HLA-A11 Epitope Loss Isolates of Epstein-Barr Virus from a Highly A11$^+$ Population

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Cytotoxic T lymphocytes (CTLs) control viral infections by recognizing viral peptides presented by major histocompatibility complex (MHC) class I molecules. Human leukocyte antigen (HLA)–A11–restricted CTLs that recognize peptide residues 416 to 424 of the Epstein-Barr virus (EBV) nuclear antigen–4 frequently dominate EBV-induced responses in A11$^+$ Caucasian donors. This epitope is conserved in type A EBV strains from Caucasians and central African populations, where A11 is relatively infrequent. However, strains from highly A11$^+$ populations in New Guinea carry a lysine-to-threonine mutation at residue 424 that abrogates CTL recognition and binding of the peptide to nascent A11 molecules. The results suggest that evolution of a widespread and genetically stable virus such as EBV is influenced by pressure from MHC-restricted CTL responses.

EBV-specific CTL memory can be demonstrated in virtually all healthy virus-carrying individuals (*1*, *2*). The identity of the EBV target antigens and the influence of host HLA class I type on antigen choice have been clarified (*3*–*5*). In Caucasian populations, where type A EBV strains are prevalent, the total virus-induced CTL response in HLA-A11–positive donors is frequently dominated by A11-restricted CTLs directed against the single nonamer epitope IVTDFSVIK (6), which represents residues 416 to 424 of the type A EBV nuclear antigen–4 (EBNA4, also known as EBNA3B) (*7*).

We sought to determine whether the above epitope was conserved among EBV strains (types A and B) of different geographical origins. Type B strains are relatively rare among healthy virus carriers in the West but are almost as prevalent as type A strains in parts of equatorial Africa (*8*). As targets for CTL assays, a panel of lymphoblastoid cell lines (LCLs) was produced either by in vitro transformation of A11$^+$ indicator B cells with different EBV isolates or (when the original virus donors were themselves A11$^+$) by spontaneous outgrowth of their cultured B cells. Because different EBV isolates encode EBNAs of slightly different sizes, the identity of the resident virus in each of these lines could be checked by immunoblotting of protein extracts with appropriate EBNA-specific reagents (Fig. 1A); such assays also confirmed expression of the EBNA4 protein in target cells.

These target cells were then tested for EBV-specific CTL recognition with polyclonal CTL preparations from five donors, each displaying a dominant EBNA4-specific A11-restricted component, as well as several CTL clones with proven specificity for the 416–424 peptide–A11 complex. Recognition was clearly type-specific in that B95.8 and most other type A virus–transformed lines were lysed, whereas type B transformants were not (Fig. 1B). However, some type A isolates (represented in Fig. 1B by WW1, AMB, and H17) were also not recognized. These differences were only apparent with EBNA4-specific, A11-restricted CTLs; the complete panel of target cells was killed by CTLs allo-specific against A11 (anti-A11) (Fig. 1B) and also by EBV-specific CTLs that were restricted through another HLA allele, A2.1 (9). Table 1 summarizes the geographical origin, type, and observed sensitivity to A11-restricted cytolysis of all EBV isolates assayed. The six type A EBV isolates that escaped recognition were all derived from New Guinea and in all but one case from coastal populations where HLA-A11 is unusually prevalent, with frequencies of 25 to >50% (*10*–*12*); four of these six New Guinea virus strains (WW1, L2, L12, and L24) were isolated from A11$^+$ donors.

To determine the genetic basis of this altered susceptibility, we sequenced the EBNA4 epitope in these six New Guinea isolates (*13*). All showed the same single-point mutation relative to the B95.8 prototype, namely an A → C mutation that produced a Lys → Thr (K → T) change in residue 424 of EBNA4 at position 9 of the CTL epitope. In contrast, four type A isolates from other countries [WIL, BL74, BL72, and MWI, which all retain CTL susceptibility (Table 1)] were identical to B95.8 across the region of interest. Three type B EBV isolates (WAN, WW2, and BL16) were indistinguishable from the AG876 prototype and, in accordance with the published sequence (*14*), showed changes relative to B95.8 at positions 420 (Phe → Leu) and 422 (Val → Ile), which

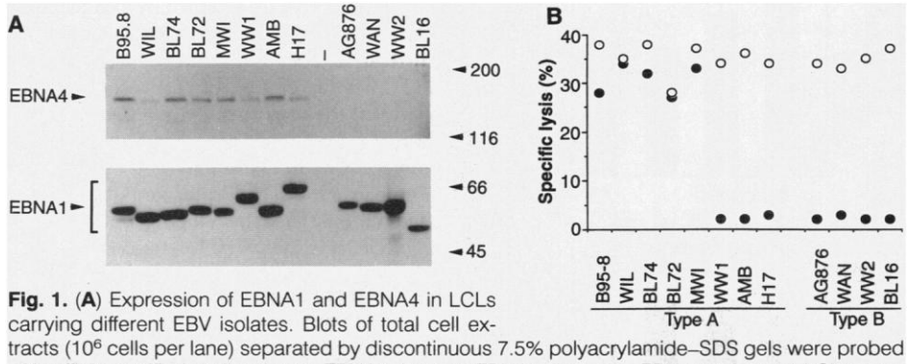

Fig. 1. (**A**) Expression of EBNA1 and EBNA4 in LCLs carrying different EBV isolates. Blots of total cell extracts (10^6 cells per lane) separated by discontinuous 7.5% polyacrylamide–SDS gels were probed with affinity-purified antibodies to EBNA4 (top) or EBNA1 (bottom). EBV-transformed LCLs were obtained by infection of freshly separated lymphocytes from the HLA A11$^+$ donor CR (HLA-A2,A11 and HLA-B7,B8) with the virus isolates listed in Table 1 with standard techniques (*28*). EBV antigen expression was detected by immunoblotting (*29*) with polyclonal human serum containing high titers of antibodies to all EBNAs or affinity-purified antibodies to EBNA1 [binding to the Gly-Ala repeat (*28*)] and EBNA4 [binding to a β-galactosidase–EBNA4 fusion protein (*30*)]. Antibody to EBNA4 is type-specific and therefore only detects the protein in type A virus–transformed lines. Molecular size standards are shown at right (in kilodaltons). (**B**) Sensitivity of LCLs carrying different EBV isolates to lysis by HLA-A11 allo-specific (○) and HLA-A11–restricted EBNA4-specific (●) CTLs. HLA-A11–restricted EBNA4-specific CTL clones were obtained by stimulation of lymphocytes from the EBV seropositive donor BK (HLA-A2,A11 and HLA-B7,B35), with the autologous B95.8 virus–transformed LCL as described (*31*). HLA-A11 allo-specific CTL clones were generated by stimulation of lymphocytes from donor WP (A2,A3 and Bw38,Bw52) with the allogeneic LCL IE-B-1 (A3,A11 and B7,B35). Single-cell cloning was done by limiting dilution. The cytotoxic activity was assayed in standard 4-hour ^{51}Cr-release assays (*31*). One representative experiment out of four performed with each effector population is shown; results are expressed as the percentage of specific lysis at 10:1 effector:target ratios.

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affected residues 5 and 7 of the epitope. We then tested the ability of the standard type A (B95.8), the altered type A (WW1), and the standard type B (AG876) peptides to sensitize $A11^+$ phytohemagglutinin (PHA)–stimulated blasts to recognition by B95.8-induced A11-restricted CTL clones (Fig. 2). The cognate B95.8 peptide was effective at subpicomolar concentrations (half-maximal lysis at 5×10^{-14} M), whereas the WW1 and AG876 peptides were virtually inactive, mediating only weak lysis at concentrations 10^7 times higher.

Although the altered type A (WW1) peptide was antigenically distinct from its B95.8 counterpart, it too might be selectively presented by A11 molecules and serve as a CTL epitope in individuals infected with WW1-like virus strains. We therefore compared the relevant B95.8, WW1, and AG876 nonamer peptides for their ability to bind to nascent HLA-A11 molecules. The cell line T2, which is defective in the supply of endogenous peptides to maturing HLA class I molecules and shows reduced surface HLA class I expression (*15*), was first transfected with an *oriP*-based expression vector carrying the HLA-A11 heavy chain gene (*16, 17*). A clone was obtained that expressed cytoplasmic A11 polypeptides, with little or no mature A11 detectable at the cell surface. Overnight exposure of this clone to a 10^{-5} M concentration of the B95.8 nonamer rescued cell surface expression of A11, whereas no such effect was observed with the WW1 nonamer (Fig. 3). This supports the view that the Lys → Thr substitution at residue 9 reduces the affinity of the peptide for the HLA-A11 groove. The AG876 peptide, altered in residues believed to interact with the T cell receptor rather than the HLA groove (*18*), did rescue A11 expression; it remains to be seen, however, whether this peptide actually represents a CTL epitope in individuals infected with type B EBV strains.

Further epidemiological studies of EBV

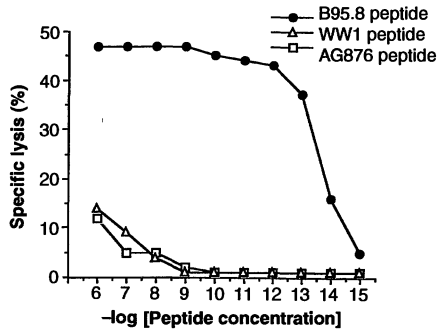

Fig. 2. Titration of synthetic peptides on PHA-stimulated blasts. Peptides synthesized by the Merrifield solid-phase method (*32*) were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10^{-2} M and further diluted in phosphate-buffered saline to obtain the indicated concentrations before the cytotoxicity assay. The peptide concentration of the DMSO stock solutions was determined by a Biuret assay (*33*). Twenty microliters of tenfold serial dilutions of peptide IVTDFSVIK, derived from the standard type A (B95.8) epitope sequence; IVTDFSVIT, derived from the mutant type A (WW1) sequence; and IVTDLSIIK, derived from the standard type B (AG876) sequence were added to 4×10^3 ^{51}Cr-labeled $HLA\text{-}A11^+$ PHA-stimulated blasts (in 20 μl of complete medium) in triplicate wells of 96 V-shaped well plates (*6*). The plates were incubated for 1 hour at 37°C before the addition of HLA-A11–restricted CTLs (BK cl.12) known to be reactive against peptide IVTDFSVIK. The percentage of specific lysis recorded at a 10:1 effector:target ratio is shown. One representative experiment out of four is shown, each involving a different clone from donor BK.

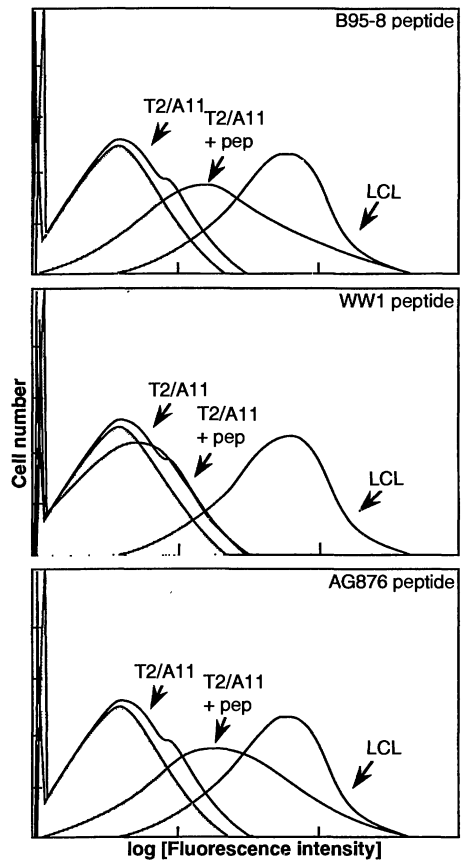

Fig. 3. Induction of surface HLA-A11 expression in A11-transfected T2 cells. An HLA-A11–carrying subline of the peptide transporter mutant T2 cell line (*15*) was obtained by transfection with a pHEBO-based HLA A11 expression vector (*16, 17*). Antibiotic-resistant clones were maintained in medium containing hygromycin-B (200 U/ml). Portions of 3×10^6 T2-A11 cells were cultured overnight at 37°C in 4 ml of complete medium or medium containing 10^{-5} M of the synthetic peptides. We detected surface HLA-A11 expression in untreated T2-A11 cells, in T2-A11 cells incubated with the indicated synthetic peptides (pep), and in a reference HLA-A11–positive EBV-transformed LCL by staining with the HLA-A11–specific monoclonal antibody AUF 5.13 (*34*). The figure shows the results of one representative experiment out of three. Shaded areas represent the fluorescence of cell incubated with the FITC conjugate clone.

Table 1. Sensitivity of LCLs carrying different EBV isolates to lysis by EBNA4-specific HLA-A11–restricted CTLs.

Virus	Origin	Virus type	CTL lysis	416–424 Sequence*	Reference
B95.8	Caucasian (United States)	A	+	WT A	(*22*)
WIL	Caucasian (Australia)	A	+	WT A	(*23*)
BK-sp	Caucasian (Sweden)	A	+	NT	†
SI-sp	Caucasian (Iceland)	A	+	NT	†
PB-sp	Caucasian (United Kingdom)	A	+	NT	(*16*)
BL74	Caucasian (France)	A	+	WT A	(*24*)
BL36	North African	A	+	NT	(*24*)
BL72	North African	A	+	WT A	(*24*)
KYU	Central African	A	+	NT	(*25*)
OBA	Central African	A	+	NT	(*25*)
MWI	Central African	A	+	NT	(*25*)
ODHI	Central African	A	+	NT	(*25*)
WW1	New Guinea coast	A	–	424 K → T	(*25*)
AMB	New Guinea coast	A	–	424 K → T	(*26*)
L2-sp	New Guinea coast	A	–	424 K → T	†
L12-sp	New Guinea coast	A	–	424 K → T	†
L24-sp	New Guinea coast	A	–	424 K → T	†
H17	New Guinea highlands	A	–	424 K → T	†
AG876	Central African	B	–	WT B	(*27*)
ELI	Central African	B	–	NT	(*25*)
WAN	Central African	B	–	WT B	(*25*)
WW2	New Guinea coast	B	–	WT B	(*26*)
BL16	Central African	B	–	WT B	(*24*)

*WT A is IVTDFSVIK; NT is nontested; 424 K → T is IVTDFSVIT; and WT B is IVTDLSIIK. †Refers to this report.

isolate prevalence need to be carried out before the biological significance of the present results can be fully assessed. However, all six virus isolates from the New Guinea populations that we examined had a mutation specifically affecting the dominant EBNA4 epitope for A11-restricted CTL responses. Such viruses may have enjoyed a biological advantage in host populations where the HLA-A11 gene frequency is unusually high. At the same time, the polyclonal nature of the EBV-induced CTL responses would ensure that A11 epitope–loss isolates of EBV remain subject to CTL recognition through other less immunogenic epitopes so that a stable, but more relaxed, virus-host balance could still be achieved.

Observations on the pathogens malaria and human immunodeficiency virus (HIV) have provided an indication that HLA-restricted CTL responses may influence the evolution of the host-pathogen relation (*19–21*). That influence may be seen in the case of malaria as determining the distribution of HLA alleles retained in the population at risk (*19, 20*) and in the case of HIV as driving virus evolution in the infected host (*21*). Our results suggest that by examining human populations with limited HLA polymorphism, one may be able to address the same issues in the context of viruses (such as EBV) that are widespread, genetically stable, and not life-threatening to the immunocompetent host.

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6. Single letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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13. A 578-bp DNA fragment comprising the coding sequence for the 416–424 EBNA4 epitope was amplified by the polymerase chain reaction with biotinylated oligonucleotide primers. The biotinylated products were separated with Dynabeads M280–streptavidin (Dynal, Oslo, Norway) and denatured with 0.1 M NaOH. The single-stranded template was annealed at 65°C for 5 min with 5 pmol of the sequencing primers in 10 mM tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and bovine serum albumin (0.1 mg/ml). The reactions were cooled at 37°C and incubated for 10 min. After further incubation for 30 min with 50 nM dATP, dCTP, dGTP, and fluore-dUTP (Boehringer Mannheim, Germany) and 5 U of T7 DNA polymerase (Pharmacia, Uppsala, Sweden), 6 mM dithiothreitol, and 45 mM NaCl were added. The reactions were then divided into four portions, mixed with one of the four dideoxynucleotides, and incubated for 5 min at 37°C. An equal volume of deionized formamide containing Blue dextran (5 mg/ml) was added, and the samples were loaded onto a 0.5-mm-thick 6% polyacrylamide–7 M urea gel and run on an A.L.F. DNA sequencer (Pharmacia, Uppsala, Sweden).
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Extracellular Access to the Na,K Pump: Pathway Similar to Ion Channel

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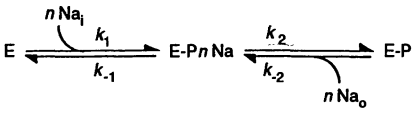
In each normal Na,K pump cycle, first three sodium and then two potassium ions are transported; in both cases, the ions become temporarily occluded in pump conformations that isolate them from internal and external solutions. A major charge movement occurs during sodium translocation and accompanies the deocclusion of sodium ions or their release to the cell exterior, or both. The nature of the charge movement was examined by measurement of the unidirectional sodium-22 efflux mediated by Na_i-Na_o exchange (Na_i and Na_o are internal and external sodium ions) in voltage-clamped, internally dialyzed squid giant axons in the absence of potassium; in this way the pump activity was restricted to the sodium-translocation pathway. Although electroneutral, the Na_i-Na_o exchange was nevertheless voltage-sensitive: increasingly negative potentials enhanced its rate along a saturating sigmoid curve. Such voltage dependence demonstrates that the release and rebinding of external sodium is the predominant charge-moving (hence, voltage-sensitive) step, suggesting that extracellular sodium ions must reach their binding sites deep in the pump molecule through a high-field access channel. This implies that part of the pump molecule is functionally analogous to an ion channel.

To probe the charge-moving steps in the Na-translocation pathway, we measured the voltage sensitivity of ^{22}Na efflux mediated by the electroneutral Na_i-Na_o exchange mode (*1*) of the pump. Pumped ^{22}Na efflux and current were determined in voltage-clamped, internally dialyzed squid giant axons as the components that are sensitive to dihydrodigitoxigenin (H_2DTG), a rapidly reversible specific inhibitor of the Na,K pump (*2, 3*). Pump-mediated ^{22}Na efflux was almost doubled by hyperpolarization from 0 to −60 mV (Fig. 1A). The accompanying pump currents were barely detectable at −60 mV and were still very small at 0 mV (Fig. 1B), confirming a predominance (>97% at −60 mV and ≥90% at 0 mV) of the electroneutral mode of Na_i-Na_o exchange.

Because the principal voltage-sensitive step is believed to occur late in the Na exit pathway (*4, 5*), the kinetic scheme below is sufficient to account for the influence of membrane potential on electroneutral Na_i-Na_o exchange:



(1)

where n is the apparent molecularity (*6*) of the interaction between Na_i or Na_o and each pump molecule (nonphosphorylated, E; phosphorylated, E-P). Voltage sensitivity arises because the first-order (*7*) rate constants k_i and k_{-i} (i = 1 or 2) may be exponential functions of membrane poten-