly HLA-B35-positive African population were sequenced across a B35-restricted epitope-containing region of the EBNA3A protein (92). However, the HLA-B35 frequency in this population was only about half that of the HLA-A11 frequency in the South-East Asian populations where A11 epitope mutations were detected; further, the HLA-B35-restricted CTL response to EBV is not usually as strong as that restricted by HLA-A11 (92). Whether the EBV-specific CTL response does in fact select for viral variants with a growth advantage within human populations bearing certain HLA alleles thus remains an unresolved issue.

Hepatitis B virus (HBV)

HBV is a hepadnavirus which, although it has a DNA genome, replicates via reverse transcription from a pregenomic RNA and thus has a high mutation rate. This virus produces acute and chronic infections in man during which the $CD8^+$ CTL response plays a key role both in virus clearance and in the pathogenesis of the associated liver disease (93). During acute HBV infection, most patients develop a strong polyclonal CTL response against multiple epitopes in different viral proteins (93). As discussed above, the likelihood of selection of escape mutant viruses under these conditions is probably low, because a mutation which provides escape from CTL of just a single specificity may not confer a significant selective advantage on the virus bearing it in the presence of strong CTL responses to many other epitopes. In contrast, the CTL response is usually much weaker during chronic HBV infection (93). In some patients it may also be more oligospecific, providing greater opportunity for the selection of epitope-specific escape mutations. Indeed, there is one report (94) of two patients who showed strong HLA-A2-restricted CTL responses narrowly focused on an epitope in the HBV core protein at amino acids 18–27 and failed to respond to any of the other HLA-A2-

restricted CTL epitopes in HBV that are frequently recognized in acutely infected patients. When the persisting virus in these patients was sequenced, the quasispecies was found to be dominated by variant virus carrying mutations within the HBV core 18–27 epitope that affected epitope recognition by the patients' CTL and could antagonize the response of certain CTL clones to the index epitope (30). Although conversion from a wild-type to the mutant sequence was not demonstrated in these patients, it is very likely that this does represent an example where escape variants were selected by the strong epitope-specific CTL response.

CTL responses restricted by other HLA alleles were not analyzed in these patients, and no information is available about how the CTL repertoire may have evolved in response to the emergence of the mutant viruses. It is thus difficult to assess how the escape variant may have impacted on the overall efficiency of CTL-mediated control of virus replication in these individuals. Other reports suggest that the emergence of virus variants bearing CTL escape mutations may not be a common event during chronic hepatitis B infection: no escape mutations were identified in a study where the CTL response against eight different HLA-A2-restricted epitopes defined in patients with acute HBV infection and the sequence of these regions in the *in vivo* viral quasispecies were analyzed in parallel in 12 patients chronically infected with HBV (95). Virus-specific CTL responses were undetectable in eight of these patients and weak in the other four; CTL-mediated pressure may thus have been too low to select for escape variants. Whether selection of escape mutations plays a critical role in HBV persistence is thus questionable (96); it is likely that the development of a weak antiviral immune response is of much greater importance in determining the course of this infection (93).

Hepatitis C virus (HCV)

HCV is a positive-stranded RNA virus, assigned to a new genus in the Flaviviridae, which is well adapted for persistence in its human host: at least 60% of infected individuals develop a chronic infection that is frequently associated with clinical hepatitis (97). As in HBV infection, the virus-specific $CD8^+$ CTL response is thought to play an important role in limiting virus replication, and simultaneously to contribute to disease pathogenesis. However, unlike the situation in HBV infection, where cell-mediated immune responses to the virus are generally low/undetectable in chronically infected individuals, HCV persistence in the presence of strong virus-specific CTL responses has been observed in both humans and experimentally infected chimpanzees (reviewed in (98)). The highly mutable nature of this virus's RNA genome raises the possibility that antigenic

variation may be one of the mechanisms via which it achieves persistence in the face of the antiviral immune response. Indeed, there is one report that in a chimpanzee which became persistently infected with HCV, the viral quasispecies underwent complete replacement with viral variants bearing a conservative amino acid substitution at residue 4 of an epitope in non-structural protein 3 (NS3) recognized by liver-derived CTL lines from this animal; this mutation abrogated recognition of the epitope by the animal's CTL (99). The CTL response in this animal recognized epitopes in multiple viral proteins (100); whether mutations were selected for in any of the other epitopes, how epitope specificity/immunodominance may have changed over the course of infection in response to viral variation, and what impact escape variant selection may have had on the overall efficiency of control of virus replication were not determined. However, this study illustrates that even though the CTL response to HCV is frequently polyclonal, escape viral variants can be selected during HCV infection. As discussed below for HIV, where more information about the co-evolution of the viral quasispecies and the immune response is available, escape mutations likely do compromise immune control of HCV infection, and are probably one of the factors which contribute to the persistence of this virus in its human host (98).

Human immunodeficiency virus (HIV)

HIV elicits strong CTL responses in most infected individuals. The early CD8+ CTL response is temporally coincident with the reduction in acute plasma viremia (63, 64); as discussed above, virus-specific CTL likely expand to extremely high frequencies during the primary immune response (53, 73). The frequency of virus-specific CTL then declines somewhat; however, high levels of activated CTL are commonly detected in chronically infected asymptomatic individuals, even in the setting of an extremely low viral load (77, 101–104). As disease progression occurs, virus-specific CTL frequencies may initially increase in response to increasing viral load, but generally fall to undetectable levels in the end stages of the infection (104). A number of lines of both direct and indirect evidence (discussed in (53)) indicate that the CD8+ CTL response does play an important role in controlling virus replication in HIV-1-infected individuals, and a variety of mechanisms have been proposed to contribute to virus persistence in the face of the host immune response (105–111). We focus here just on the role that the selection of CTL escape virus variants may play during this infection.

The high viral turnover during HIV-1 infection, rapid mutation rate of this virus and strong virus-specific CTL responses provide conditions under which CTL escape mutants would be

likely to emerge. Indeed, as indicated earlier, there have been numerous reports of variant viruses within the quasispecies in different patients which are able to escape recognition by epitope-specific CTL. Escape virus variants may be selected at different stages of infection and correspondingly impact on the virus-host balance in different ways. During primary HIV-1 infection, when the virus undergoes an intensive burst of replication and CTL directed against dominant epitopes may reach extremely high frequencies, escape variant selection may be particularly favored. We have observed replacement of the viral quasispecies by variants with mutations in an epitope targeted by the primary CTL response in the early stages of infection in two patients analyzed (53) (P. Borrow, H. Lewicki, X. Wei, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. A. Oldstone, G. M. Shaw, unpublished data), and there has also been another study documenting CTL escape variant emergence in a seroconverter (54). Interestingly, the two patients in whom we observed early selection of CTL escape variant viruses both underwent rapid disease progression: studies of larger numbers of patients are required to reveal how frequently early selection of mutations in epitopes recognized during the primary CTL response in fact occurs, and whether this phenomenon is always associated with rapid progression to AIDS. The emergence of viral variants with mutations that confer resistance to control by dominant CTL responses in the early stages of infection may potentially impact on the subsequent course of infection in two ways. First, as a viral variant will only be selected for if it has a replicative advantage, the emergence of CTL escape variants must be associated with an increase in early virus replication and spread. Consequences of this may include increased loss of CD4+ T cells and establishment of a larger pool of latently infected cells or a higher setpoint level of persisting virus: the latter has been demonstrated to be a good predictor of the subsequent rate of disease progression (112). Second, because (as discussed above) mutations in immunodominant CTL epitopes may promote evolution of the immune response to recognize subdominant epitopes, the host may be left after the acute phase of the infection not only with a higher level of persisting virus, but also with a CTL response that is less adequate to contain it.

In mid-HIV infection, mutations which affect recognition by epitope-specific CTL have been demonstrated in the persisting viral quasispecies in a number of studies (e.g. (15, 77, 113)); however, clear examples of such variants being selected for and fixed in the viral quasispecies over time are difficult to find. This is likely because CTL responses to multiple viral epitopes are frequently present and, as discussed earlier, under such conditions a complex cycle of fluctuations in the domi-

nance of CTL responses directed against different viral epitopes and corresponding shifts in the epitopic composition of the viral quasispecies may occur (78, 79). There are also examples of strong CTL responses being exhibited over long periods of time in the absence of appearance of mutations in the epitopes to which they are directed (76, 77). CTL epitope mutation during chronic HIV infection thus does not seem to be essential for the continued persistence of virus; however, when it does occur it may allow higher levels of virus replication, and hence promote disease progression. In the later stages of the infection, when the immune system is failing, the impact of viral variation on immune control may become more pronounced. The capacity of the immune response to evolve to focus on unmutated epitopes as escape variants are selected may be more limited at this time. Simultaneously, the reliance of the host on CTL lysis as a means of controlling virus replication and spread may be increasing, particularly if syncytium-inducing viruses resistant to control by chemokines such as RANTES, MIP1α and MIP1β appear. CTL escape variants may thus grow out and contribute to the escalating viremia. A recent paper described two examples of patients where mutations conferring evasion from a previously stable immunodominant CTL response became fixed in the viral quasispecies in the late stages of the infection (80), providing a clear illustration that this can occur. CTL escape viral variants may thus influence the immune system's ability to control virus replication at all stages of HIV infection, and likely do have an important impact on the overall disease course in at least a proportion of infected individuals.

Conclusions

The preceding discussion illustrates that viral variants which are able to escape recognition by host CTL emerge during a number of different virus infections, and clearly have a biologically significant impact on the balance between virus replication and its control by the immune response in at least some of these cases. The latter include several infections that are of clinical significance in humans; it is thus important that immune-based prophylactic and therapeutic strategies to combat these infections should be designed to minimize the likelihood of CTL escape variant selection. There are examples in the literature of both prophylactic (114) and therapeutic strategies (115) which have led to the selection of viral variants able to escape recognition by the CTL response which was intended to mediate a beneficial effect. As described in this review, studies in both human virus infections and murine model systems have illustrated the conditions under which CTL escape viral variants are likely to emerge: this information should be considered in future vaccine/therapy design to prevent a similar outcome from occurring.

Given that escape-conferring mutations will not be selected for if they have detrimental effects on virus replication, and that escape mutations emerge most rapidly when CTL pressure is predominantly focused onto a single highly immunodominant epitope, vaccines should aim to induce broad immune responses that recognize multiple co-dominant viral epitopes, at least some of which should lie in conserved regions of viral proteins on which there are stringent functional constraints. Peptide or minigene-based vaccination strategies are probably not the optimal choice for use in such infections: even if they include a carefully chosen cocktail of epitopes that can be recognized in association with multiple HLA alleles, there is a high probability that they will induce mono- or oligospecific immune responses in individuals of certain HLA types. Particularly if the infecting virus shows sequence variation compared to the antigens used for vaccination, there will be a danger that the vaccine might prime for an immune response that could be more detrimental than the immune response that may have been elicited naturally following infection. Vaccines that include multiple viral antigens are preferential immunogens for the induction of beneficial multispecific antiviral immune responses.

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