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Epstein-Barr virus isolates with the major HLA B35.01-restricted cytotoxic T lymphocyte epitope are prevalent in a highly B35.01-positive African population*

An influence of cytotoxic T lymphocyte (CTL) response over Epstein-Barr virus (EBV) evolution was first suggested by the finding that virus isolates from highly HLA-A11-positive Oriental populations were specifically mutated in two immunodominant A11-restricted CTL epitopes. Here we turn to a second HLA allele, B35.01 and show that B35.01-restricted CTL responses in Caucasian donors reproducibly map to a single peptide epitope, YPLHEQHGM, representing residues 458–466 of the type 1 EBV nuclear antigen 3A protein (B95.8 strain). In this case, however, most EBV isolates from a highly B35.01-positive population (in The Gambia) either retained the CTL epitope sequence or carried a mutation (P → S at position 2) which conserved antigenicity; changes leading to reduced antigenicity (Y → N at position 1) were found in only a minority of cases. Furthermore, CTL recognizing the YPLHEQHGM epitope could be reactivated from the blood of some B35.01-positive Gambian donors by *in vitro* stimulation with the synthetic peptide, indicating that epitope-specific immunity does exist in this population. Possible differences between the A11-based and B35.01-based studies are discussed.

1 Introduction

Epstein-Barr virus (EBV), a B lymphotropic herpes virus widespread in all human populations, elicits strong cytotoxic T lymphocyte (CTL) responses in the majority of infected individuals [1, 2]. These HLA class I-restricted, CD8-positive CTL are believed to play an important role in controlling the virus during primary infection, and also in maintaining an asymptomatic life-long virus carrier state. Thus EBV-specific memory CTL can be reactivated from the blood of immune donors by *in vitro* stimulation with the autologous virus-transformed lymphoblastoid cell line (LCL), suggesting that some or all of the EBV latent proteins expressed within an LCL, namely the six nuclear antigens (EBNA 1, 2A, 3A, 3B, 3C, LP) and two latent membrane proteins (LMP 1,2), can act as targets for CTL responses. Using the B95.8 virus as a representative of the type 1 EBV strains which are prevalent in most human populations, including Western societies, the antigenic specificity of such EBV-induced CTL has been assigned to individual latent proteins and in several cases to individual peptide epitopes [3–9]. As in other viral systems, target choice was critically dependent on the donor's HLA type, with individual alleles focusing the response on a small number of allele-specific epitope regions. It was also noted that certain HLA alleles, where present, tended to be the dominant restricting element. For example, individuals who were B8-positive mounted a strong response to an epitope in EBNA 3A (residues 325–333) [5], those who were A11-positive mounted a strong response to an epitope in EBNA 3B (residues 416–424) [7] and those who were B27-positive mounted a strong response to an epitope in EBNA 3C (residues 258–266) [8]. Because the EBNA 3 proteins are polymorphic between the type 1 and type 2 families of EBV isolate, with only 72–84% sequence conservation between types [10], some of these responses proved to be type 1 specific, whereas others were cross-reactive [1].

One particularly interesting example of EBV sequence polymorphism affecting CTL recognition was noted using A11-restricted effectors from Caucasian donors. When tested against a panel of A11-positive target LCL carrying different virus isolates, CTL recognition was clearly type 1-specific but did not extend to type 1 strains derived from S. E. Asia (China and Papua New Guinea) [11, 12]. Sequencing of the immunodominant EBNA 3B 416–424 epitope region showed conservation of the B95.8 sequence in type 1 viruses from Caucasian and African populations, whereas New Guinean and Chinese isolates carried mutations within this epitope which specifically affected the anchor residues at positions 2 and 9 that were critical for peptide binding to the A11 molecule. The majority of the Chinese isolates and all New Guinean isolates studied also carried mutations at positions 1 and 2 in the second most immunodominant A11-restricted epitope, EBNA 3B residues 399–408, that again abrogated B95.8-reactivated CTL recognition. Furthermore, Chinese donors naturally

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Abbreviations: **LCL:** Lymphoblastoid cell line **LMP:** Latent membrane protein **EBNA:** EBV nuclear antigen

Key words: Epstein-Barr virus / Cytotoxic T lymphocyte / HLA B35 / Epitope

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infected with such virus strains failed to mount detectable A11-restricted responses when stimulated *in vitro* with an autologous LCL carrying their endogenous EBV isolate, thus indicating that such isolates do not carry an alternative A11-restricted immunodominant epitope.

These observations were of particular interest because the HLA-A11 allele, though relatively infrequent in Caucasians (approximately 10% positivity) and virtually absent from African populations, is present at an unusually high frequency in the coastal regions of New Guinea (25–50%) and in China (>50%) [12]. The results, therefore, suggested that EBV variants lacking the immunodominant A11-restricted epitopes have enjoyed a selective advantage in these highly A11-positive populations. The ability of CTL to select for "epitope loss" variants has been observed previously in prospective studies on individual hosts infected with genetically unstable viruses such as lymphocytic choriomeningitis virus [13] and HIV [14]. However, the studies with EBV at the level of human populations provided the first indication that CTL responses may influence the longer-term evolution of a genetically more stable virus. To investigate this issue further, we have turned to a second HLA allele, B35.01, which also acts as a restricting determinant for T cell responses to EBV and which, though present in many human populations, is particularly common in West Africa (The Gambia) where at least 30% individuals are B35.01-positive [15, 16].

2 Materials and methods

2.1 Cell lines

LCL were generated from donors of known HLA type by *in vitro* transformation of peripheral B lymphocytes with the standard type 1 EBV isolate B95.8 [17], and cultured by feeding twice weekly with RPMI 1640 containing 10% FCS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (growth medium). The fibroblast cell line was established from a small skin biopsy sample taken from a donor of HLA type A11,24 B7,35 (donor 2) and cultured in DME-M supplemented as described above. PHA blasts were obtained from donors of known HLA type by stimulating PBMC with 1 μg/ml PHA in growth medium, then cultured by feeding twice weekly with growth medium supplemented with 25% MLA 144 supernatant as a source of IL-2.

2.2 Recombinant vaccinia viruses

A panel of recombinant vaccinia virus vectors coding for individual EBV latent proteins (EBNA 2, 3A, 3B, 3C, LP and LMP 1,2) has been described previously [3]. The EBNA 1ΔGA vaccinia recombinant was generated in the same manner using the EBNA 1 open reading frame BKRF 1 [18] but with the internal glycine-alanine repeat sequence (amino acid residues 91–325) deleted and replaced with a single amino acid triplet (Gly-Ala-Gly). High level expression of the truncated EBNA 1 protein was confirmed by Western blotting of infected cell preparations using an EBNA 1-specific rat mAb (1H4, F. Grasser et al. manuscript in preparation). For cytotoxicity assays, fibroblasts were infected with the recombinant vaccinias for 2 h at a multiplicity of infection of 10:1. The cells were then incubated in culture medium for a further 16 h and subsequently trypsinized and used as targets in the assay.

2.3 Synthetic peptides

Peptides were synthesized by J. Fox (Alta Bioscience, University of Birmingham, Birmingham, GB) using fluorenylmethoxycarbonyl chemistry. They were then dissolved in DMSO and protein concentrations measured by a modified biuret assay [19].

2.4 Cytotoxic T lymphocytes

EBV-specific polyclonal CTL lines were generated from five EBV-immune Caucasian donors by co-culturing peripheral blood mononuclear cells with the autologous B95.8-transformed LCL (γ-irradiated) at a responder to stimulator ratio of 40:1. The cells were then expanded in growth medium supplemented with IL-2 as described previously [20], and tested in cytotoxicity assays. The HLA types of the donors used in this study were as follows: donor 1 = HLA A2,11 B35,40; donor 2 = HLA A11,24 B7,35; donor 3 = HLA A3,11 B35,40; donor 4 = HLA A2,24 B27,35; donor 5 = HLA A2,24 B7,35. The evidence strongly suggests that all five donors are B35.01, the B35 subtype most common in Caucasian populations. Isoelectric focusing of HLA class I heavy chains from the donor's LCL identified a band in the position occupied by B35.01; furthermore, PCR typing using a probe which distinguishes B35.01 from the other major B35 subtypes gave a B35.01-positive result in all five cases (data from S. Ali and A. V. S. Hill, John Radcliffe Hospital, Oxford, GB). CTL were also generated from a number of Caucasian and Gambian donors by stimulating PBMC with a synthetic peptide representing the B35.01-restricted target epitope in EBNA 3A (residues 458–466). The cells were first incubated with peptide at 100 μg/ml for 1 h, washed and plated out at 2×10^6 cells/2-ml well. Peptide-stimulated PBMC were cultured in growth medium and after 3 days, 10% lymphocult T (Biotest) added. The cells were cultured for a further 7–9 days and then tested in a cytotoxicity assay.

2.5 Cytotoxicity assays

Target cells were labeled for 1–2 h with $^{51}CrO_4$, washed and incubated with CTL in a standard 5-h chromium release assay at known effector:target ratios. Assays which included vaccinia-infected target cells were harvested into 1% formaldehyde before counting. To study CTL recognition of synthetic peptides, labeled targets were preincubated with peptide or the appropriate dilution of DMSO solvent (control) in 100 μl for 1 h before the addition of a further 100 μl of CTL. The concentration of peptide recorded refers to the final concentration after the addition of CTL.

2.6 DNA sequencing by polymerase chain reaction (PCR)

LCL were incubated in a proteinase K lysis solution containing 100 μg/ml proteinase K, 10 mM Tris-HCl

(pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.005% Tween 80 for 1–2 h at 55 °C to extract the DNA. Crude DNA (10–100 ng) was amplified in a 100-µl reaction mix as described previously [10] by using the following oligonucleotide primers: 5′-GAAACCAAGACCAGAG-GTCC-3′ (coordinates 93596–93615) and 5′-TCCCAGGG-CCGGACAATAGG-3′ (coordinates 93871–93852) according to the published DNA sequence of the prototype B95.8 virus strain [18]. Following amplification, aliquots of the PCR mix were run out on 1.5% low-melting-point agarose (Ultrapure) gels and PCR products were purified using a Magic PCR Preps DNA Purification System Kit (Promega) according to the manufacturer's instructions. Cycle sequencing was carried out with the Amplitaq Cycle Sequencing Kit (Perkin-Elmer Cetus) using the above PCR primers which were ^{32}P end-labeled with T4 polynucleotide kinase (Boehringer Mannheim).

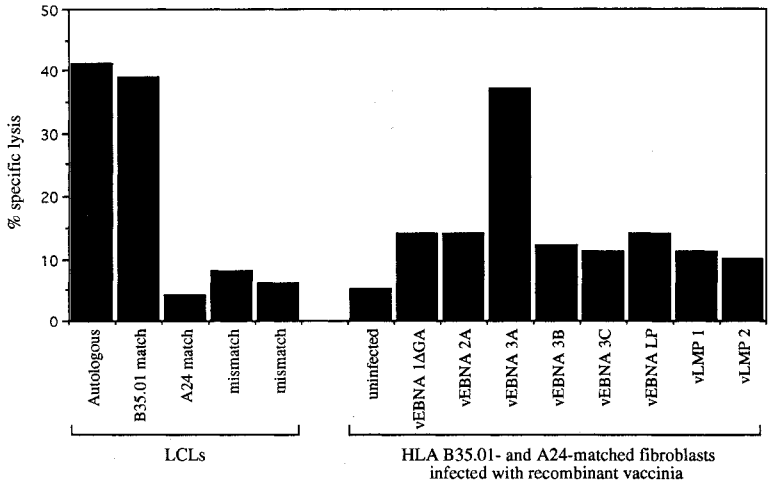

Figure 1. HLA B35-restricted EBV-specific polyclonal CTL from donor 4 (A2,24; B27,35.01) recognize the viral protein EBNA 3A. Polyclonal CTL were tested in a 5-h cytotoxicity assay on fibroblast targets (matched through HLA A24 and B35.01) expressing individual EBV (B95.8 strain) latent proteins from vaccinia viral vectors. Control targets included B95.8 EBV-transformed LCL, either autologous, B35.01-matched, A24-matched or completely mismatched. Results are shown as percent specific lysis observed at an effector/target ratio of 10:1.

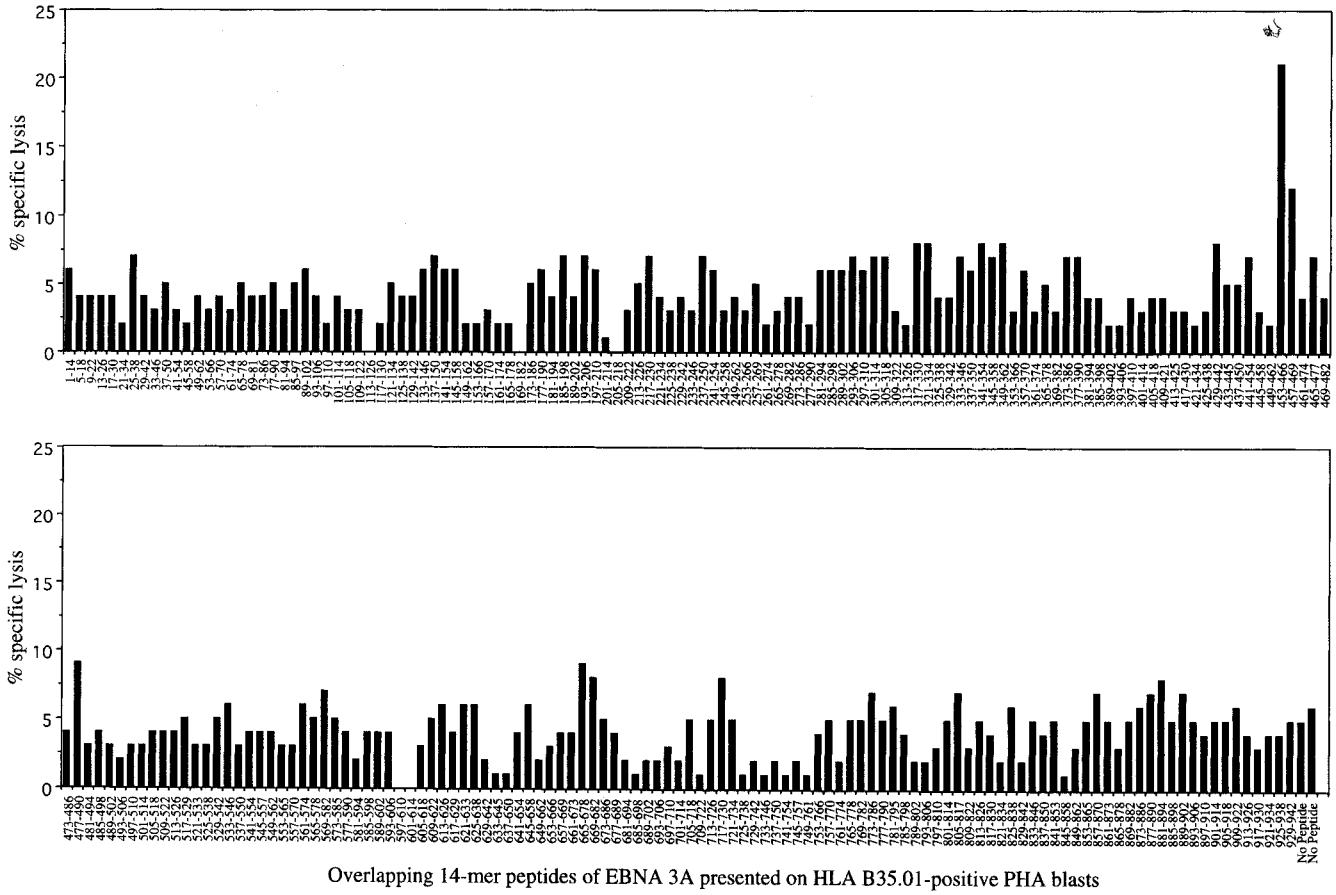

Figure 2. Identifying the target epitope of B35-restricted EBNA 3A-specific CTL using overlapping synthetic peptides. Polyclonal CTL from donor 1 were tested on HLA B35-matched PHA blast targets preincubated with a 2×10^{-7} M concentration of 13- and 14-mer peptides from the EBNA 3A sequence (amino acid residue numbers as shown) or to an equivalent dilution of DMSO solvent (no peptide) as a control. Results are shown as percent specific lysis observed in a 5-h chromium-release assay at an effector/target ratio of 10:1.

3 Results

3.1 Mapping the HLA B35.01-restricted response to a defined epitope in EBNA 3A

Effector CTL were generated from five EBV immune Caucasian donors, previously identified as HLA B35.01-positive (see Sect. 2.4), by *in vitro* stimulation of PBMC with the autologous B95.8-transformed LCL. Polyclonal CTL populations were analyzed for lysis of the autologous LCL and of allogeneic LCL either sharing individual HLA class I alleles with or HLA-mismatched with the effector cells. All five individuals showed evidence of a B35.01-restricted response, although we noted that this was frequently not as strong as the accompanying A11-restricted component for donors 1–3 or the B27-restricted component for donor 4 ([21] and data not shown). The antigenic specificity of the B35.01-restricted response was therefore analyzed by testing on B35.01-matched fibroblast targets expressing individual EBV latent proteins from recombinant vaccinia virus vectors. Fig. 1 shows the results from one representative assay of this kind using polyclonal effector cells from donor 4. The B35.01-restricted effectors within this population preferentially recognized target cells expressing EBNA 3A. Similar results were obtained for B35.01-restricted CTL from all the donors analyzed.

To identify the target epitope recognized by these CTL, synthetic peptides (13- and 14-mers, overlapping by 10 amino acids) were synthesized representing the entire B95.8 EBNA 3A protein sequence and tested for their ability to sensitize HLA B35.01-matched PHA blasts to lysis. The results obtained in such an experiment using polyclonal CTL effectors from donor 1 are shown in Fig. 2, where significant lysis of target cells above background was observed with a single peptide representing EBNA 3A residues 453–466. In addition there was weak recognition of target cells coated with the overlapping 13-mer peptide (EBNA 3A residues 457–469). To define the target epitope more precisely, we used the same approach to test a new series of overlapping peptides of between 8 and 12 amino acids in length from the region spanning residues 453–469. These peptides were tested at two concentrations (2×10^{-7} M and 2×10^{-8} M) in an experiment using polyclonal CTL from three B35.01-positive donors (donors 1–3). The results are shown in Table 1. Significant target cell lysis was obtained not only with the original 14-mer (453–469) and 13-mer (457–469) peptides, but also with the overlapping 11- and 12-mer peptides 456–466, 457–467 and 458–469. However, only background levels of lysis were obtained with the immediately adjacent 11-mers 455–465 and 459–469, indicating the importance for CTL recognition of the tyrosine at position 458 and the methionine at position 466. The highest levels of target cell lysis were observed with the 10-mer peptide (457–466, representing the precise region of overlap between the original 14-mer and 13-mer), and with the related 9-mer peptide (458–466), whereas 8-mers from this region were never recognized.

Table 1. Screening of peptides in EBNA 3A region 449–474[a)]

EBNA 3A peptide (residues)	Peptide Sequence	Donor 1		Donor 2		Donor 3	
		$2x10^{-7}$M	$2x10^{-8}$M	$2x10^{-7}$M	$2x10^{-8}$M	$2x10^{-7}$M	$2x10^{-8}$M
449-462	IHLAAQGMAYPLHE	6[a]	13	ND	ND	ND	ND
453-466	AQGMAYPLHEQHGM	24	20	ND	ND	ND	ND
457-469	AYPLHEQHGMAPC	21	15	ND	ND	ND	ND
461-474	HEQHGMAPCPVAQA	7	5	ND	ND	ND	ND
453-463	AQGMAYPLHEQ	6	7	1	3	0	0
454-464	QGMAYPLHEQH	11	9	10	10	0	0
455-465	GMAYPLHEQHG	7	7	5	3	1	1
456-466	MAYPLHEQHGM	29	37	37	60	19	34
457-467	AYPLHEQHGMA	31	24	51	66	36	17
458-469	YPLHEQHGMAPC	21	18	41	53	16	18
459-469	PLHEQHGMAPC	6	5	3	2	6	3
457-466	AYPLHEQHGM	43	38	51	69	40	53
458-466	YPLHEQHGM	51	55	55	75	31	45
458-465	YPLHEQHG	10	8	21	4	12	6
459-466	PLHEQHGM	12	7	13	6	2	4
No peptide		8	7	0	0	1	4

a) Values represent % specific lysis of peptide-coated B35.01-matched PHA blasts tested in a chromium release assay with HLA B35.01-restricted EBNA 3A-specific polyclonal CTL from three donors at an effector/target ratio of 10:1. ND = not done.

These results were confirmed in titration experiments where peptides that had given significant levels of lysis above were tested over a wider concentration range (2×10^{-5} M to 2×10^{-13} M). Fig. 3 shows the results of one such experiment using CTL from donor 1. Both the 14-mer (453–466) and 13-mer (457–469) peptides mediated low levels of target cell killing, with half maximal lysis achieved at a peptide concentration of approximately 10^{-8} M. A similar result was obtained with the 11-mer (457–467), whereas the other 11-mer (456–466) and the 10-mer (457–466) were recognized more strongly with half maximal lysis at approximately 10^{-9} M. However, the 9-mer peptide (458–466) not only gave the highest levels of target cell lysis, but was also recognized the most efficiently, with half maximal lysis achieved at approximately 10^{-10}M. Note that with some of these peptides there was a reduction in the level of target cell lysis when tested at higher peptide concentrations. We have noticed this in other peptide epitope titrations and attribute the effect to increased lysis of the effector CTL population itself through peptide-mediated T cell-T cell killing [9, 22, 23]. The above work clearly identified the peptide YPLHEQHGM (EBNA 3A residues 458–466) as the dominant target epitope for the B35.01-restricted response to EBV.

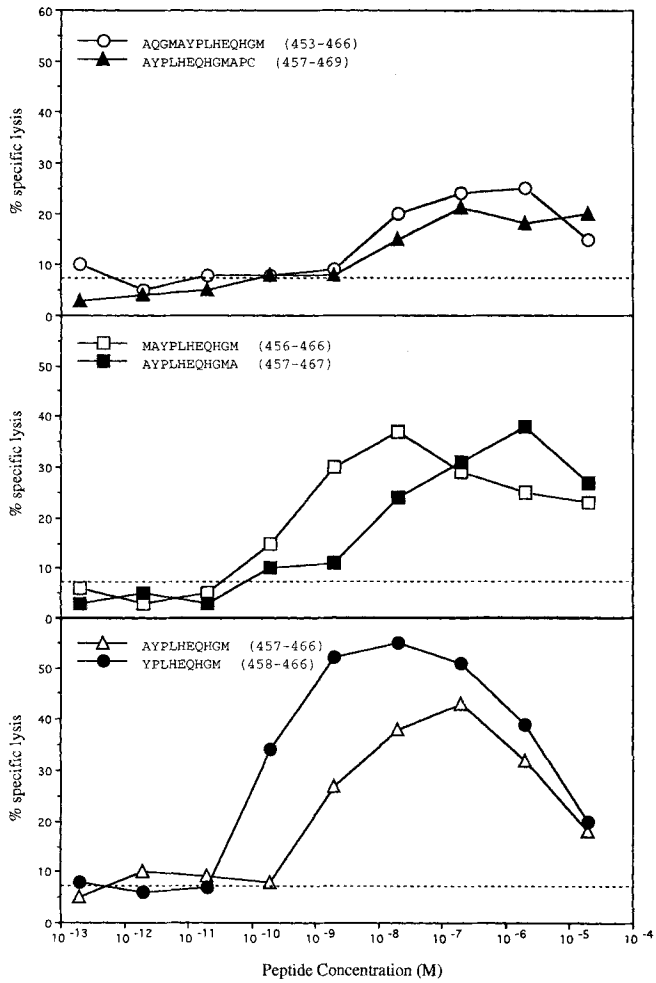

Figure 3. Determining the minimal target epitope by titration of different length peptides from the EBNA 3A epitope region. Polyclonal CTL from donor 1 were tested on B35-matched PHA blast targets pre-exposed to a series of tenfold dilutions of peptides covering the EBNA 3A epitope region. Peptide sequences (with amino acid residue numbers) are shown. Results are shown as percent specific lysis observed in a 5-h chromium release assay at an effector/target ratio of 10:1; the dotted line represents the mean level of background lysis for targets pre-exposed to an equivalent dilution of DMSO solvent as a control. For clarity, the data from one representative experiment are presented on three graphs.

3.2 Analyzing conservation/mutation of the HLA B35.01-restricted epitope in a range of EBV isolates

The B35.01-restricted target epitope described above was identified using CTL stimulated with the reference type 1 EBV isolate B95.8. We next sought to determine to what extent this epitope was conserved amongst other EBV isolates, and began by comparing the B95.8-derived epitope sequence with the corresponding sequence in EBNA 3A of the reference type 2 EBV isolate Ag876 [10]. This showed three amino acid substitutions relative to the B95.8 sequence, with a tyr → his mutation at position 1, a glu → gln mutation at position 5 and a gly → ser mutation at position 8. To assess the effect of such changes on CTL recognition we synthesized three 9-mer peptides, each carrying one of these amino acid substitutions, plus a 9-mer representing the Ag876 epitope sequence itself. B95.8-reactivated CTL were then tested for their ability to lyse B35.01-matched PHA blasts preexposed to tenfold dilutions of these peptides. The results from one such assay are summarized in Table 2, showing the maximum levels of target cell lysis obtained with each peptide and the minimum peptide concentration required to achieve half maximal lysis. The amino acid substitutions at positions 1 or 8 had very little effect by themselves on CTL recognition, whereas the single mutation at position 5 (glu → gln) increased 500-fold the concentration of peptide required to achieve half maximal lysis. However, when targets were preexposed to a peptide representing the Ag876 epitope sequence which combines all three of these mutations, CTL recognition was almost completely eliminated. These results suggests that B35.01-restricted CTL targeting the EBNA 3A 458–466 epitope are type 1 virus-specific, and this was indeed confirmed when such effectors were tested on paired B95.8 and Ag876 virus-transformed LCL targets (data not shown).

Table 2. Titration of peptides representing the EBNA 3A 458–466 epitope sequence carried by the standard type 1 (B95.8) and type 2 (Ag876) EBV isolates

EBV type	Peptide sequence	Max. % lysis[a)]	Min. peptide concentration for half max. lysis
Type 1	Y P L H E Q H G M	55	10^{-10} M
	H P L H E Q H G M	53	3×10^{-9} M
	Y P L H **Q** Q H G M	52	2×10^{-7} M
	Y P L H E Q H **S** M	52	3×10^{-9} M
Type 2	**H** P L H **Q** Q H **S** M	18	$>10^{-6}$ M

a) Maximum % specific lysis observed of B35-matched PHA blasts pre-exposed to tenfold dilutions of peptide (2×10^{-5} M to 2×10^{-13} M) and tested in a 5-h chromium-release assay with B95.8-reactivated CTL from donor 1 (effector/target ratio = 10:1).

We then turned to the analysis of the B35.01 epitope in type 1 virus isolates from different world populations, with special emphasis on populations with a high frequency of the B35 allele. In this context, previous work had shown that approximately 30% of the Gambian population are B35-positive by serological typing [15] and that the great majority of these (>90%) appear to be B35.01-positive by PCR analysis using a B35 subtype-specific probe (A. V. S. Hill, personal communication). Furthermore, type 1 viruses are clearly prevalent within the Gambian population since all of the LCL of Gambian origin that were available to us (derived by spontaneous *in vitro* transformation from the circulating lymphocytes of Gambian donors) carried type 1, as opposed to type 2, EBV strains. Alongside these Gambian isolates, we also included isolates from an East African population (Kenya) where a lower proportion (15%) is B35.01-positive (A. V. S. Hill, personal communication) and from South East Asian populations (Southern China and New Guinea) where the B35 allele is very rare [16].

Altogether, the relevant region of the EBNA 3A gene was amplified by PCR and sequenced from two Caucasian, eleven Gambian, eight Kenyan and five South East Asian isolates. Table 3 summarizes the sequence data obtained across the EBNA 3A 458–466 epitope. We found that six of the Gambian strains retained the B95.8 (Caucasian) epitope sequence, whilst three showed a Tyr → Asn change at position 1 and two showed a Pro → Ser change at position 2. Of the Kenyan isolates, three retained the B95.8

Table 3. Sequence of the B35.01-restricted CTL epitope region of EBNA 3A (residues 458–466) in geographically distinct type 1 EBV isolates

Isolate	Origin	B35.01 Epitope Sequence [a]
B95-8	Caucasian	
RT	Caucasian	
GA1	Gambian	
GA2	Gambian	TAC CCA TTA CAT GAA CAA CAC GGC ATG
GA3	Gambian	
GA4	Gambian	Y P L H E Q H G M
GA5	Gambian	
GA6	Gambian	
ODH	Kenyan	
OGA[b]	Kenyan	
MKN[b]	Kenyan	
GA7	Gambian	AAC CCA TTA CAT GAA CAA CAC GGC ATG
GA8	Gambian	N* P L H E Q H G M
GA9	Gambian	
GA10	Gambian	TAT TCA TTA CAT GAA CAA CAC GGC ATG
GA11	Gambian	Y S* L H E Q H G M
NYA	Kenyan	
IGU	Kenyan	
CHG	Kenyan	TAC ACA TTA CAT GAA CAA CAC GGC ATG
WAI	Kenyan	Y T* L H E Q H G M
MTK	Kenyan	
QX	Chinese	
QY	Chinese	
DH	Chinese	TAC CCA TTA CAT GAA CAA CAC GGC AGG
WW1	New Guinean	Y P L H E Q H G R*
H17	New Guinean	

a) Nucleotide changes with respect to the B95.8 sequence are underlined; amino acid changes are marked with an asterisk.
b) Two EBV isolates showed a conservative mutation (CAA → CAG) relative to B95.8 in codon 6 of the epitope region.

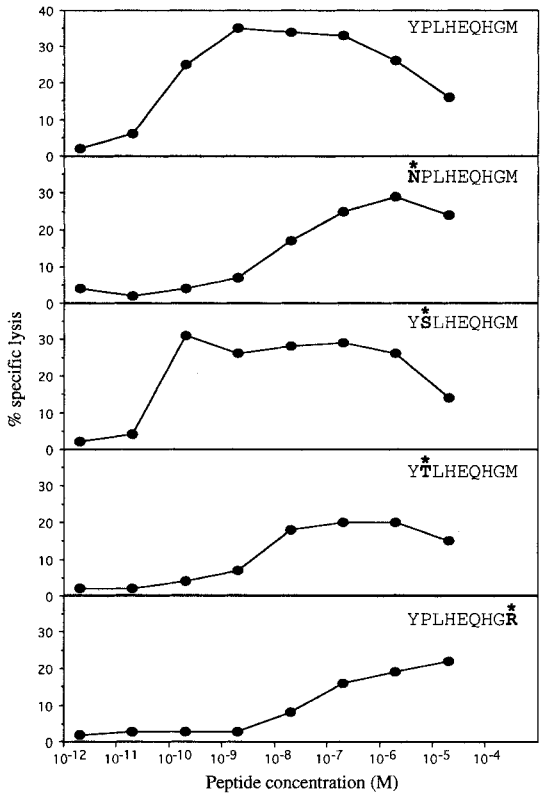

Figure 4. CTL recognition of the YPLHEQHGM peptide epitope (amino acid residues 458–466) from the B95.8 EBNA 3A sequence and of variant 9-mer peptide sequences from other EBV isolates. Effector CTL from donor 1 were tested as in Fig. 3 on B35.01-matched PHA blasts pre-exposed to a series of tenfold peptide dilutions. The amino acid changes relative to the B95.8 sequence are marked with an asterisk. For clarity, the data from one representative experiment are presented on five graphs.

sequence, whilst the rest showed either a Pro → Ser or a Pro → Thr change at position 2. One S. E. Asian isolate showed the same Pro → Thr change but the remaining four carried a different mutation changing Met → Arg at position 9. To determine the extent to which these changes affected CTL recognition, peptides representing each of the four variant sequences were synthesized and compared against the B95.8 sequence in peptide sensitization assays using B95.8-reactivated CTL effectors. Fig. 4 shows the pattern of results reproducibly obtained in such experiments. The Pro → Ser mutation at position 2 had little if any effect on CTL recognition, whereas peptides with the Tyr → Asn mutation at position 1, the Pro → Thr mutation at position 2 or the Met → Arg mutation at position 9 were all recognized 100-1000 fold less efficiently than the B95.8 sequence. Nevertheless, by these criteria none of the EBV isolates tested carried an epitope sequence that completely abrogated CTL recognition. Thus, despite the relatively high frequency of HLA B35.01 in The Gambia, all EBV isolates sequenced from this population carried a B35.01-restricted epitope that to various degrees was recognized by B95.8-reactivated CTL.

3.3 Studies with West African responders

We next sought to determine whether the B35.01-restricted EBNA 3A epitope actually serves as a target for CTL

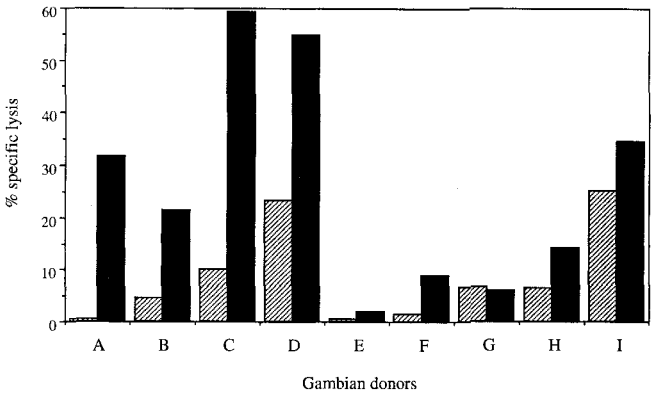

Figure 5. CTL specific for the YPLHEQHGM peptide epitope can be reactivated from the blood of B35.01-positive Gambian donors. PBMC from B35.01-positive Gambian donors A to I were stimulated *in vitro* with the B95.8 EBNA 3A peptide epitope and 10–12 days later tested in a cytotoxicity assay against B35.01-matched LCL targets (▨) and against the same targets pre-exposed to a 10^{-5} M concentration of the YPLHEQHGM peptide (■). The results represent percent specific lysis observed in a 5-h chromium-release assay and effector : target ratios usually were between 20 : 1 and 80 : 1 depending upon the number of effector cells available. Levels of lysis in excess of 20% are considered significant.

responses within the Gambian population. To address this issue specifically, we elected to stimulate PBMC with the B95.8 epitope peptide rather than with the autologous LCL. Preliminary studies on relevant Caucasian donors indicated the feasibility of this approach and it was, therefore, used to screen nine B35.01-positive Gambian donors. Freshly isolated PBMC were pulsed with peptide, then washed and cultured for 10–12 days (with IL-2 added on day 3) before harvesting to provide effector cells for cytotoxicity assays. Fig. 5 shows the results in such experiments, in each case testing the effectors both against a B35.01-matched target LCL and against the same target preincubated with the epitope peptide. This was carried out because, in other contexts, we have found that lysis of LCL targets by EBV-specific effectors is often improved by preincubation of these targets with the cognate peptide (A. Hill, S. P. Lee et al., submitted). At least four of the Gambian donors tested (A–D) gave evidence of an epitope-specific response, and another one (I) showed significant lysis of the LCL target that was slightly improved in the presence of peptide.

4 Discussion

Recent studies on the CTL response to EBV have identified a number of immunodominant target epitopes in the viral proteins expressed during latent infection [5, 7, 8]. One unusually strong epitope, located at residues 416–424 in EBNA 3B and presented in association with HLA A11, is highly conserved amongst most type 1 EBV isolates of different geographic origins but is selectively mutated in isolates from New Guinea and China, where HLA A11 is very common [11, 12]. This raised the possibility that such "epitope loss" mutants may have enjoyed a selective advantage in these particular populations. To investigate further the possible influence of CTL on the evolution of EBV, we sought to identify the target epitope(s) recognized in association with a second HLA allele, HLA B35.01, which also shows significant differences in prevalence between different human populations.

Using B35.01-positive Caucasian donors and stimulating *in vitro* with cells carrying the reference type 1 EBV isolate B95.8, we detected a B35.01-restricted CTL response which reproducibly mapped to a single EBV latent protein. EBNA 3A, in all five individuals tested. Using overlapping synthetic peptides representing the entire B95.8 EBNA 3A protein sequence, we then showed that the response focused on a peptide epitope YPLHEQHGM (residues 458–466) that was recognized by all of the donors; recognition of this same epitope by a B35.01-restricted CTL clone has also been recently reported elsewhere [24]. Work in other viral systems first showed that the anti-viral CTL response is often dominated by reactivities to a few immunodominant epitopes, with MHC type acting as a key determinant of target epitope choice [25, 26]. The identification of one dominant B35.01-restricted CTL epitope in EBNA 3A re-emphasizes the degree to which EBV-induced CTL responses are also concentrated on a small number of epitope peptides [3, 4, 8, 9] even though the transformed B cells which elicit this response express at least eight potentially immunogenic viral proteins differing in size from 40–140 kDa [27].

The target epitope that we have identified (YPLHEQHGM) accords well with two recent reports describing peptide binding motifs for HLA B35.01 [28, 29]. Thus, the epitope is nine amino acids long with a Pro residue at the important anchor position 2. Describing the first of these motifs, Hill et al. [28] reported that the only residue favored at the other anchor position 9, was Tyr, whereas our findings are in line with the suggestion of Falk et al. [29] that this position can, in addition to Tyr, also accommodate hydrophobic residues (Phe, Met, Leu, Ile). Of the other positions, Tyr at 1, Leu at 3, Glu at 5 and Gln at 6 have also been found in peptide sequences naturally eluted from B35.01 [28, 29]. We have in fact observed strong binding of the B95.8 epitope peptide to B35.01 molecules in an HLA refolding assay [30] using a B35.01 transfectant of the processing mutant LCL, T2 (unpublished observations). Interestingly, the corresponding sequence of EBNA 3A in type 2 EBV (Ag876) is mutated at positions 1, 5 and 8 and parallel refolding assays with this peptide indicated much reduced binding to B35.01; certainly there was little or no cross-recognition of this Ag876 peptide by type 1-reactivated CTL, the change from Glu → Gln at position 5 being apparently the most important mutation in this respect. From these results, we infer that the EBNA 3A 458–466 region is unlikely to constitute a strong B35.01-restricted epitope in type 2 viral strains.

Our specific interest in viral isolates from the Gambia was encouraged by two prior observations. One was that the frequency of B35.01 in the Gambian population is unusually high, with some 30% of the population possessing this allele [15]. The other was that the EBV isolates resident in that population appear to be predominantly type 1, at least on the basis of the virus strains present in LCL derived from Gambian donors by spontaneous transformation. The greater *in vitro* transforming ability of type 1 as compared to type 2 EBV strains [31] introduces the possibility of some bias towards the former in virus isolations based on LCL

outgrowth; however, the fact that all of the available LCL carried type 1 virus strongly suggests that in the Gambia, as observed elsewhere in equatorial Africa [32], this virus type is in the majority. We, therefore, felt that the analysis of Gambian viral isolates for conservation/mutation of the B35.01-restricted type 1 epitope sequence was a valid extension of the earlier work based on the A11-restricted type 1 epitope sequence in South East Asian isolates.

The results show that the B35.01 epitope was in fact conserved in six out of eleven Gambian isolates studied. Of the remainder, two isolates showed a Pro → Ser mutation at position 2 which did not appear to affect antigenicity (note that Ser at position 2 has also been observed in a B35.01-restricted CTL epitope in *Plasmodium falciparum* [28]) while three isolates showed a Tyr → Asn mutation at position 1 which reduced recognition significantly in peptide sensitization assays. However, we never observed changes which totally destroyed antigenicity. This stands in contrast to the findings made on the A11 epitope in South East Asian viruses where all of the isolates tested were specifically mutated in key residues (with a range of different mutations observed) and all displayed complete epitope loss. Interestingly, in the present work, the best example of reduced antigenicity in the B35.01-restricted epitope (a Met → Arg mutation at anchor position 9) was observed in four out of five South East Asian isolates. Such a change is unlikely to have arisen from immune CTL pressure, since B35.01 is very rare in South East Asian populations, and is probably coincidental, representing another of the increasing number of sequence polymorphisms which now appear to distinguish South East Asian viruses from Caucasian and African isolates [9, 33, 34].

Although the sequence data suggested that antigenicity of the B35.01-restricted epitope was largely conserved in isolates from The Gambia, it remained important to determine whether or not this epitope actually served as a target for CTL responses in that population. We therefore attempted to screen B35.01-positive Gambian donors for evidence of epitope-specific CTL memory by short-term *in vitro* stimulation of PBMC with the epitope peptide. Preliminary studies indicated that peptide-specific responses could indeed be elicited in this way from some of our EBV-immune B35.01-positive Caucasian donors; moreover such an approach has been used successfully to reactivate HIV-specific memory CTL from the blood of HIV-infected individuals without ever eliciting a primary response in PBMC cultures from unexposed donors (S. Rowland-Jones et al, submitted). Stimulation with peptide rather than with the LCL was also more easily applicable in the context of a field study in the Gambia since it allowed us to test individuals without requiring prior establishment of their autologous LCL. Although the present study was limited in scope, we did observe clear evidence of a CTL response to the B95.8 epitope peptide in several B35.01-positive Gambian donors as measured by effector cell recognition of peptide-coated *versus* uncoated LCL targets. Note that the ability of the cognate peptide to enhance lysis even of LCL cells already expressing the EBV target antigen is not just a feature of peptide-induced effectors but can often be observed with EBV-specific CTL generated by conventional LCL stimulation (A. Hill, S. P. Lee et al., submitted). We, therefore, feel that the peptide stimulation results genuinely reflect the presence of epitope-specific CTL memory in Gambian individuals, thus confirming that many Gambian EBV isolates are capable of eliciting a B35.01-restricted response.

The evidence for CTL responses influencing the evolution of EBV isolates in particular communities is therefore much less clear in the context of B35-positive West African populations than of A11-positive South East Asian populations. The present discordant results do not necessarily invalidate the concept of immune selection, however, since there are some potentially important quantitative differences between the B35.01-based and the A11-based studies. In particular, the relative frequency of the B35.01 allele in The Gambia is only half that of the A11 allele in South East Asia where, remarkably, >50% of all individuals are A11-positive [12, 15]. Equally important may well be the relative strength of the B35.01- and A11-restricted responses. In this regard, work to date with Caucasian donors indicates that A11-positive individuals mount unusually strong memory CTL responses to EBV with A11-restricted EBNA 3B epitope-specific clones constituting one of the major reactivities [7]. By comparison, B35.01-restricted responses, though frequently detectable in individuals with this allele, tend not to be as strong, at least as measured in polyclonal effector populations reactivated *in vitro* ([21] and data not shown). More recently, we have begun to study the fine specificity of the primary CTL response to EBV infection (*i.e.* the point at which CTL-mediated immune pressure on competing virus strains might be exerted) and have again found dominant A11-restricted reactivities in PBMC effectors tested immediately *ex vivo* but, to date, much weaker B35-restricted killing (N. M. Steven et al., data to be published). In the context of the earlier A11-based studies [11, 12], therefore, it may have been the combination of these two special features, the extremely high frequency of the allele in South East Asian populations and the extremely potent nature of A11-restricted responses to many type 1 virus isolates, which allowed the longer-term effects of CTL-mediated immune pressure to be observed.

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