T Cell Responses and Virus Evolution: Loss of HLA A11-restricted CTL Epitopes in Epstein-Barr Virus Isolates from Highly A11-positive Populations by Selective Mutation of Anchor Residues

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Summary

Epstein-Barr virus (EBV) is a B lymphotropic herpesvirus of humans that elicits strong HLA class I-restricted cytotoxic T lymphocyte (CTL) responses. An influence of such responses on virus evolution was first suggested by our finding that EBV isolates from the highly HLA A11-positive Papua New Guinea (PNG) population carried a lys-thr mutation at residue 424 of the nuclear antigen EBV-encoded nuclear antigen (EBNA4) that destroyed the immunodominant target epitope for A11-restricted CTL recognition. Here we turn to a much larger population, Southern Chinese, where the A11 allele is again present in over 50% of the individuals. Each of 23 EBV isolates analyzed from this population were also mutated in the EBNA4 416-424 epitope, the mutations selectively involving one of the two anchor residues in positions 2 (417 val-leu) or 9 (424 lys-asp, -arg or -thr) that are critical for A11-peptide interaction. The majority of the Chinese isolates and all 10 PNG isolates also carried mutations affecting positions 1 and 2 of the next most immunodominant A11-restricted epitope, EBNA4 residues 399–408. These changes clearly affected antigenicity since A11-positive lymphoblastoid cell lines (LCLs) carrying these mutant EBV strains were not recognized by A11-restricted CTLs raised against the prototype B95.8 virus. Furthermore, Chinese donors naturally infected with these mutant viruses did not mount detectable A11-restricted CTL responses on in vitro stimulation with autologous LCL cells carrying either the B95.8 or their endogenous EBV strain. In two different highly A11-positive populations, therefore, immune pressure appears to have selected for resident EBV strains lacking immunodominant A11-restricted CTL epitopes.

CTLs appear to play an important role in the control of virus infections (1, 2). Such virus-induced CTLs recognize short peptides, typically 8–11 amino acids in length, that are derived by proteolytic degradation of viral proteins and that are selected for presentation at the surface of infected cells by complexing with nascent MHC class I molecules (3). Despite the antigenic complexity of most viruses, the CTL response to viral infection is in many instances dominated by reactivities directed against a limited number of immunodominant peptide epitopes, the identity of these epitopes being strongly influenced by the particular MHC class I alleles of the host (4, 5).

The possibility that viruses might escape CTL surveillance in vivo through mutations in an immunodominant CTL epitope was first formally demonstrated in studies of lymphocyte choriomeningitis virus (LCMV) infection in a T cell receptor transgenic mouse model (6). Later, an apparently similar T cell driven selection of escape mutants was documented in man during the course of natural infection with HIV (7). Thus, in special circumstances involving highly genetically unstable viruses, prospective studies on individual carriers can reveal viral adaptation to the prevailing CTL response. It remains to be seen, however, what influence the CTL system has had over the evolution of those viruses that are genetically stable and that have a long history of coexistence with their host species.

Here we examine one such agent, EBV, a lymphotropic herpesvirus with growth transforming ability, which is widespread in human populations and which elicits strong CTL responses. Studies on healthy virus carriers have shown that EBV-specific CTL memory is predominantly HLA class I-restricted and directed against the limited number of viral pro-

teins that are constitutively expressed in virus-transformed lymphoblastoid cell lines (LCLs)[1] (8–10). Using recombinant vaccinia viral vectors and synthetic peptides in CTL sensitization assays, a number of immunodominant CTL epitopes have now been identified within these EBV products, each presented in the context of a specific HLA class I molecule (9, 11–15). In particular, we have shown that the HLA A11 allele, which mediates unusually strong EBV-specific CTL responses in Caucasian donors, focuses reactivity on a small number of epitopes in the EBV-encoded nuclear antigen (EBNA)4 (13). During the course of the above work we identified natural EBV isolates that were not recognized by A11-restricted effectors; when compared with the B95.8 prototype strain, all showed a lys-thr change at position 9 of the most immunodominant CTL epitope, EBNA4 residues 416–424, which abrogated binding of the peptide to A11.

It is interesting to note that these "epitope-loss variants" of EBV were all derived from a Papua New Guinea (PNG) population in which the A11 allele itself is unusually prevalent. This raised the possibility that viral strains lacking the major A11-restricted CTL epitope had enjoyed a selective advantage in this particular population due to their failure to elicit strong CTL responses. It has to be remembered, however, that PNG populations have an unusually long history of isolation and are probably derived from small founder groups; the prevalence of epitope-loss EBV variants may therefore be a coincidence arising from founder effects rather than the specific result of T cell–mediated selection. Furthermore, such viruses, though lacking the immunodominant epitope of the B95.8 strain, might nevertheless be capable of inducing strong A11-restricted CTL responses via other immunodominant epitopes peculiar to the PNG isolates. To address these issues further, we have turned to a much larger population group, Southern Chinese, which also shows a high frequency of the HLA A11 allele and from which individuals are more readily available for analysis of both their resident EBV isolates and their virus-specific CTL memory.

Materials and Methods

EBV Isolates and Cell Lines. A total of 63 type A EBV isolates of different geographic origin were included in this study. 29 isolates (9 Caucasian, 5 African, 7 Chinese, and 8 PNG) were derived from EBV carrying LCLs established by spontaneous outgrowth from lymphocytes of normal EBV seropositive individuals cultured in the presence of 0.1 μg/ml cyclosporin A (16). 17 isolates (6 Caucasian, 9 African, and 2 PNG) were obtained from previously established tumor cell lines derived from EBV carrying Burkitt's lymphomas and 17 isolates (1 African and 16 Chinese) were directly sequenced from EBV carrying nasopharyngeal carcinoma biopsies.

B95.8 EBV transformed LCLs were obtained by infection of lymphocytes from HLA class I typed donors with spent culture supernatants of the virus producer B95.8 line (17). All cell lines were maintained in RPMI 1640 supplemented with 100 mg/ml streptomycin 100 IU/ml penicillin and 10% FCS (complete medium).

DNA Sequencing by PCR. Appropriate oligonucleotide primers were chosen flanking the DNA region coding for the 399–408 and 416–424 EBNA4 epitopes. One of the primers was biotinylated at the 5′ end. PCR-amplified biotinylated material was separated by incubation with Dynabeads M280-streptavidin (Dynal, Oslo, Norway). The immobilized double stranded DNA was denatured with 0.1 M NaOH at room temperature for 6 min. The biotinylated template DNA was annealed at 65°C for 5 min with 5 pmole of the sequencing primers 5′ATAATTGTTGAGGATGACG3′; 5′CTCTGCTCCATGACTTCA3′ in 14 μl buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and 0.1 mg/ml BSA. After further incubation for 30 min with 50 mM dATP, dCTP, dGTP, fluore-dUTP (Boehringer Mannheim, Mannheim, Germany) and 3 U T7 DNA polymerase (Pharmacia LKB, Uppsala, Sweden), 6 mM dithiothreitol, and 45 mM NaCl were added. The reactions were divided in 4 aliquots, mixed with one of the 4 dideoxinucleotides, and incubated for 5 min at 37°C. After addition of an equal volume of deionized formamide containing 5 mg/ml of blue dextran, the samples were loaded onto a 0.5-mm thick 6% polyacrylamide gel with 7 M urea and run on an A.L.F. DNA sequencer (Pharmacia LKB). The same protocol was used for sequencing of the nonbiotinylated strands recovered in the supernates of the NaOH denaturation step after neutralization with HCl.

Synthetic Peptides. Peptides, synthesized by the Merrifield solid phase method (18), were purchased from Alta Bioscience (The University of Birmingham, School of Biochemistry, Birmingham, UK). The peptides were dissolved in DMSO at the concentration of 10^{-2} M and further diluted in PBS to obtain the indicated concentrations before the cytotoxicity assays. The protein concentration of the DMSO stock solutions was determined by a Biuret assay (19).

Generation of CTL Cultures and Clones. EBV-specific CTLs were obtained by autologous B95.8 LCL stimulation (20) of lymphocytes from 12 EBV seropositive Caucasian donors: BK (HLA A2,11 B7,35), EA (HLA A10,11 B35,51), SI (HLA A2,11 B7,27.03), JAC (A1,11 B49,55), KS (HLA A2,11 B35,40), SW (A11,24 B7,35), DT (A2,11 B44,55), CMC (A2,11 B8,44), SG (A3,11 B35,40), MO (HLA A3,11 B35,40), PB (HLA A1,11 B22,37), and KK (HLA A11,28 B14,40) and 3 EBV seropositive Chinese donors: QYY (HLA A2,11 B13.38), DH (HLA A2,11 B27.04,40), and QJZ (HLA A11, B13,51). After two or three consecutive restimulations, the cultures were expanded in complete medium supplemented with 10 U/ml recombinant IL-2 and 30% (vol/vol) culture supernatant from the gibbon lymphoma line MLA144 (21). Single cell cloning was done by limiting dilution in 96-well plates in 200 μl medium containing 30% MLA144 culture supernatant, 10 U/ml human recombinant IL-2, and 10⁵ irradiated (3,000 rad) allogeneic PHA-activated PBLs as feeder (20). Growing cultures were transferred into 48-well plates and were fed twice a week by replacing half of the medium. HLA A11–restricted EBV-specific CTL clones were expanded into 24-plates and restimulated weekly with irradiated autologous LCLs in IL-2 containing medium. The EBV specificity and HLA class I restriction of the CTL preparations was investigated by testing their cytotoxic activity against a panel of EBV positive and negative targets including the autologous LCLs, allogeneic LCLs matched through single HLA class I alleles, at least two cell lines for each allele, PHA activated blasts, HLA mismatched LCLs, and the prototype NK sensitive target K562.

Cytotoxicity Assay. The cytotoxic activity was assayed in standard 5-h ⁵¹Cr release assays (20). The targets were labeled with Na⁵¹CrO₄ (0.1 μCi/10⁶ cells) for 1 h at 37°C. The cytotoxicity

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[1] *Abbreviations used in this paper:* EBNA, EBV-encoded nuclear antigen; LCL, lymphoblastoid cell line; PNG, Papua New Guinea.

tests were routinely run at 10:1, 3:1, and 1:1 effector/target ratios in triplicate. To test the ability of synthetic EBNA4 peptides to sensitize the targets to CTL lysis, 20 μl of each peptide were added to 4 × 10³ ⁵¹Cr-labeled targets (in 20 μl complete medium) in triplicate wells of 96 V-shaped well plates. The plates were incubated for 1 h at 37°C before addition of CTLs (4 × 10⁴/well in 100 μl). Peptide toxicities were checked in each assay and were always ≤3%.

Results

Epitope Sequence in EBV Isolates of Different Geographic Regions. We have previously shown that five epitope regions of the B95.8 EBNA4 protein are capable of inducing A11-restricted CTL responses (13). These regions differ considerably in immunogenicity and the immunodominant epitope has been defined as the 9-mer peptide IVTDFSVIK, EBNA4 residues 416–424. CTLs against this epitope, are present as the major component in polyclonal effector populations from A11-positive Caucasian donors; this is illustrated by results obtained using CTLs from donor BK (Fig. 1 *A*) and from the derived clone BK cl.112 (Fig. 1 *B*) which was representative of most clones from the polyclonal population. The second most common A11-restricted component, detectable in polyclonal preparations from most but not all A11 positive donors, was originally mapped to EBNA4 residues 396–410. We have recently identified the cognate peptide in this region as the 10-mer AVFDRKSDAK, representing EBNA4 residues 399–408 (Levitsky, V., unpublished results). Results illustrating
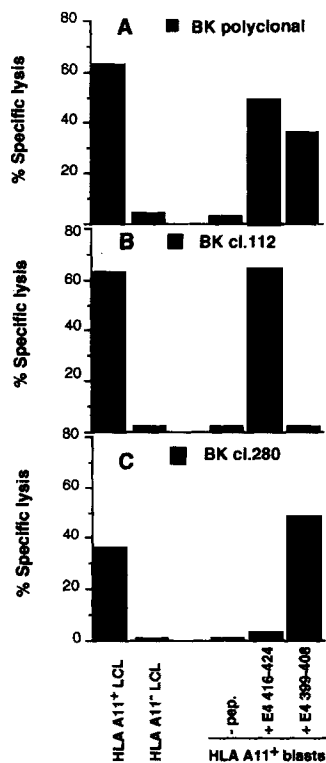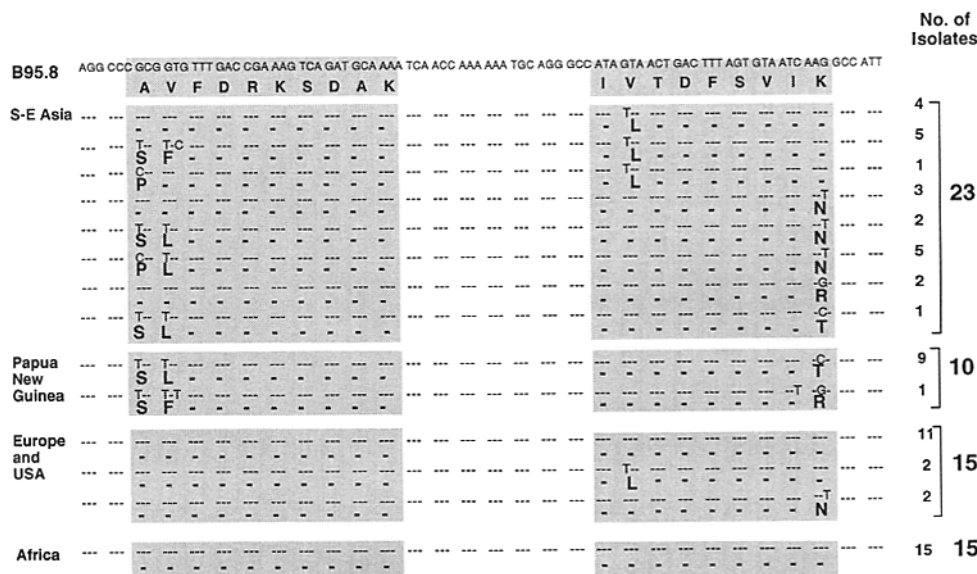

Figure 1. EBNA4 peptides 416–424 and 399–408 are the cognate epitopes of HLA A11–restricted EBV-specific CTLs. HLA A11 positive PHA blasts (donor QJZ) were preincubated for 1 h with a 10⁻⁷ M concentration of the synthetic peptides EBNA4 416–424 and 399–408 and then tested for lysis by a polyclonal CTL culture from donor BK, previously shown to contain a predominant A11-restricted component, and by the CTL clones BK cl.112 and BK cl.280 derived from the polyclonal culture by cloning in limiting dilution. The percent specific lysis recorded at a 10:1 effector/target ratio is shown. One representative experiment out of four is shown.

recognition of the cognate 399–408 epitope are shown in Fig. 1 *A* for the BK polyclonal population and Fig. 1 *C* for a representative clone BK cl.280. Sequencing of EBV isolates across the relevant region of the EBNA4 gene could therefore be undertaken with precise knowledge of the positions of these two adjacent epitopes.

To test the generality of our earlier observation on EBV isolates from the highly A11-positive PNG population, we took advantage of the fact that the A11 allele is also unusually prevalent in other South East Asian populations (22, 23). In particular, the Southern Chinese represent a large population group in which more >50% of the individuals are A11-positive (23). Furthermore, a number of Southern Chinese donors (several of whom proved to be A11-positive) were available to us for virus isolation and CTL generation. Spontaneous LCLs carrying the individual's resident EBV strain were established from seven such Chinese donors and the relevant region of the EBNA4 gene was then PCR amplified and sequenced; work was also extended to include 16 EBV isolates from Chinese nasopharyngeal carcinoma biopsies, an enlarged panel of 10 PNG virus isolates and, for comparison, 15 Caucasian and 15 African isolates.

Fig. 2 shows the different sequence patterns observed, with mutations being identified relative to the B95.8 prototype sequence. All 23 Chinese isolates examined showed changes in the immunodominant 416–424 epitope region; furthermore, though a number of different mutations were observed, these all selectively involved one or the other of the two anchor positions, amino acids 2 and 9 of the peptide epitope, which are known to be important determinants for A11 binding (24). In almost half of the cases the val in position 2 was substituted by a leu (L2 mutation), while in the remaining cases the lys in position 9 was substituted by asp or more rarely by arg or thr (N9, R9, T9 mutations, respectively). In addition, 13 of the 23 Chinese isolates also showed mutations that altered positions 1 and 2 of the 399–408 epitope from ala-val to either ser-leu, pro-leu, or ser-phe (S1L2, P1L2, and S1F2 mutations, respectively). One isolate showed a single substitution of ala in position 1 to pro. In confirmation of earlier work, most of the PNG isolates possessed the T9 mutation in the 416–424 epitope while a single isolate in the extended panel carried the R9 mutation; it is interesting to note that every one of these PNG isolates also showed the ser-leu or ser-phe mutation in positions 1 and 2 of the 399–408 epitope. By contrast, only a minority of Caucasian isolates (from Europe and USA) showed any mutation relative to the B95.8 sequence; when present, these exclusively involved the immunodominant 416–424 epitope and were either of the L2 or N9 type already represented among the Chinese viruses. The majority of Caucasian isolates and, interestingly, all 15 African isolates tested were identical to the B95.8 sequence throughout this whole region of EBNA4.

Sensitivity of Chinese EBV Isolates to A11-restricted B95.8 EBV-induced CTLs. To examine the effect of such mutations on immunological recognition, A11-restricted CTL clones of the kind illustrated in Fig. 1 (i.e., specific for the EBNA4 416–424 and 399–408 epitopes of the B95.8 virus) were tested on target
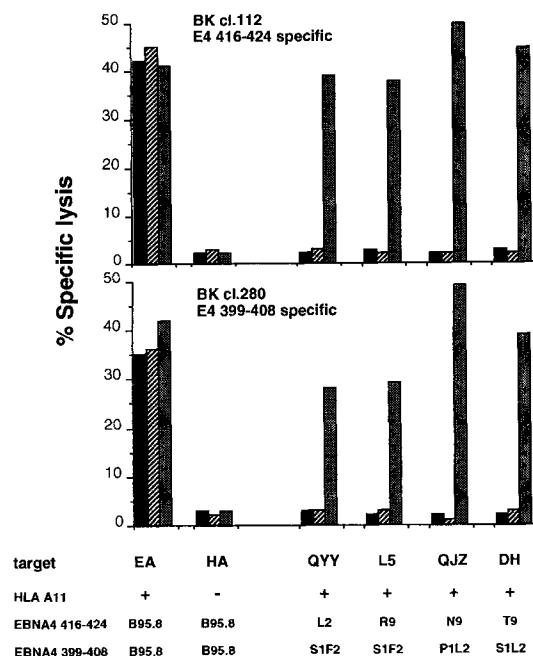
No. of
Isolates

B95.8 AGG CCC GCG GTG TTT GAC CGA AAG TCA GAT GCA AAA TCA ACC AAA AAA TGC AGG GCC ATA GTA ACT GAC TTT AGT GTA ATC AAG GCC ATT
       A V F D R K S D A K   I V T D F S V I K

S-E Asia ...

Papua New Guinea

Europe and USA

Africa

Figure 2. Sequence of A11-restricted EBNA4 epitopes in EBV isolates of different geographic origin. Nucleotide and amino acid changes relative to the B95.8 sequence are indicated. Shadowed boxes identify the sequence of the EBNA4 399–408 and 416–424 epitopes.

LCLs carrying the endogenous EBV strain that had been established from A11-positive Chinese or PNG donors by spontaneous in vitro outgrowth. The panel of target LCLs, each confirmed by immunoblotting to be expressing the endogenous virus-encoded EBNA4 protein (not shown), included representatives of each of the mutations observed in the EBNA4 416–424 epitope (QYY-LCL, L2; QJZ-LCL, N9; L5-LCL, R9; DH-LCL, T9; note that these lines also carried the S1L2, S1F2, or P1L2 double mutation in the EBNA4 399–408 epitope.

Fig. 3 shows representative results obtained with the 416–424 epitope specific clone BK cl.112 and the 399–408 epitope-specific clone BK cl.280 when tested on the above LCL panel. There was no significant lysis of any of the spontaneous Chinese LCLs by these effectors (solid bars). However, when the B95.8 EBNA4 protein was expressed in these cells via a recombinant vaccinia virus vector, all four target lines became susceptible to A11-restricted CTLs (hatched bars); infection of these cells with a control vaccinia recombinant had no such effect (stripped bars). This strongly suggested that lack of recognition of the spontaneous LCLs reflected an altered antigenicity of the resident virus strain rather than some subtle polymorphism of the A11 restriction element between Caucasian and Chinese donors.

Further analysis of this altered antigenicity concentrated on those mutations observed of the immunodominant 416–424 epitope on CTL recognition. Peptide sensitization assays were carried out to compare the four different epitope variants with the cognate B95.8 sequence. Fig. 4 shows a typical set of results obtained in assays testing the 416–424-specific CTL clone BK cl.112 on QYY, QJZ, L5, and DH spontaneous LCL targets either alone or after preincubation with 10^{-7} M concentrations of the relevant synthetic peptides. There was again no recognition of the LCLs alone but addition of the B95.8 epitope peptide mediated strong target cell lysis. Interestingly, synthetic peptides corresponding to the N9 and T9 mutations were not able to sensitize the target cells whereas those

corresponding to the L2 and R9 mutations were recognized as efficiently as the cognate B95.8 epitope sequence. Sensitization with the L2- and R9-mutated peptides was observed with all four A11-positive LCLs tested, including those where

target	EA	HA	QYY	L5	QJZ	DH
HLA A11	+	−	+	+	+	+
EBNA4 416-424	B95.8	B95.8	L2	R9	N9	T9
EBNA4 399-408	B95.8	B95.8	S1F2	S1F2	P1L2	S1L2

Figure 3. Lysis of HLA A11 positive LCLs carrying Chinese EBV isolates by B95.8 EBNA4-specific CTLs. Spontaneous LCLs from A11-positive Chinese donors carrying EBNA4 mutated EBV strains were tested for sensitivity to lysis by CTL clones specific for the B95.8 EBNA4 epitopes 416–424 (BK cl.112) and 399–408 (BK cl.280). The targets were either untreated ■, or had been infected for 6 h at a multiplicity of infection of 10:1 with a control recombinant vaccinia virus ▨ or with a vaccinia recombinant expressing the B95.8 EBNA4 gene ■ We have previously shown that cells infected under these conditions expressed high levels of the gene encoded by the vaccinia recombinant (8). The percent specific lysis recorded at a 10:1 effector/target ratio is shown. One representative experiment out of four is shown.
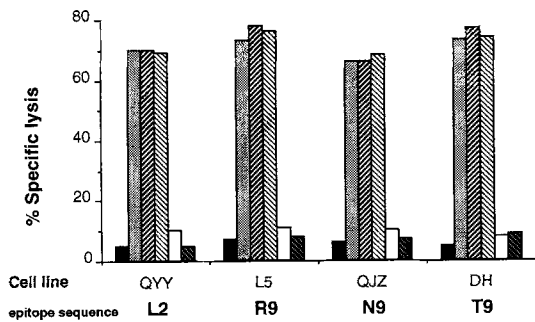
Figure 4. CTL recognition of mutated EBNA4 416–424 peptides. Spontaneous LCLs from A11-positive Chinese donors carrying EBNA4 mutated EBV strains were tested for lysis by the B95.8 EBNA4 416–424 specific CTL clone BK cl.112 either untreated or after preincubation with 10⁻⁷ M of synthetic peptides corresponding to the B95.8 416–424 epitope sequence or peptides representing the L2, R9, N9, and T9 mutations. The percent specific lysis recorded at a 10:1 effector/target ratio is shown. One representative experiment out of three is shown. ■ Untreated; ▨ E4 416–424; ▨ L2; ▧ R9; □ N9; ■ T9.

the endogenous virus contained the same mutation. This suggested that, even when the mutation in the resident virus had not destroyed the antigenicity of the EBNA4 epitope, presentation of the mutated peptide was in some way impaired.

Comparison of A11-positive Caucasian and Chinese Donors to Autologous LCL Stimulation. In a first set of experiments,

HLA A11 positive Caucasian and Chinese donors were compared for their ability to generate A11-restricted EBV-specific CTLs after stimulation with autologous LCL cells transformed by the reference Caucasian EBV isolate B95.8. Several independent CTL activations were carried out from each donor and in each case the allele-specific component reactivities within the overall response were analyzed by testing on the autologous LCL and on a panel of B95.8 transformed allogeneic LCLs either matched with the donor through a single HLA class I allele or completely HLA class I mismatched. Of 12 Caucasian donors tested, 10 yielded CTL preparations that showed clear HLA class I–restricted EBV-specific memory responses with lysis of mismatched LCLs and EBV negative targets always below 20% of the autologous LCL lysis. Two other Caucasians (KK and PB) failed to mount a detectable EBV specific CTL response. As illustrated by representative results obtained with 4 donors (BK, SI, EA, JAC; Fig. 5), an A11-restricted CTL component was present in all 10 cases where an EBV-specific response was obtained. When the strength of each individual allele-specific reactivity is compared with the level of autologous LCL lysis, it is clear that the A11-restricted component is in each case the dominant reactivity. By contrast, A11-restricted reactivities were either not detectable above background (i.e., above the lysis of HLA-mismatched LCL targets) or represented only a minor component within CTL preparations from the three A11-positive Chinese donors tested. These individuals yielded an HLA class
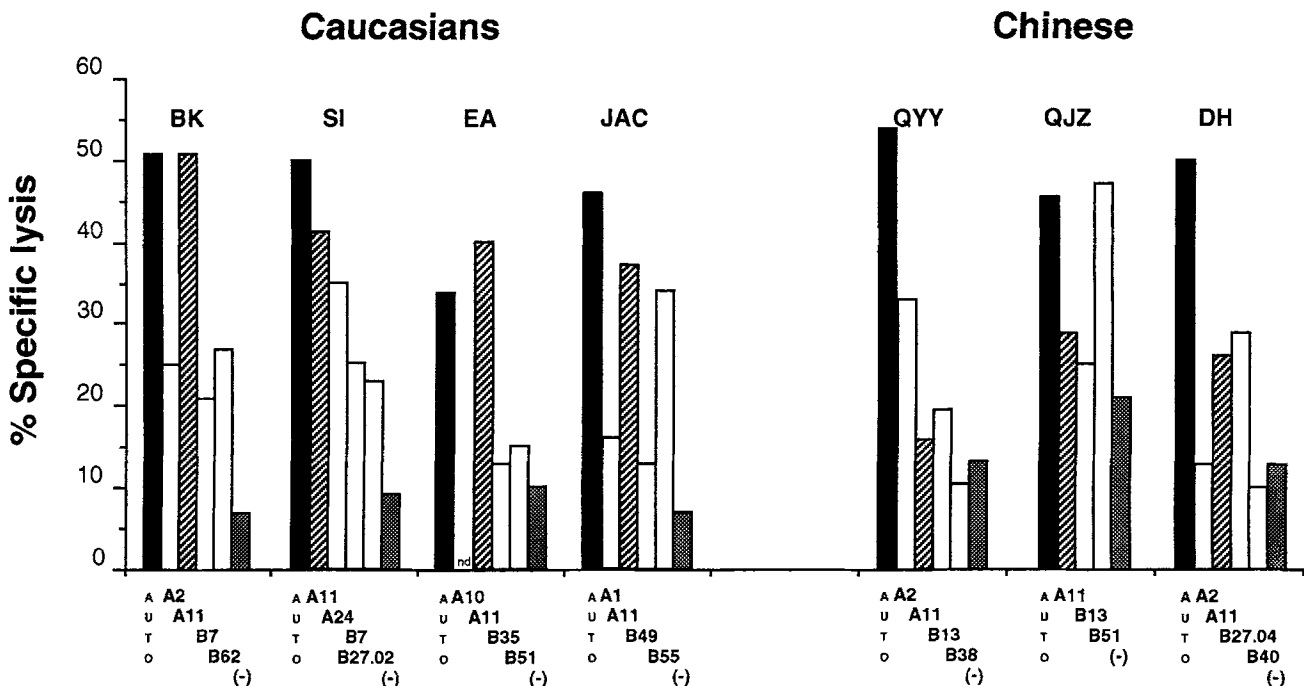
Figure 5. Response of HLA A11 positive Caucasian and Chinese donors to autologous B95.8 transformed LCLs. Lymphocytes from the HLA A11 positive Caucasian donors BK, SI, EA, and JAC and A11-positive Chinese donors QYY, QJZ, and DH were stimulated with autologous B95.8 virus transformed LCLs. Polyclonal CTL cultures obtained after three consecutive restimulations were tested for lysis against a panel of B95.8 virus transformed LCLs including the autologous LCL and allogeneic LCLs either matched through a single HLA class I allele or completely mismatched. At least two single matched LCLs for each allele and two mismatched LCLs (–) were included in the panel. The mean percent specific lysis at 10:1 effector/target ratio in 10–20 tests performed with CTLs from 3–10 independent stimulations for each donor is shown.

I-restricted response to autologous B95.8 LCL stimulation which appeared to be predominantly restricted through the B51 allele in donor QJZ and through the A2 allele in donor QYY, but which for the other donor, DH, showed no one dominant reactivity.

We then compared the CTL responses of these Chinese donors to in vitro stimulation with their B95.8 LCL to that induced by the spontaneous LCL carrying their endogenous EBV strain. For all three individuals, there was no significant difference in the pattern of cytotoxicity observed; in particular the spontaneous LCL never induced an obvious A11-restricted response. Representative results from donors QJZ and QYY are shown in Fig. 6; CTL component restricted through the B51 (donor QJZ) and A2 (donor QYY) alleles appeared to dominate the response to both spontaneous and B95.8 transformed LCLs. Most notably, stimulation with the spontaneous LCL failed to induce A11-restricted CTL components specifically directed against A11-matched LCLs carrying Chinese or PNG virus isolates. Thus, in the case of donor QJZ shown in Fig. 6, lysis of the QXL spontaneous LCL (an A11-matched line carrying the same N9 mutation in the 416–424 epitope as the CTL donor) was only slightly above background. Finally, we tested such spontaneous LCL-induced CTL preparations for their ability to recognize syn-thetic peptides representing the mutated version of the 416–424 and 399–408 epitopes known to be present in the donor's endogenous virus strain; no epitope specific lysis was ever observed (data not shown).

Discussion

The existence of EBV-related B lymphotropic herpesviruses in many old world primate species clearly indicates that these agents have coevolved with their host species over millions of years. Only in special circumstances, therefore, might one be able to discern a specific influence of host immune responses upon the evolution of such genetically stable agents. Our ob-servation that all six EBV isolates studied from a highly HLA A11 positive PNG population carried a single point muta-tion affecting the antigenicity of the major A11-restricted CTL epitope (25) was possibly one such example. To examine this possibility more carefully and to assess the generality of our initial findings we focused upon EBV isolates from a second much larger population group, Southern Chinese, in which the A11 allele is highly prevalent.

Having identified the two most immunodominant pep-tide epitopes for A11-restricted responses in Caucasians as EBNA4 416–424 and EBNA4 399–408 (13, Fig. 1), we set
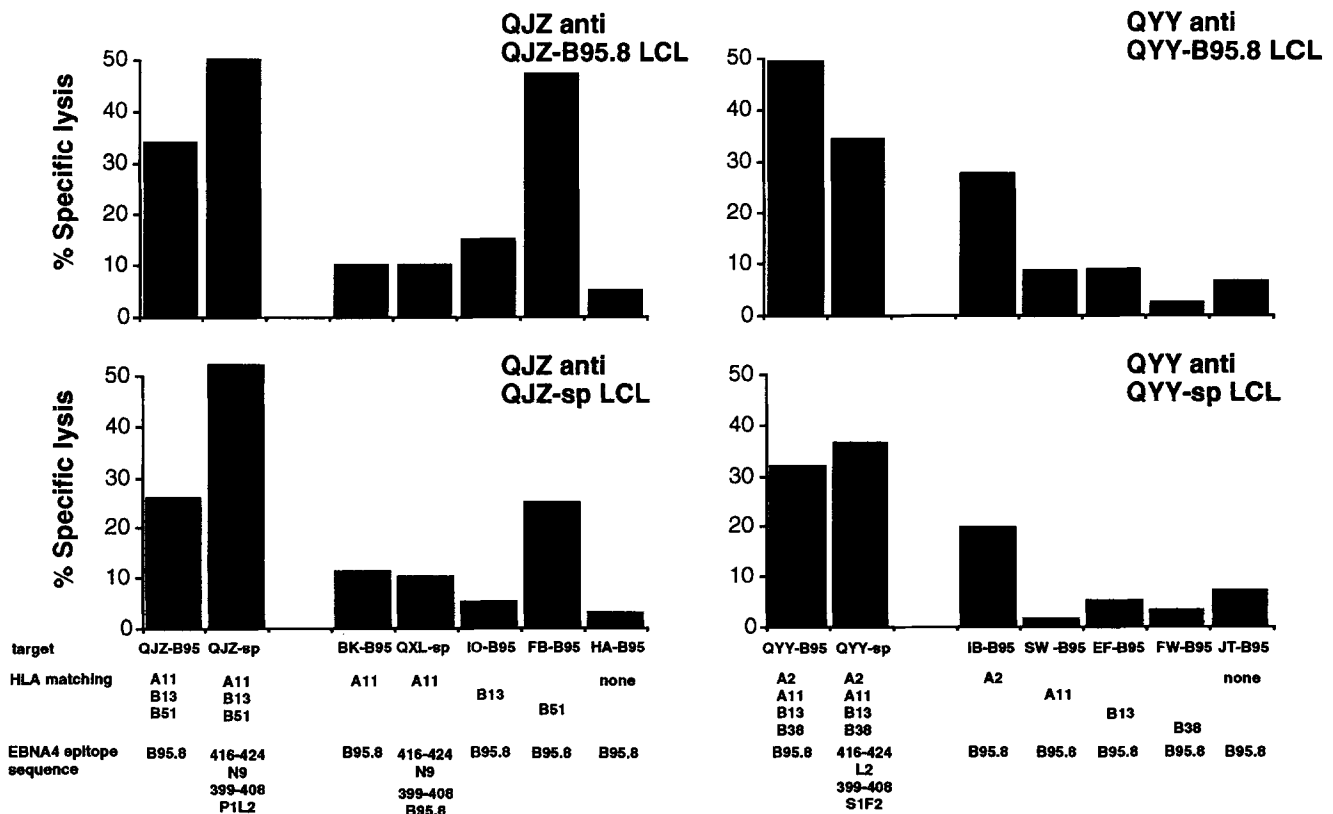
Figure 6. Cytotoxic activity of polyclonal CTL preparations obtained by in vitro stimulation of PBLs from the Chinese donors QJZ and QYY with autologous LCLs carrying the B95.8 virus or the endogenous EBV strain. The percent specific lysis at 10:1 effector/target ratio is shown. One representative experiment out of three is shown.

out to sequence this region of the EBNA4 gene in virus isolates from Southern China and, as a reference, from additional PNG, African, and Caucasian isolates. All 33 Chinese and PNG isolates sequenced carried mutations within 416–424 epitope that selectively involved one or the other of the two anchor residues in positions 2 and 9. Moreover, about half of the Chinese isolates and all the PNG isolates showed additional mutations in the 399–408 epitope, again selectively affecting both residue 1 and anchor residue 2.

An important feature of these results is the multiplicity of variants seen amongst Chinese viruses. Thus, we identified four independent mutations (L2, R9, T9, and N9) affecting the 416–424 epitope in this group of isolates, whereas isolates from PNG were more homogeneous (all but one were T9). The multiplicity of Chinese strains is further stressed by the fact that both the L2 group and the N9 group of isolates can be further divided into subgroups that either did or did not contain mutations in the second epitope. The separate origin of these subgroups is confirmed by additional sequence data which consistently revealed the presence of two additional coding mutations (at residues 380 ser-ala and 461 gln-his) in all viruses with mutations in the second epitope (not shown). In contrast, viruses lacking changes in the second epitope were identical to B95.8 over the whole region except for the single mutation in the first epitope. This reemphasizes the selective nature of the mutations observed, and is consistent with the view that CTL responses directed against the immunodominant 416–424 epitope, which was regularly mutated in the Chinese isolates, exert the strongest selective pressure.

A common feature of the mutations described in this work is that, like the original mutation found in PNG isolates (25), they all abrogated recognition by CTLs raised against the B95.8 epitope. Thus, A11 positive spontaneous LCLs carrying the L2, R9, N9, or T9 mutations were not lysed by B95.8-induced CTL clones (Figs. 3 and 4). We would have predicted

this result on structural ground in the case of the N9 and T9 mutations since substitution of the lys anchor residue at position 9 by asn or thr is likely to abrogate binding to the A11 groove; as indeed has been shown to be the case for the T9 mutant peptide (25). However, the val-leu substitution at position 2 and the lys-arg substitution at position 9 represent more conservative changes that are unlikely to impair binding. Indeed, synthetic peptides representing the L2 and R9 mutant sequences (but not T9 and N9) were able to sensitize target cells to lysis by CTLs raised against the cognate B95.8 epitope sequence (Fig. 4). The L2 and R9 mutations identified in Chinese isolates, therefore, do not abrogate the antigenicity of the peptide epitope per se; the cells carrying these isolates nevertheless seem unable to present this epitope from the endogenously expressed EBNA4 protein. The mechanism of this defect is currently under investigation.

Another important goal of the present study was to assess whether such "A11 epitope-loss" EBV variants did indeed induce weaker A11-restricted CTL responses in their infected hosts. We initially observed that three HLA A11 positive Chinese donors failed to mount an A11-restricted CTL response when challenged with autologous B95.8 virus transformed B cells, even though EBV-specific responses restricted via other alleles were detected (Fig. 5). Furthermore, in vitro stimulation of these donors' T cells with autologous cells carrying the resident EBV isolate still failed to uncover an A11-restricted component within the CTL memory (Fig. 6). Although the number of Chinese donors studied is small, the contrast with A11-positive Caucasian donors is already very striking since A11 is the preferred restriction element of EBV-specific responses in the latter group.

Two coincidental features of the HLA A11 allele were key factors in the planning of the present study. One was its proven capacity to mediate immunodominant CTL responses to EBV (26, 27); the other was the marked variation that exists in A11 frequency between different human populations. Fig. 7
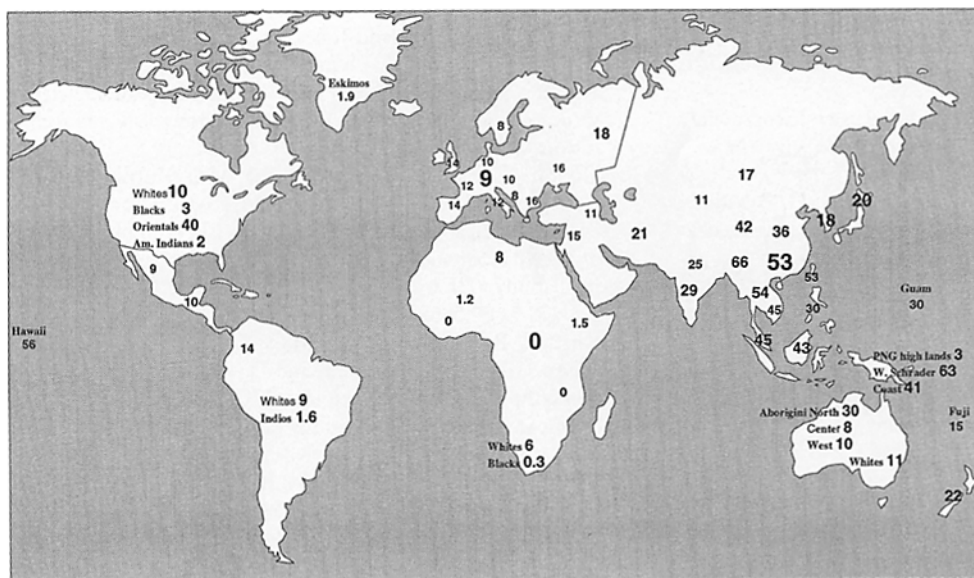
Figure 7. HLA A11 antigen frequency in different ethnic groups. Data of HLA A11 antigen frequency in different human populations were collected from references 22, 23, 28–30. Antigen frequency was calculated from gene frequency data by the formula: $AF = 1-(GF-1)^2$. The figures include both the HLA A11.01 and A11.02 subtypes. Large bold numbers represent the average antigen frequency in the indicated area/ethnic group.

illustrates the degree of this variation on a world map, emphasizing the remarkably high allele frequencies which are seen in various South East Asian populations. We would also stress that these comparisons are unlikely to be greatly affected by polymorphism within the A11 allele family itself. Thus of the three known subtypes of A11, the only one as yet found in Caucasian populations (A11.02) is also by far the most common subtype present in South East Asia. We therefore view as very significant the fact that two different South East Asian populations both consistently yielded EBV strains with mutations selectively affecting critical residues in A11-restricted epitope regions. Such mutations were never seen in African populations, where A11 is virtually absent, and occurred in only a small fraction of isolates from Caucasian populations where the A11 frequency is roughly 10%. Our studies indicate that these South East Asian viruses were indeed epitope-loss mutants since they were neither recognized by B95.8-induced A11-restricted CTLs nor capable of themselves eliciting A11-restricted responses. We infer that A11 epitope loss had conferred a selective advantage to EBV isolates in these particular host communities.

The mechanism by which such CTL-mediated selection occurs remains to be determined. One interesting possibility is that CTL responses may be able to influence the type of viral strain which becomes established in the B cell reservoir of an individual. Thus, in certain cases, a particularly strong primary CTL response, perhaps dominated by reactivity to an immunodominant HLA class I-EBV peptide complex, may be capable of eliminating virus-infected cells from the body early in the infection before virus persistence is established in the B cell reservoir; clearly viral strains lacking the relevant target peptide would have a greater chance of successful transmission to such individuals. Another possibility is that CTL responses exert their influence during the life-long persistent infection by controlling the size of the virus-infected B cell pool, and thereby the size of the reservoir from which transmissible infectious virus is ultimately derived. If this were the case, CTL epitope loss mutants would have a greater chance of being reactivated as infectious virus in the oropharynx, and hence a greater chance of being transmitted within the community.

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