Kaposi’s Sarcoma-Associated Herpesvirus Cytotoxic T Lymphocytes Recognize and Target Darwinian Positively Selected Autologous K1 Epitopes

Justin Stebbing,1,2 Dimitra Bourboulia,1 Margaret Johnson,3 Stephen Henderson,1 Ian Williams,4 Natalie Wilder,1 Mervyn Tyrer,3 Mike Youle,3 Nesrina Imami,2 Toru Kobu,2 Wolfgang Kuon,5 Joachim Sieper,3 Frances Gotch,2 and Chris Boshoff1*

Cancer Research U.K. Viral Oncology Group, The Wolfson Institute for Biomedical Research,1 Ian Charleson Centre,3 and Department of Sexually Transmitted Diseases,4 The Royal Free and University College Medical Schools, University College London, and Department of Immunology, Imperial College of Science, Technology and Medicine,2 London, United Kingdom, and Medical Department, Klinikum Benjamin Franklin, Freie Universität Berlin, Berlin, Germany5

Received 15 August 2002/Accepted 3 January 2003

Kaposi’s sarcoma-associated herpesvirus (KSHV) is the infectious cause of Kaposi’s sarcoma (KS) and certain lymphoproliferations particularly in the context of human immunodeficiency virus (HIV) type 1-induced immunosuppression. The introduction of effective therapies to treat HIV has led to a decline in the incidence of KS, suggesting that immune responses may play a role in controlling KSHV infection and pathogenesis. Cytotoxic-T-lymphocyte (CTL) activity against KSHV proteins has been demonstrated; however, the identification of KSHV CTL epitopes remains elusive and problematic. Although the herpesvirus genomic layout is generally conserved, KSHV encodes a unique hypervariable protein, K1, with intense biological selection pressure at specific amino acid sites. To investigate whether this variability is partly driven by cellular immunity, we designed K1 peptides that match only the unique viral sequence for every individual studied here (autologous peptides). We identified functional CTL epitopes within K1’s most variable areas, and we show that a given individual responds only to autologous peptides and not to peptides from other individuals. Furthermore, these epitopes are highly conserved sequences within KSHV isolates from a specific strain but are not conserved between different strains. We conclude that CTL recognition contributes to K1, and therefore to KSHV, evolution.

Cytotoxic-T-lymphocyte (CTL) responses against viral infections are often limited to reactivity against a few immunodominant epitopes, the identity of these being strongly influenced by the major histocompatibility complex (MHC) class I alleles of the host (17, 35). The majority of these epitopes have been identified in conserved sites, and this may be due in part to our inability to detect non-cross-reactive epitopes in variable domains (30). The importance of CTLs in controlling viral replication is supported by models of CD8-depleted animals with AIDS in which decreased numbers of CTLs result in high viral loads (28) and by the frequent selection of human immunodeficiency virus (HIV) or simian immunodeficiency virus mutants in vivo that are no longer recognized by CTLs and therefore escape immune surveillance (5, 24). Kaposi’s sarcoma-associated herpesvirus (KSHV) can establish chronic infections, as can other herpesviruses, such as Epstein-Barr virus (EBV), varicella-zoster virus, and cytomegalovirus (26). CTLs against KSHV proteins in HIV-1-positive (22) and HIV-1-negative (32) individuals have been demonstrated; however, there are only four MHC class I-restricted epitopes in KSHV that have been identified thus far (1, 20, 31, 33).

The KSHV K1 open reading frame (ORF-K1) encodes a 46-kDa transmembrane glycoprotein that has been shown to induce foci of transformation in rat fibroblasts (14). enhance KSHV lytic replication (12), cooperate in NF-κB signaling in vitro (27) and in vivo (25), and potentially maintain latent infection by blocking B-cell activation and therefore the induction of lytic replication (13). ORF-K1, located at the extreme left-hand end of the KSHV genome, has positional homology with the gene encoding transforming protein LMP-1 of EBV. K1 is known to contain the two most variable regions (VR1 and VR2) across the entire viral genome, which are used to classify KSHV into four clades (A, B, C, and D) (6, 16, 36). Every viral isolate studied thus far is unique to an infected individual. Unlike the situation in retroviruses, where proteins targeted by the host immune system undergo CTL escape during infection, K1 mutations have not been detected within an infected individual over time (29, 36). There are various hypotheses explaining K1’s variability, but evidence for a mechanism that drives its variability has not yet been shown (3, 6, 16, 36). Here, we investigate whether an immunological mechanism is partly responsible for this unique variability.

Initially, we performed computational analyses of K1 variability at the amino acid or codon level using three different measures not previously applied. We investigated the diversity and substitution rates at each amino acid site and then established the ratio of nonsynonymous substitutions (amino acid altering) to synonymous substitutions (silent, noncoding) at each codon position. This analysis allowed us to test whether
CTL epitopes are specifically located within positively selected codon sites. 

Previously, Osman et al. used a recombinant modified An- 
kara vaccine expressing K1 (A clade) and identified the pres- 
ence of CTLs against the whole K1 protein (22). Here, we 
investigated the regions of K1 that elicit CTL responses, and 
for this, the patient’s own (autologous) K1 sequences were 
required. Overlapping peptides that corresponded to an indi-
 vidual’s exact K1 sequence were synthesized. The immuno-
genic epitopes of K1 were found to lie only within the highly 
positive selected region of VR1. We discuss the possibility of 
diversity in a DNA viral gene being driven by CTL recognition 
as opposed to CTL escape.

MATERIALS AND METHODS

**Subjects.** One hundred twenty HIV-1-positive individuals were identified by positive antibody tests (HIV-1 enzyme-linked immunosorbent assay and HIV-1 Western blotting). All patients were receiving highly active antiretroviral ther-
apy. DNA was extracted from peripheral blood mononuclear cells (PBMCs) for 
HLA class I typing and nested PCR for ORF-K1. ORK-K1 cells was amplifiable from 
20 patients, and with 10 of these, overlapping peptides were generated for each 
individual’s K1 sequence. The patients were selected on the basis of shared common 
HLA class I alleles and the availability of PBMCs for multiple immunological assays. 
All patients signed informed-consent agreements, and the study was approved by the 
University College and Royal Free Hospital Ethics committees.

**PCR and sequencing.** DNA was extracted from 5 × 10^8 PBMCs by using a 
QIAamp blood kit (Qiagen, Hilden, Germany). K1 gene fragments were amplified 
by nested PCRs with 50-μl mixtures of buffer containing 2 μl of template 
DNA, 4.0 mM (first round) or 1.5 mM (second round) MgCl2, 1.0 U of Expand 
polymerase (Roche, Mannheim, Germany), and 10 pmol of the following prim-
 ers: for the first round with K1 VR1, 5′-GTCTGCGACGCTATGTGC-3′ and 
5′-GTAACTCATGCTGACCAAGAC-3′; for the second round with VR1, 5′-CTG 
GCCGCCCTTGTTGAAAC-3′ and 5′-GACTGTGGTTGATGGCTGTGC-3′; for the 
first round with K1 VR2, 5′-GCAGTCGGCTGTAACCT-3′ and 5′-A 
CTGTTGGCTAGTATCCTCC-3′; and for the second round with VR2, 5′-G 
TATATGTTTTGGGCGCTTG-3′ and 5′-CCGTGGCAACATCTGTAG 
GG-3′. Alternative primers were also used if VR1 PCR was unsuccessful (in the 
first round, 5′-GGTCTGCGACGCTATGTGC-3′ and 5′-ACACAAGTGCATT 
GTGGTTG-3′, and in the second round, 5′-TCAGGCTTTGGACATCTC 
3′ and 5′-ACACAAGTGGGTTAAGAC-3′). The conserved area between VR1 
and VR2 from nucleotides 324 to 480 was not amplified; however, overlapping 
peptides were made for this area. PCR conditions were as follows: 95°C for 30 s, 52 to 58°C for 45 s, and 72°C for 1 min; and 72°C for 
10 min. PCR products were purified with a QIAgen PCR product purification 
kit prior to sequencing with a CEQ dye terminator cycle sequencing kit (Beck-
 man Coulter, Fullerton, Calif.).

HLA class I and II typing were performed by the Anthony Nolan Trust (Royal 
Free Hospital) by using amplification refractory mutation system-PCR with se-
quence-specific primers (2). Plasma HIV-1 viral loads were determined by the 
Bayer (Berkshire, United Kingdom) HIV-1 RNA 5.0 (branched DNA) assay or 
by PCR assay (Cobas Amplicor HIV-1 Monitor test version 1.5; Roche Diag-
nostics, Sussex, United Kingdom) with a lower level of detection of fewer than 
50 HIV-1 RNA copies/ml.

**IFN-γ ELISPOT assay, CD4 and CD8 depletion, and flow cytometry.** PBMCs 
were separated from heparinized whole blood by standard density gradient 
centrifugation on Ficoll-Hypaque (Pharmacia, St. Albans, United Kingdom), and 
gamma interferon (IFN-γ) release ELISPOT assays (MABTECH, Stockholm, 
Sweden) were carried out as previously described (7). In depletion experiments, 
assays were carried out with fresh PBMCs after depletion of T cells by using CD4 
or CD8 MACS-positive selection beads (Mitenyi Biotec, Auburn, Calif.). Flow 
cytometry, following labeling with anti-CD4 fluorescein isothiocyanate and anti-
CD8 phycoerythrin antibodies (Mitenyi Biotec), was performed on a MoFlow 
(Fort Collins, Colo.) apparatus, which confirmed that cell subset depletions were 
>95% in all experiments. Results were considered positive if the number of spot-
forming cells per million PBMCs in peptide-stimulated wells was twofold 
higher than the number of spots per million PBMCs in control wells and if at least 
50 spots per million PBMCs were present. The peptide concentration between 
PBMCs was 5 μM. 

**Synthetic peptides.** Peptides were synthesized by conventional fluorenylme-
 thoxycarbonyl (f-moc) solid-phase chemistry and analyzed for purity (>95%) by 
re-verse-phase high-pressure liquid chromatography (Shimadzu, Chester, United 
Kingdom) and mass spectrometry (Thermo Finnigan AQA, San Jose, Calif.). In 
total, 130 K1 peptides were synthesized, as 50% of these were shared with more than 
one patient (15-mers overlapping by 5 or 10 nucleotides or 9-mers and 10-mers). 

**Chromium release assay.** Quantitation of CTL activity was determined by the 
chromium release assay as previously described (22). Briefly, patients’ PBMCs 
were cultured in 10% AB plasma-RPMI 1640 medium at 2 × 10^6 cells/ml and 
stimulated with a 50 μM concentration of the relevant peptide and 50 U of interleukin 2 (Roche Diagnostics Ltd.) per ml, which was added on days 0, 3, and 
day 6. Effector cells were assessed for specific killing of 5^1Cr-labeled EBV 
transformed autologous cell targets (positive selection of the effec-
tor-to-target ratios of 10:1, 20:1, 40:1, and 80:1 on day 10. Autologous B-cell lines 
and HLA-matched and -mismatched targets were tested with and without pep-
tide. A spontaneous release of ≤15% of maximum was considered acceptable. 

**B^27 binding assay.** To confirm that the B^27 peptide identified bound HLA-
B^27, we tested binding in an assembly assay with the TAP-defective RMA-S-
B^27 line (4, 9). HLA-B^27 molecules stabilized by peptides were monitored by 
direct staining with fluorescein isothiocyanate-coupled anti-HLA-B^27-spe-
cific monoclonal antibodies (HLA-ABC3; Serotec, Eching, Germany).

**Analysis of protein variability at the single-site level.** One hundred forty-five 
unique nucleotide sequences encoding all of ORF-K1 were collected from the 
National Center for Biotechnology Information (GenBank: accession nos. 10, 11, 19). Se-
quences that contained in-frame insertions (including the putative K1-D strain 
sequences thus far reported) were excluded from the present analysis, and sequences that clustered weakly within K1-A, -B, and -C strains were also excluded. The amino acid sequences deduced from the nucleotide sequences 
were aligned with each other by using CLUSTAL X, version 1.6. Alignment 
was successful for all 289 amino acid sites in 118 different K1 sequences, and 
no regions were excluded from analysis. Sequences were split into K1-A, -B, 
and -C strains by computer-based multialign and phylogenetic-tree programs from 
the National Institutes of Health website (http://bimas.dcrt.nih.gov/molbio 
/index.html), thus confirming previous reports (6, 16, 36). Programs to analyze 
changeability and diversity were a kind gift from Yumi Yamaguchi-Kabata (Na-
tional Institute of Genetics, Mishima, Japan) (34).

In order to analyze the number of amino acid substitutions (changeability), we 
calculated the number of amino acid changes that have arisen throughout the 
phylogeny. To further evaluate the level of amino acid variation in this popula-

tion, we defined the diversity of amino acids at a given position as the propor-
tion of pairs having different amino acids in two sequences randomly chosen from 
the sample (34). This value was estimated from the frequencies of different amino 
acids or gaps at each position. This quantity is analogous to the concepts of 
nucleotide diversity and heterozygosity or gene diversity in population genetics. 

The amount of diversity estimated by these methods is independent of 
the number of sequences if the sequences are randomly sampled. Because some 
sequences in K1-C sequences included gaps (within VR2), the diversity of gaps (D_g) was 
estimated as

\[ D_g = \frac{1}{x_i} - \frac{1}{x_i^2} \left( \sum_{i=1}^{x_i} \frac{1}{x_i^2} \right) \]

The diversity of amino acids was also estimated as

\[ D_a = \frac{1}{x_i} - \frac{1}{x_i^2} \left( \sum_{i=1}^{x_i} \frac{1}{x_i^2} - \sum_{i=1}^{x_i} \frac{1}{x_i^2} \right) \]

\( x_i \) represents the proportion of the i_th amino acid (aa) at a given position, and \( \frac{1}{x_i^2} \) represents the proportion of the gap at a given position.

**Elucidation of positively selected individual codon sites in K1.** An excess of 
non synonymous (dn) over synonymous (ds) codon mutations was used to identify 
sites of positive selection with the CODEML program from Yang’s Phylodetic 
Analysis Maximum Likelihood (PAML) software (http://abacus.gen.ucl.ac.uk /research.html) (21). K1-A, -B, and -C sequence data sets were analyzed separa-
tely to ascertain possible differences. PAML corrects the raw dn and ds values of 
each site (compared to those of a single consensus sequence) for the likely 
transition-to-transversion ratio, the empirical base frequency in the sequence 
groups, and the biased likelihood of each codon to undergo either synonymous 
or non synonymous change. After this correction, the dn-to-ds ratio is termed ω. 
Previsous analyses have observed changes by K1 region but not by site (6, 16). We 
first tested the null hypothesis by comparing two distinct models of the distribu-
tion of synonymous codon sites. The first was a neutral-selection model (at each 
peptide) (null hypothesis) that fixes dn/ds at either 0 or 1, and the second was an alternative 
model with a discrete distribution of ω values (eight classes), including positive

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selection of values ($\omega > 1$). The likelihood-score ratio of each model was compared to a chi-square distribution with 5 df (as the discrete model has six extra parameters). The alternative model was found to produce a significantly superior fit of the data for the K1-A, -B, and -C data sets. We obtained sequences for the MHC class I B locus (exons 2 and 3 of HLA-B subtypes), KSHV ORF-26 (8), HIV-1 nef (18), and HIV-1 env (subtype B) (34) from GenBank and calculated the dn/ds ratios with the above-described methods (Table 1). The sequences used were obtained by using accession numbers corresponding to different individuals, and all sequences were confirmed to be different.

## RESULTS

### Analysis of K1 variability by substitutions, diversity, and dn-to-ds ratios

We established a phylogenetic analysis and relationship among the three major divergent K1 strains (A, B, and C), as has been previously done with 63 K1 sequences (36); however, in this study we used 118 complete K1 DNA sequences obtained from an updated GenBank database (Fig. 1A). Following translation of the DNA sequences to proteins, the cumulative substitution rates and diversity of the encoded protein at each position in sequence alignments (Fig. 1B) were determined. This analysis showed that all three strains have a high rate of amino acid substitution and diversity across K1 (strain K1-B > K1-A > K1-C for substitutions and diversity).

After separating K1 sequences into their respective strains, we created a map of positive selection and calculated the dn/ds ratio (or $\omega$) per codon (21) (Fig. 1C). We observed that the
FIG. 1. (A) Predicted phylogenetic relationships among the 118 complete KSHV ORF-K1 protein sequences (A strain in blue \(n = 45\); B strain in red \(n = 21\), and C strain in green \(n = 52\)). (B) Cumulative substitution rates and diversity across KSHV ORF-K1 determined with a radial phenogram constructed with alignment (CLUSTAL X, version 1.6) and treeview (Treeview, version 1.5) programs show that A and C strains are the most closely related. For substitutions, the gradient is offset by \(-1\), so a gradient of 0 is equivalent to no substitution. (C) \(d_n\)-to-\(d_s\) ratios (\(\omega\)) across the K1 gene for A, B, and C strains as measured per codon by PAML software. Shown are the results of an analysis of \(\omega\) values at each position across K1 in all three strains, and specific peaks indicate highly positively selective areas. (D) Cumulative \(\omega\) values among the three strains showing an overall increase in \(\omega\), i.e., the K1 protein is under positive selection throughout the whole sequence (\(\omega > 1\)). Previously postulated areas of VR1 and VR2 are indicated by light lines; darker dashes (codons 55 to 71) indicate our updated VR1 on the basis of analysis of 118 complete K1 sequences.
highest probabilities of positive selection in K1 are clustered primarily between codon positions 53 and 94 (VR1) (36), particularly between codons 55 and 71. Figure 1D shows the cumulative dn/ds ratios for all three strains. Overall, there are higher dn/ds ratios for A strains than for B and C strains. The analyses, performed for the first time at the single amino acid level, also show that B strains have more diffuse scattering of positive selection across K1 than A and C strains, where mutations occur specifically in VR1 and VR2 (Fig. 1C). K1-C strains had the highest numbers of substitutions, greatest diversity, and highest probabilities of positive selection but only for the VR2 region (amino acids 191 to 228).

Our comparison of the mean dn/ds ratios among ORF-K1, other viral genes, and human MHC class I-B sequences demonstrates that the biological selective pressure observed in K1 is high and comparable to that of the variable areas of other genes studied (Fig. 2). Complete HIV-1 env genes from subtype B strains (and the KSHV ORF-26 gene) were negatively selected. No individual codon sites in the KSHV ORF-26 gene had a dn/ds ratio of $>1$ (Fig. 2).

**Immunogenic epitope analysis by ELIspot.** We synthesized from 10 individuals autologous K1-overlapping peptides covering the whole protein and performed IFN-γ release ELIspot assays (Fig. 3A and B). Two out of 10 did not show any responses (1 of these 2 individuals had received recent systemic cytotoxic treatment for non-Hodgkin’s lymphoma). For the eight remaining patients, we observed responses against autologous viral peptide sequences and all responses were in peptides at sites in and around the most variable region of K1 VR1 (Fig. 3A). Furthermore, there were no responses when patients’ PBMCs were stimulated with nonautologous viral epitopes and all 10 patients were tested with pooled nonautologous peptides corresponding to the K1 sequence of other patients (Fig. 3B and C).

**Chromium release assay.** Confirmation of cell killing of targets presenting all four peptides was determined by using a standard chromium release assay (Fig. 3C). K1-specific CTL responses were greatest to the B*27-restricted nonamer HRQSIWITW, with a mean specific cytotoxicity of 43% ($\pm$5%) at an effector/target ratio of 80:1 (Fig. 3D). The mean specific cytotoxicities for the other three peptides at this effector/target ratio ranged from 17 to 34% (Fig. 3C). The correlation between ELIspot results and KSHV-specific cytotoxicity in the chromium release assay was 0.373 ($r$ value). Responses were greatest for effector-mediated killing of autologous B-cell targets followed by HLA-matched targets. Background counts were observed by using effectors against HLA-unmatched targets, with and without peptides (Fig. 3D).

**Eptope frequency among strains.** To investigate whether the four epitopes identified here were shared among the three strains, we aligned 118 K1 protein sequences from the GenBank database. It appears that all the epitopes are relatively conserved within each strain and are not shared with the other strains; e.g., the B*27-restricted epitope exists in over 90% of
FIG. 3. Epitopes that elicit CTL responses as demonstrated by IFN-γ release ELISPOT assays, with HLA class I restriction, were identified in eight HIV-1-infected patients on highly active antiretroviral therapy. (A) Patients’ K1 amino acid sequences from codons 50 to 101. Epitope sequences that elicit responses and HLA class I-restricted alleles (Cw3, B*27, B*51, and B*55) are shaded. The viral strain of each patient is also indicated. The same class II HLA types were not shared between individuals that responded to the same epitope, and CD8+ depletion eliminated responses. (B) ELISPOT data for 10 patients in whom autologous peptides were made. (C) Summary of ELISPOT and chromium release assays. The number of responding patients tested is shown in brackets with the number of spot-forming cells per 10⁶ PBMCs (ELISPOT) and the highest mean percentage of specific lysis (51Cr release) for each patient. E:T, effector-to-target ratio. (D) Representative Cr release assay demonstrating percentage of specific lysis with the B*27-restricted peptide HRQSIWITW (data for patient 4 are shown). Results of experiments with an autologous effector and target (top), an effector and target matched at B*27 (middle), and an unmatched effector and target (with and without the peptide) are shown.

<table>
<thead>
<tr>
<th>Patient</th>
<th>K1/VR1 PATIENT AMINO ACID SEQUENCE (amino acids 50 to 101)</th>
<th>HLA CLASS I TYPE</th>
<th>A</th>
<th>B</th>
<th>Cw</th>
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<tr>
<td>1 (A4)</td>
<td>WYCNTRLFLRTERTTLEFVTIPCNFTCQVEQSGHQS1WITWHAQFPLT.LIA</td>
<td>01.24 08.15 03.07</td>
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<tr>
<td>2 (A1)</td>
<td>WYCNNTRLFLRTERTTLEFVTIPCNFTCQVEQSGHQRSLWITWHAQFPLT.LCA</td>
<td>02.88 40 02.03</td>
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</tr>
<tr>
<td>3 (A)</td>
<td>WYCNNTRLFLRTERTTLEFVTIPCNFTCQVEQSGHQRSLWITWHAQFPLT.LCA</td>
<td>02.86 27.38 03.04</td>
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<tr>
<td>4 (A1)</td>
<td>WYCNNTRLFLRTERTTLEFVTIPCNFTCQVEQSGHQRSLWITWHAQFPLT.LCA</td>
<td>02 29.51 02.14</td>
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<tr>
<td>5 (C3)</td>
<td>WYCNTRLELLQPTLTVSLIKNFQCVGFQHRS1WITWHAQFPLT.LCA</td>
<td>01.31 51.55 06.16</td>
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<tr>
<td>6 (C3)</td>
<td>WYCNTRLELLQPTLTVSLIKNFQCVGFQHRS1WITWHAQFPLT.LCA</td>
<td>06 51 15</td>
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<td></td>
<td></td>
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<tr>
<td>7 (A1)</td>
<td>WYCNNTRLFLRTERTTLEFVTIPCNFTCQVEQSGHQRSLWITWHAQFPLT.LCA</td>
<td>11.23 44.55 03.04</td>
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<tr>
<td>8 (C3)</td>
<td>WYCNTRLELLQPTLTVSLIKNFQCVGFQHRS1WITWHAQFPLT.LCA</td>
<td>11.32 44.56 03.05</td>
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<tr>
<th>Epitope sequence</th>
<th>Number of patients tested with this epitope</th>
<th>Number of patients with the exact epitope sequence</th>
<th>Number of patients that responded to this epitope</th>
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<tr>
<td>FRILERTLF</td>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>HRQSIWITW</td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>YPQVLTLL</td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>QPVLQTLLCA</td>
<td>10</td>
<td>2</td>
<td>2</td>
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<table>
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<tr>
<th>Epitope sequence</th>
<th>Number of SFCs/10⁶ PBMCs (ELISPOT) for each responding patient [Mean ± SEM]</th>
<th>Highest mean % specific lysis in chromium release assay [E:T of 80:1]</th>
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<tr>
<td>FRILERTLF</td>
<td>135 ± 5 (patient 1) 216 ± 38 (p2)</td>
<td>34%</td>
</tr>
<tr>
<td>HRQSIWITW</td>
<td>222 ± 4 (p3) 306 ± 3 (p4)</td>
<td>43%</td>
</tr>
<tr>
<td>YPQVLTLL</td>
<td>185 ± 25 (p5) 190 ± 10 (p6)</td>
<td>17%</td>
</tr>
<tr>
<td>QPVLQTLLCA</td>
<td>118 ± 10 (p7) 98 ± 8 (p8)</td>
<td>32%</td>
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</table>

**A**

**B**

**C**

**D**

**FIG. 3.** Epitopes that elicit CTL responses as demonstrated by IFN-γ release ELISPOT assays, with HLA class I restriction, were identified in eight HIV-1-infected patients on highly active antiretroviral therapy. (A) Patients’ K1 amino acid sequences from codons 50 to 101. Epitope sequences that elicit responses and HLA class I-restricted alleles (Cw3, B*27, B*51, and B*55) are shaded. The viral strain of each patient is also indicated. The same class II HLA types were not shared between individuals that responded to the same epitope, and CD8+ depletion eliminated responses. (B) ELISPOT data for 10 patients in whom autologous peptides were made. (C) Summary of ELISPOT and chromium release assays. The number of responding patients tested is shown in brackets with the number of spot-forming cells per 10⁶ PBMCs (ELISPOT) and the highest mean percentage of specific lysis (51Cr release) for each patient. E:T, effector-to-target ratio. (D) Representative Cr release assay demonstrating percentage of specific lysis with the B*27-restricted peptide HRQSIWITW (data for patient 4 are shown). Results of experiments with an autologous effector and target (top), an effector and target matched at B*27 (middle), and an unmatched effector and target (with and without the peptide) are shown.
the sequences from the A strain but is not present in either B or C strains (Table 2). However, two amino acid changes within this epitope resulted in a 9-mer, highly conserved in clade C strain viruses (90.4%), which did not induce any CTL responses in two tested patients carrying the A strain.

B*27 binding assay. Transfected RMA-S-B*27 cells incubated at 37°C without peptide showed no HLA-B*27 expression but did so at 27°C. However, when the temperature was raised from 27 to 37°C, we found that after 8 h, the cell line no longer showed empty heavy-chain expression (i.e., it was thermostable). In contrast, when the RMA-S-B*27 line was incubated with exogenous peptide at 27°C, stable expression of heavy chains was maintained at 37°C (i.e., it was thermostable). Incubation with an unrelated control peptide with HLA-B*27 revealed no stabilization of HLA-B*27 on the surface, whereas the B*27 nonamer (sequence HRQSIWITWH) and decamer (sequence HRQSIWITWHL), both of which induce IFN-γ release in ELIspot assays with samples from B*27-positive patients infected with KSHV strains that contain this epitope, showed stabilization of the molecule (Fig. 4).

We identified HLA class I restriction within B*27, B*51, B*55, and CW*3 alleles (Fig. 3), as responses to these epitopes were observed in more than one patient possessing that allele and were not observed in mismatched cases (Fig. 3A). ELIspot, chromium release, and B*27 binding assays confirmed that these epitopes are immunogenic. We also used an HLA-binding, motif-based epitope prediction algorithm which indicated high scoring of these epitopes (23). In CD8 depletion experiments, all positive responses were eliminated (data not shown), CD8 depletions were confirmed by fluorescence-activated cell sorter analysis (data not shown).

**DISCUSSION**

In this study, we explored the relationship between positive selection in KSHV K1 and sites of CTL recognition. It was previously shown that K1 has two hypervariable regions (3, 6, 16, 36). Here, we mapped the positive selection and variability of K1 at the codon rather than averaging within a window (16) (Fig. 1B and C). This enabled us to compare precisely sites of positive selection by exhaustive CTL epitope mapping. Given this level of analysis, we suggest a redefinition of VR1 as extending from codons 55 to 71 rather than the previously described extension from codons 53 to 94 (Fig. 1D). The positive selection within VR1 is comparable to, or possibly higher than, a number of well-known positively selected immunogenic regions in other genes studied (Fig. 2).

We next tested whether this variability is partly driven by cellular immunity. To identify class I-restricted epitopes within K1, we required autologous peptides corresponding exactly to the patient's own viral sequence and individuals of an appropriate HLA class I type that were infected with a specific viral strain. We tested the entire K1 protein of 10 individuals by using autologous overlapping peptides, yet we could find no CTL responses outside the VR1 region (Fig. 3A). We show that CTL responses occurred in 8 out of 10 patients tested and that the responses were specifically against positively selected sites within KSHV as identified by ELIspot, chromium release, and binding assays (Fig. 1C, 3, and 4). Epitopes located at equivalent K1 regions in other KSHV strains did not show binding (or antagonism in ELIspot assays) when the peptides were tested in patients with a different K1 strain and HLA class I allele (Fig. 3B). This result provides evidence to suggest that interstrain antigenic diversity between K1 subtypes exists and may promote viral-host “coevolution.”

We also aligned 118 K1 protein sequences from the GenBank database to investigate whether the four epitopes identified here are conserved among the three strains. We observed that all the epitopes were relatively conserved within each strain and were not shared with the other strains (Table 2). This finding suggests intraspecies conservation of epitope antigenicity and might partly explain why each strain predominates in a specific population. KSHV A strain is found in the United States and in northwestern Europe, C is found mainly in Mediterranean and Middle Eastern populations, and B dominates in sub-Saharan Africa (6, 16, 36).

We and others have thus far been able to identify only four other epitopes that are all HLA-class I A*02 restricted in the complete KSHV genome. This was done by using peptides that overlap a number of conserved proteins or by using computer programs to predict HLA-binding epitopes (1, 20, 31, 33). We also evaluated K1 using a binding prediction algorithm program to identify peptides that could bind HLA-A*02 alleles, but the scores were low (23). Previous attempts to identify HLA-binding motifs by computational analysis with consensus K1 sequences also failed to predict CTL responses (1). Further studies will show why uncommon alleles are preferentially selected for CTL responses against K1 and whether this is a result of a viral immune evasion mechanism towards common HLA class I molecules.

To confirm that K1 does not change over time in an individual, we sequenced K1 in DNA samples from various time points (up to 5 years apart) in 16 patients, and no change in their specific K1 nucleotide sequences was observed (data not shown). Furthermore, K1 does not appear to change in different tumor sites within an individual. For example, in one patient with KSHV-infected primary effusion lymphoma, Castleman's disease, and KS, identical K1 sequences were reported in all three tumors (29).

The genetic changes occurring in K1 are probably a result of

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**TABLE 2. Intrastrain conservation of identified epitopes**

<table>
<thead>
<tr>
<th>Patients</th>
<th>HLA Class I allele restriction</th>
<th>Epitopes tested</th>
<th>Epitope frequency per viral strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2</td>
<td>B*2702</td>
<td>HRQSIWITWH</td>
<td>93.3</td>
</tr>
<tr>
<td>3 and 4</td>
<td>B*51</td>
<td>YPQVIQTLGA</td>
<td>91.1</td>
</tr>
<tr>
<td>5 and 6</td>
<td>B*55</td>
<td>QVQVIQTLG</td>
<td>64.4</td>
</tr>
<tr>
<td>7 and 8</td>
<td>B*80</td>
<td>QVQVIQTLGCA</td>
<td>91.1</td>
</tr>
</tbody>
</table>

*Epitope occurrence within the three viral strains as deduced from the analysis of the 118 complete K1 protein sequences. 9-mer peptides correspond to the epitopes identified in this study, and the HLA class I restriction is indicated. Amino acids in bold represent changes in alternative peptide sequences in the patients indicated as determined by ELIspot assay. Peptides corresponding to KSHV strain A are in blue; alternative peptides corresponding to strain C are in green.*
mutations that accumulated over epochs of viral evolution. CTL recognition is clearly associated with K1 variability, but further studies will show whether other immunological factors like humoral responses also contribute to this. Herpesviruses have evolved through cospeciation with their hosts. Evasion of all host immune control mechanisms will lead to overwhelming viral infection with subsequent death of the host and, therefore, of the virus. For viruses to persist as latent infections without causing harm, a pathogen-host equilibrium must be established (35). K1 is an early-lytic-phase antigen; given the cell-mediated responses shown here, it is possible that continual immune surveillance, even in the context of HIV-1 infection, serves to recognize cells reactivating from latency, which limits viral dissemination, thus preserving the host and therefore the virus. Our data show a clustering of functional CTL epitopes in the most positively selected region of the most variable gene of KSHV. We consider it possible that the pressure causing this selection could be a mechanism associated with virus-host natural adaptation and, therefore, survival.

ACKNOWLEDGMENTS

We thank Tony Drinkwater, Zoe Cuthbertson, Pat Byrne, and Diana Aldam for recruiting study subjects and Mary Collins, Robin Weiss, and Paul Klenerman for discussion. We thank Richard Campbell for help with peptide synthesis. We also thank Anne-Margaret Little and Tawna Pitts for HLA typing and fluorescence-activated cell sorter analysis. We are grateful to Yumi Yamaguchi-Kabata and Zhong Yang for computational programs and to the patients who participated in this study.

J.S. and N.W. are Medical Research Council Ph.D. students, and the work was also supported by Cancer Research U.K. and The Leukemia Research Fund.

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