HIV-1 evolution and adaptation to the host during the course of infection
Gijsbers, E.F.

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DYNAMICS OF VIRAL ESCAPE FROM HLA-B27-RESTRICTED CTL RESPONSES IN HIV-1 INFECTED INDIVIDUALS

Esther F. Gijsbers and Neeltje A. Kootstra

Department of Experimental Immunology, Sanquin Research, Landsteiner Laboratory, and Center for Infectious Diseases and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

Manuscript in preparation
ABSTRACT

HLA-B27 has been associated with prolonged AIDS-free survival after HIV-1 infection. Loss of control of viremia in HLA-B27 patients has been associated with CTL escape at position 264 in the immunodominant KK10 epitope. This CTL escape mutation has a high fitness cost and it has been hypothesized that this mutation is not viable on its own, but requires the presence of compensatory mutations before emerging.

We here studied sequence evolution within HLA-B27-restricted CTL epitopes in the viral Gag protein during disease progression in four HLA-B27+ patients. Longitudinal Gag sequences obtained at different time points around AIDS diagnosis were obtained and analyzed for the presence of CTL escape mutations in epitopes restricted by HLA-B27 and potential compensatory mutations. Sequence variation was most predominant in the immunodominant HLA-B27-restricted KK10 epitope and CTL escape mutations in this epitope were observed in sequences obtained from 3 out of 4 patients. The R264K mutation was observed in sequences from 1 patient and the R264G escape mutation in sequences from 2 patients. These CTL escape mutations were accompanied by their respective compensatory mutations S173A and E260D. Comparing viral sequences, a higher number of total mutations in Gag was observed for viruses containing the R264K escape mutation, which may represent additional compensatory mutations that increase replication capacity and impact disease progression.
INTRODUCTION

Expression of certain HLA class I alleles is known to be associated with lower viral load and prolonged survival after HIV-1 infection, of which HLA-B57 and HLA-B27 are the most studied [1-5]. Cytotoxic T lymphocytes (CTL) play an important role in the protective effect of these HLA alleles and the presence of HIV-specific CTLs restricted by HLA-B57 and HLA-B27 in early infection better defines disease progression than HLA genotype alone [2]. However, HIV-1 is able to escape from CTL responses through selection of mutations that abrogate CTL-mediated killing [6]. Individuals carrying HLA-B57 show CTL escape from the immunodominant TW10 epitope through the T242N CTL escape mutation early after infection. The T242N mutation is known to have a negative effect on the replication capacity of the virus, leading to lower viral load [6,7]. In patients carrying the HLA-B27 allele on the other hand, CTL escape in the immunodominant KK10 epitope occurs late in the infection and is associated with loss of control of viremia [8-13]. The CTL escape mutation in this epitope is located at the R264 position and is most usually mediated by a mutation toward a lysine (K), however, mutation toward glycine (G), threonine (T), or glutamine (Q) have also been described [13-17]. It has been hypothesized that the R264 escape mutation is not viable on its own and requires the presence of compensatory mutations before emerging and is therefore only observed late in infection during disease progression [13].

We here studied the dynamics of viral escape from HLA-B27-restricted CTLs in four HLA-B27 positive patients during disease progression. Longitudinal Gag sequences obtained at different time points around AIDS diagnosis were analyzed for the presence of CTL escape mutations from HLA-B27-restricted CTL responses and potential compensatory mutations.

RESULTS

Characteristics of four HLA-B27+ patients who progressed to AIDS

Four HLA-B27 positive patients, who were followed longitudinally in the Amsterdam Cohort Studies (ACS), were analyzed in the present study. Patient characteristics are summarized in Table 1. All patients progressed to AIDS without the use of antiretroviral therapy and over the course of infection an increasing viral load and declining number of CD4+ T cells were observed (Figure 1). For all patients, serum samples from time points close to disease progression (between 2 years prior and 6 months after AIDS diagnosis) were available for analysis.

Sequence variation in CTL epitopes restricted by HLA-B27

The Gag region of HIV-1 present in serum from the patients was amplified by PCR and cloned into the pGEM T easy vector. Per time point, 1-10 gag sequences were generated to study sequence evolution over time (Table 2).

First, the sequence variation in the 5 Gag CTL epitopes restricted by HLA-B27 was investigated (Table 2). Inside epitope IK9, CTL escape mutations were only observed in viral sequences obtained from patient ACH19490. In this patient, the K26S/A escape mutation was observed in viral sequences obtained close to and after AIDS diagnosis. Additionally, a single viral sequence obtained from patient ACH19689 at 94.6 months after seroconversion contained
the R20F mutation in the IK9 epitope; this mutation has no effect on peptide presentation and was not positively selected for in this patient (Table 2).

Within the EL9 CTL epitope, no sequence variation was observed in patients ACH19490 and ACH19689. In patient ACH19974, we observed 3 mutations (E42G, R43Q and V46L) that were only present in 1 viral sequence at a single time point. Of these mutations, only the R43Q is located at an anchor residue and may have disrupted presentation of the epitope by HLA-B27, however the viral variant containing this mutation was not selected during the course of infection. In patient ACH19778, all viral sequences contained the V46L mutation, which is not associated with escape from presentation by HLA-B27. Additionally, the L50P mutation was observed in a single viral sequence obtained at 64.8 months after seroconversion. This mutation may represent an escape mutation located at the P9 anchor residue of the HLA-B27 EL9 epitope, but viral variants containing this mutation were not selected for over time (Table 2).

The previously described CTL escape mutation at position 264 within the HLA-B27 KK10 epitope [8-17] was observed in 3 out of 4 patients. The most common R264K escape mutation emerged in all viral sequences from patient ACH19978 at 85.4 months after seroconversion. In patients ACH19490 and ACH19689, the R264G escape mutation was observed in a minority of the viral sequences at the first time point analyzed, before the onset of AIDS, and this escape mutation was present in all viral sequences obtained at later time points. In patient ACH19778 and ACH19689 we additionally observed the L268M mutation, which has previously been associated with early escape from TCR recognition [18]. The L268M mutation has been described to have a negative impact on replication kinetics of the R264G variant, but not the R264K variant [15]. Indeed, mutations L268M and R264G were never seen in combination in a single viral sequence in patient ACH19689. Furthermore, the emergence of CTL escape variants containing R264G mutation might explain the loss of viral variants containing the L268M in ACH19689 during the course of infection. In patient ACH19490, we observed an additional mutation in the KK10 epitope (L266V) in a minority of sequences obtained from the first time point analyzed. Surprisingly, we did not observe any sequence variation in the KK10 epitope in patient ACH19974 (Table 2).

CTL escape was also observed in epitope DR11 in 3 out of 4 patients (ACH19974, ACH19490 and ACH19689). The R286K escape mutation was present in all viral sequences obtained at all time points from patient ACH19974 and in the majority of the viral sequences obtained at 105.9

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**Table 1. Patient characteristics.**

<table>
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<tr>
<th>Patient</th>
<th>Seroconversion or entry date</th>
<th>AIDS diagnosis * (months after seroconversion or entry)</th>
<th>HLA alleles</th>
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<td>06-03-1986 (S)</td>
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<td>A*02</td>
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<td>ACH19689</td>
<td>19-12-1984 (E)</td>
<td>118.2</td>
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* AIDS diagnosis according to CDC 1993 definition [24] or CD4+ T cell counts <200 cells/µl.
S: Date of seroconversion for HIV-1 antibodies during active follow-up.
E: HIV+ entry into the cohort.
NA: not available.
Figure 1. Longitudinal CD4+ T cell counts (grey) and HIV-1 RNA viral load measurements (black) for patients ACH19974, ACH19778, ACH19490 and ACH19689. Arrows indicate the time points from which serum was obtained. Dotted lines indicate time of AIDS diagnosis according to the CDC AIDS definition 1993 [24] or CD4+ T cell counts below 200 cells/µl.
Table 2. Sequence variation in CTL epitopes restricted by HLA-B27 and at positions 173 and 260 that have been associated with the R264K and R264G mutation, respectively.

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<th>Time after SC or entry (months)</th>
<th>number of sequences</th>
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<th>EL9 42-50</th>
<th>S 173</th>
<th>E 260</th>
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¹ AIDS diagnosis according to CDC 1993 definition [24] or CD4+ T cell counts <200 cells/µl. NA: not available.
and 112 months after seroconversion in ACH19490. However, the escape mutation was only present in the minority of viral sequences obtained at 94.6 months after seroconversion in ACH19689 and was not observed in viral sequences obtained from later time points.

No sequence variation resulting in escape from presentation by HLA-B27 was observed in CTL epitope DA9 in any of the patients under study. Only 1 viral variant obtained from patient ACH19974 contained a mutation in this epitope (F300L; Table 2).

Compensatory mutations associated with the R264K/G CTL escape mutation
The HLA-B27-restricted CTL epitope KK10 has been described as the immunodominant CTL epitope in most patients [19] and the previously described CTL escape mutation at position 264 was observed in viral sequences from 3 out of 4 patients. Mutations at this position are associated with a fitness cost, which may be compensated for by mutation S173A for R264K, or E260D in the case of R264G [12,13]. Indeed, the S173A (patient ACH19778) and E260D (patients ACH19490 and ACH19689) mutations emerged in viral sequences obtained from time points close to disease progression (Table 2). In the case of the R264G escape mutation in patients ACH19490 and ACH19689, we observed viral variants containing the R264G alone, or in combination with the E260D compensatory mutation. In the case of the R264K mutation in patient ACH19778, we only observed the combination of the escape mutation and the S173A compensatory mutation. Neither of the compensatory mutations was observed in sequences obtained from patient ACH19974, in which no escape at position R264 occurred. Other mutations that have been hypothesized to serve as (partially) compensatory mutations in literature, for instance Q136R, I267V and R275K, were not observed in viral sequences obtained from our patients [13,20,21].

To study possible compensation of the fitness cost associated with the R264K/G escape mutation by other mutations in Gag, viral sequences obtained from the four patients under study were compared to subtype B consensus sequence and the number of amino acid differences was determined. Comparing viral sequences with or without the R264K or R264G escape mutation, we observed a significantly higher number of total mutations in Gag for the sequences containing R264K compared to sequences lacking R264K/G escape (Figure 2). However, no difference in the total number of mutations was observed in viral sequences with R264G or without the R264K/G escape mutation (Figure 2). The higher total number of mutations in the sequences containing the CTL escape mutation at position R264K may represent additional compensatory mutations that increase replication capacity, and may impact disease progression.

DISCUSSION
HLA-B27 has been associated with prolonged AIDS-free survival after HIV-1 infection [1-3]. In patients carrying this HLA allele, CTL escape in the immunodominant KK10 epitope occurs late in the infection and is associated with loss of control of viremia [8-13]. We here studied sequence evolution in HLA-B27-restricted CTL epitopes in the viral Gag protein during disease progression in four HLA-B27 positive patients. In agreement with previous reports [8-17], CTL escape was predominant in the immunodominant HLA-B27-restricted KK10 epitope in three out of four patients. No escape at the KK10 epitope was observed in any of the sequences
from patient ACH19974 obtained from 56.0 to 82.7 months after seroconversion, despite disease progression. This indicates that development of the R264K/G escape mutation is not a prerequisite for progression to AIDS in HLA-B27 positive patients [11-15,21].

Escape from the immunodominant KK10 CTL response restricted by HLA-B27 via the R264 position can be accomplished by mutation toward lysine (K), glycine (G), threonine (T), or glutamine (Q) [13-17]. All these mutations decrease the binding affinity of the KK10 epitope to the HLA-B27 molecule, thereby limiting epitope presentation and preventing subsequent CTL-mediated lysis. The R264K is the predominant escape mechanism, whereas mutations R264G, R264T and R264Q occur with lower frequency. All four of the described amino acid changes at the R264 position incur a fitness cost on the virus, with the most substantial decrease in viral replication capacity associated with the R264K mutation [12]. Introduction of mutations that can compensate this fitness cost have been previously reported [12,13]. Interestingly, mutations that are able to compensate for the fitness cost associated with either R264K or R264G are located at a different position, with S173A and E260D serving as compensatory mutations for R264K and R264G, respectively. We here observed the emergence of the R264G mutation in two of our patients and the R264K mutation was observed in viral sequences from one patient. The presence of the R264G escape mutation was not accompanied by the E260D compensatory mutation at the initial emergence in both patient ACH19490 and ACH19689, but this compensatory mutation emerged later during the course of infection. In sequences from patient ACH19778, the R264K escape mutation was only seen in combination with the S173A compensatory mutation. Furthermore, we observed an increasing number of mutations in the Gag protein in viral variants containing R264K. This suggests that besides the known compensatory mutation (S173A), additional mutations may have emerged that further

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**Figure 2.** The number of amino acid mutations in Gag in the absence or presence of the R264K or R264G CTL escape mutation in viral sequences from patients ACH19778, ACH19490 and ACH19689. Statistical significance was assessed by Student’s T test.
compensate for the fitness cost associated with escape in the KK10 epitope. More research is
needed to evaluate the effect of these mutations on viral replication.

The R264G escape mutation is usually seen much less frequent as compared to the
predominant R264K mutation. Here, we observed the R264G mutation in sequences obtained
from two of our patients, who also show the longest AIDS-free period after infection (more than
9 years). The emergence of the R264G mutation occurred 14.8 and 23.6 months before AIDS
diagnosis, respectively, whereas the R264K mutation was only observed after AIDS diagnosis
(patient ACH19778). These findings suggest that the emergence of the R264G escape variant is
not directly associated with progression to disease. The fitness cost associated with the R264G
escape mutation is less severe as compared to the decrease in viral replication fitness associated
with the R264K mutation [12]. However, compensation of the R264K mutation by S173A results
in a higher replication capacity as compared to compensation of the R264G mutation by E260D
[12]. This may suggest that the lower replication capacity associated with the R264G/E260D viral
variants in patients ACH19490 and ACH19689 might account for the prolonged period between
the emergence of the CTL escape mutation in the KK10 epitope and the progression to AIDS.
Furthermore, sequences with or without the R264G mutation contained a similar number of
total mutations in Gag, in contrast to sequences containing R264K, which may suggest the
presence of a low number of compensatory mutations in the sequences containing R264G that
can restore the viral replication capacity.

It has also been reported that the R264G mutation is better able to disrupt binding of the
KK10 viral epitope to the HLA-B27 molecule as compared to the R264K mutation [15]. Since the
R264G mutation was observed in two patients with a prolonged AIDS-free infection time, it is
tempting to speculate that these 2 long-term nonprogressors experienced a sustained strong
immune pressure on the KK10 epitope, thereby selecting for the R264G mutation instead of
R264K, as this mutation confers better escape from CTL pressure. Moreover, the strongly
controlled viral replication in these patients may have prevented the development of the R264K
escape variant, which can only replicate when the S173A compensatory mutation is present as
well. Additional patients need to be included to test our hypotheses.

Although the KK10 CTL epitope is usually immunodominant in patients carrying HLA-B27,
escape from CTL responses in the KK10 epitope was not observed in patient ACH19974,
despite progression to AIDS. Viral sequences obtained from this patient did contain the R286K
escape mutation in the HLA-B27 DR11 CTL epitope, which may suggest immunodominance of
this epitope in this particular patient. We also observed a high number of mutations in Gag
sequences obtained from this patient at all time points studied as compared to the consensus
subtype B sequence (data not shown). Further studies are needed to determine whether
these additional mutations in Gag may compensate for the potential fitness costs that may
be associated with the R286K mutation, or whether these mutations might entail CTL escape
mutations in epitopes restricted by other HLA alleles.

It has previously been shown that the protective effects of HLA-B27 and HLA-B57 occur at
different times of infection [2]. HLA-B57-mediated protection occurs early after infection and is
associated with a delayed CD4+ T cell count decline, whereas the protective effect of HLA-B27
occurs late in infection when CD4+ T cell counts have already declined, and is associated
with a delay in AIDS-defining illnesses [2]. Both these HLA alleles present epitopes from the
conserved Gag protein and exert strong HIV-specific CTL responses. Viral escape from the immunodominant HLA-B57-restricted CTL response against the TW10 epitope occurs via the T242N mutation. This CTL escape mutation is seen early after HIV-1 infection and comes at a fitness cost to viral replication fitness. The accumulation of compensatory mutations that compensate the fitness cost associated with the T242N CTL escape mutation is associated with disease progression in HLA-B57+ patients [7,22,23]. In HLA-B27+ patients, the R264K/G escape mutation in the immunodominant KK10 epitope occurs late in infection and is associated with higher viral load and disease progression [8-13]. The strength of the CTL responses against the immunodominant HLA-B57 TW10 and HLA-B27 KK10 epitopes and the extent of the fitness defects associated with the T242N and R264K/G CTL escape mutations may partially explain the difference in protective mechanism of these HLA alleles.

In conclusion, we here analyzed the sequence variation in HLA-B27-restricted CTL epitopes in longitudinal Gag sequences from four HLA-B27+ patients around the time of AIDS diagnosis. Viral escape from CTL responses was predominant in the KK10 epitope in three out of four patients (R264K/G). The presence of the R264K/G escape mutation was accompanied by known compensatory mutations (S173A or E260D, respectively), which suggests that the fitness costs associated with the R264K/G mutation was restored. Furthermore, we observed a higher number of total mutations in Gag for viral sequences carrying the R264K CTL escape mutation, which may represent additional compensatory mutations that increase replication capacity and impact disease progression. Additional studies are required to gain more insight in the underlying mechanism of the large variation in HIV-1 disease progression in patients carrying the protective HLA-B27 allele.

**METHODS**

**Patient selection**

Four participants of the Amsterdam Cohort Studies (ACS), who were followed longitudinally and from whom serum samples were available, were analyzed in this study (Table I). All participants had routine 3 monthly visits for blood donation and physical examination. All four patients reached AIDS diagnosis (CDC 1993 definition [24] or CD4+ T cell counts <200 cells/µl) without the use of antiretroviral treatment. Selection of serum samples was done on the basis of a viral load above 1000 copies/ml around the time of disease progression (between 2 years prior and 6 months after AIDS diagnosis).

The ACS has been conducted in accordance with the ethical principles set out in the declaration of Helsinki and written informed consent was obtained prior to data collection. This study was approved by the Amsterdam Medical Center institutional medical ethics committee.

**RNA isolation, RT-PCR and PCR amplification**

Viral RNA was isolated from 150µl of serum with the Qiamp Viral Mini Kit according to manufacturer’s protocol. Subsequently, viral RNA was transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) and the gag-specific primer Gag outer reversed (5’-GCCTGTCTCTCAGTAC-3’). DNA was amplified by a nested PCR with outer primers Gag forward (5’-CGACGCAGGACTCGGTGCTG-3’) and Gag outer reversed (5’-GCCTGTCTCTCAGTAC-3’).
and inner PCR primer combinations: Gag BssHII fw (5'-TGCTGAAGCGCCCGCACGGC-3') in combination with Gag Apal rev (5'-TTCCCTAGGGCCCTGCAA-3'). High fidelity Taq polymerase (Roche) was used for amplification to limit possible amplification errors.

**Molecular cloning and sequencing**

Second-round PCR products were cloned into the pGEM T Easy Vector System (Promega), transformed into competent DH5α *E. coli* (Invitrogen) and plated on LB agar using blue/white screening. White colonies were picked at random. The vector primers T7 and SP6 were used to amplify the cloned gag regions. PCR products were purified with ExoSap-IT (USB), and sequenced with the ABI prism BigDye Terminator sequencing kit (Perkin Elmer) on an ABI 3130 XL DNA sequencer according to the manufacturer’s protocol. Sequences were analyzed using CodonCode Aligner software. The nucleotide sequences of the gag region were translated and analyzed with the BioEdit program (BioEdit v 7.0.5).

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