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Unusually High Frequency of Epstein-Barr Virus Genetic Variants in Papua New Guinea That Can Escape Cytotoxic T-Cell Recognition: Implications for Virus Evolution

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Cytotoxic T lymphocytes (CTLs) which recognize viral antigens in association with human leukocyte antigens (HLAs) play an important role in controlling persistent virus infections. These viruses use several mechanisms to evade the immune response, including mutations that affect either T-cell receptor recognition or binding of viral epitopes to the HLA. It has recently been proposed that the distribution of HLA frequencies and the specific CTL response may influence the long-term evolution of Epstein-Barr virus (EBV) by selecting variants which lack immunodominant CTL epitopes. To test this hypothesis, we have studied EBV isolates from two genetically distinct Papua New Guinea (PNG) populations, residing in coastal and highland regions, for polymorphism within seven viral CTL epitope sequences restricted through several class I HLAs. Surprisingly, all EBV isolates analyzed displayed identical amino acid substitutions within HLA A11-, B35-, and B8-restricted CTL epitope sequences which completely abrogated CTL recognition and binding of synthetic peptides to HLA molecules. Furthermore, these substitutions revealed no correlation with the contemporary distribution of HLAs in the different PNG populations, which argues for a minimal influence of immune pressure. The sequence homology between EBV isolates from coastal and highland PNG suggests that the virus may have had a single origin and, more importantly, that these isolates are genetically distinct from those present in a Caucasian population.

The importance of major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTLs) in controlling persistent viral infection is well established (7, 17, 22, 33). The potency of this antiviral immune response is such that the persistence of many viruses in the immunocompetent host relies on some form of immune escape. Evasion of the immune response often involves alteration in amino acid sequences within epitopes recognized by CTLs. These epitope changes could block correct endogenous processing of the antigen, MHC binding, and/or optimal recognition of the peptide-MHC complex by T-cell receptors (3, 19, 23, 24). Indeed, in genetically unstable viruses such as human immunodeficiency virus and lymphocyte choriomeningitis virus, epitope loss variants have often been isolated from individual hosts (8, 23, 24). It is not clear at this stage the degree to which CTL responses influence the long-term evolution of genetically more stable viruses. This question has been recently brought into focus by observations involving Epstein-Barr virus (EBV), a herpesvirus with a long-established association with *Homo sapiens* and a well-defined CTL response (11, 12). These studies showed that natural EBV isolates from the highly human leukocyte antigen (HLA) A11-positive coastal Papua New Guinea (PNG) population consistently display variations within CTL epitope sequences restricted through HLA A11, and thus it was inferred that the epitope loss had conferred a selective advantage to variant isolates in this particular host community.

PNG is ideally suited for undertaking studies of this type, since different communities within the country show pronounced genetic differentiation over short geographical distances (15, 18, 28, 30). These differences are exemplified by the frequency distributions of HLAs A11, A24, and B27, which show significant spatial heterogeneity between coastal and highland populations (4, 29). For example, HLA A11 is considered a marker for the coastal population, where its frequency is >50%, whereas its frequency in the highlands is <5% (9, 29, 31). This contrast in frequency distribution between PNG populations provides an excellent opportunity to further investigate the hypothesis that HLA diversity and CTL responses may influence the long-term evolution of genetically stable viruses such as EBV. To address this issue, we have analyzed sequence polymorphism within seven different CTL epitopes restricted through a range of class I HLAs, including HLA A11, using EBV isolates from the coastal and highland regions of PNG. This analysis shows that EBV isolates from the two regions display unusually high frequencies of identical amino acid substitutions within CTL epitope sequences with no apparent correlation with the distribution of HLA frequencies. These observations also suggest that EBV infection in PNG populations (both coastal and highland) is dominated by a single virus strain which is genetically distinct from isolates from a Caucasian population.

MATERIALS AND METHODS

Virus isolates from Caucasian and PNG donors. Lymphoblastoid cell lines (LCLs) were established from a panel of unrelated healthy, EBV-seropositive Caucasian (Australian) and PNG (coastal and highland) donors by spontaneous outgrowth from peripheral lymphocytes cultured in the presence of 0.1 μg of cyclosporin A per ml (25). A total of 28 spontaneous LCLs (10 Caucasian, 10 highland PNG, and 8 coastal PNG) were used to recover the resident EBV isolate from each individual. In addition, two virus isolates from coastal PNG were recovered from Burkitt's lymphoma cell lines. These isolates were classified as type 1 EBV on the basis of the DNA sequence divergence within the *Bam*HI WYH and E regions of the genome (26, 27). Coastal PNG samples were obtained from the Madang Province (Madang) and the East Sepik Province (Wewak), while the highland samples were collected from the Eastern Highlands Province (Goroka), between 1967 and 1985. To ensure the origins of the virus isolates, all of the donors confirmed their places of birth and present residence.

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Consistent with published HLA frequencies, none of the spontaneous LCLs from the highland individuals were HLA A11 positive, while four of the spontaneous LCLs from coastal donors expressed this antigen. LCLs were also established by exogenous virus transformation of peripheral B cells using type 1 (B95.8 and IARC-BL74) or type 2 (Ag876) EBV isolates (22). Cell lines were routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml plus 10% fetal calf serum (growth medium).

PCR and DNA sequencing of CTL epitopes. A total of seven MHC class I-restricted CTL epitopes from different EBV latent antigens were included in this study (5, 6, 13, 14, 16) (Table 1). Specific oligonucleotide primers flanking the DNA region encoding each of these epitopes were selected for PCR amplification (Table 1). The resulting PCR products were purified with QIAquick spin columns (Qiagen, Inc., Chatsworth, Calif.) and sequenced in both directions by using a PRISM ready reaction Dyedeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) according to the manufacturer's protocol.

Source and generation of EBV-specific CTL clones. EBV-specific CTL clones recognizing epitopes listed in Table 1 were established from healthy, seropositive donors as described previously (3). The EBV and antigen specificities of each CTL clone were confirmed by using autologous LCLs (transformed with either B95.8 or IARC-BL74 virus) and recombinant vaccinia virus constructs encoding individual EBV antigens (16). The specificity of each clone was also determined by using overlapping peptides from the relevant target antigen.

Synthesis of peptides. Peptides synthesized by the Merrifield solid-phase method (34) were purchased from Chiron Mimotopes (Melbourne, Australia), dissolved in dimethyl sulfoxide, and diluted in serum-free RPMI 1640 medium for use in standard CTL assays.

Cytotoxicity assay. Each of the peptide epitopes listed in Table 1 and peptides based on the wild-type variants from spontaneous LCLs were titrated on autologous phytohemagglutinin (PHA) blast target cells for CTL recognition. Briefly, ^{51}Cr-labelled target cells (PHA blasts) were preincubated for 1 h with peptide epitope serially diluted at 10-fold concentrations. Following peptide sensitization, target cells were exposed to the respective EBV-specific CTL clone and specific lysis was determined.

MHC stabilization assays. To assess MHC binding by the HLA B35-restricted epitope variants from PNG and Caucasian isolates, T2.B35 cells (2×10^5) were incubated with 200 μl of each of the peptides (200 μg/ml) at 26°C for 14 to 16 h and then incubated at 37°C for 2 to 3 h. After the incubations, HLA B35 expression was measured by fluorescence-activated cell sorter (FACS) analysis using a monoclonal HLA Bw6 antibody (SFR8 Bw6).

RESULTS

To obtain insight into the influence of HLA frequencies and CTL responses on the evolution of EBV, isolates from PNG and Caucasian donors were sequenced in the region of seven MHC class I-restricted CTL epitopes (Table 1). The nucleotide and deduced amino acid sequences were determined and compared with those of the B95.8 or IARC-BL74 isolate, used to define the reference EBV epitopes.

Sequence analysis of HLA A11-restricted epitopes in virus isolates from PNG populations displaying different HLA A11 frequencies. Previous studies have suggested that immune pressure within the coastal PNG population has selected variant EBV isolates lacking immunodominant HLA A11-restricted CTL epitopes (11, 12). To further evaluate the influence of HLA frequencies on the evolution of EBV, we have taken advantage of the low frequency of HLA A11 (<5.0%) in the PNG highlands. Spontaneous LCLs were established from highland donors, and the relevant HLA A11-restricted CTL epitope sequences (IVTDFSVIK and AVFDRKSDAK) from EBV nuclear antigen 4 (EBNA4) were compared with corresponding sequences for coastal PNG and Caucasian virus isolates. Consistent with earlier findings, all EBV isolates from the coastal region of PNG showed changes in these two epitope regions (Table 2). However, sequencing of EBV isolates from the highland region also revealed identical substitutions within HLA A11-restricted epitopes (Table 2). In all PNG isolates, lysine at position 9 of the IVTDFSVIK epitope is substituted with threonine, while the AVFDRKSDAK epitope is altered at positions 1 and 2 from alanine and valine to serine and leucine, respectively (Table 2). Furthermore, immunological recognition analysis of these variant sequences, used as synthetic peptides, revealed dramatically reduced target cell lysis by HLA A11-restricted, EBV-specific CTL clones raised against the B95.8 isolate (Fig. 1).

Sequence analysis of the HLA A11 epitopes in Caucasian EBV isolates also revealed interesting results. Only three Caucasian isolates showed the prototypic B95.8 sequence for the IVTDFSVIK epitope, whereas the remaining isolates displayed changes affecting one or more of the anchor residues (Table 2). In three isolates, lysine at position 9 was substituted with asparagine, while in the remaining isolates valine at position 2 and/or lysine at position 9 was substituted with leucine and/or arginine, respectively (Table 2). It is important to note here that changes within the IVTDFSVIK epitope, seen in Caucasian isolates, have also been reported previously for EBV isolates from Southeast Asian populations (11). Although substitution of arginine for lysine at position 9 (IVTDFSVI<u>R</u>) had a minimal effect on peptide-mediated CTL recognition, other variant peptides (IVTDFSVI<u>N</u>, I<u>L</u>TDFSVIK, and I<u>L</u>TDFSVI<u>R</u>) were significantly less efficient at sensitizing

TABLE 1. EBV CTL epitopes used in this study to investigate sequence polymorphism

CTL epitope sequence	HLA restriction	EBV antigen specificity	EBV type specificity	Primers used for PCR	Primer positions in EBV genome (nt)
IVTDFSVIK[a]	A11	EBNA4	1	TTGTTGAGGATGACGACG	96494–96511
				CAGTAGGGTTGCCATAAC	97005–96988
AVFDRKSDAK[a]	A11	EBNA4	1	TTGTTGAGGATGACGACG	96494–96511
				CAGTAGGGTTGCCATAAC	97005–96988
FLRGRAYGL[b]	B8	EBNA3	1	TGGGCACCACTAGTATCCAA	93226–93245
				TTTCACCGGTAGCACCTTCG	93378–93359
YPLHEQHGM[c]	B35	EBNA3	1	GACGAGACAGCTACCAG	93624–93640
				GAGATACAGGGGGCAAG	93780–93764
QAKWRLQTL[c]	B8	EBNA3	1	ATGTATGCCATGGCCATTCGA	92736–92756
				AGATGTACGAATGTGGGAGTC	92917–92897
EENLLDFVRF[d]	B44	EBNA6	1 and 2	TGTGTGGCTCTCTGCACCAC	99241–99260
				GATTGTCTTGTGAAACCAGT	99374–99355
QPRAPIRPIPT[e]	B7	EBNA6	1 and 2	AGGCCCACCACGTCTTCAAC	101015–101035
				ATGCTGTACCTGATGAGTCA	101187–101168

[a] Reference 22.
[b] Reference 23.
[c] Reference 24.
[d] Reference 25.
[e] Reference 26.

TABLE 2. Sequences of HLA A11-restricted epitopes in EBV isolates from PNG and Caucasian individuals

Virus origin	Epitope sequence[a]	HLA A11 restriction and frequency (%)	No. of isolates
B95.8	^{96676}ATA GTA ACT GAC TTT AGT GTA ATC AAG^{96702} I V T D F S V I K		
Highland PNG	--- --- --- --- --- --- --- --- -C- - - - - - - - - T	3.0	10
Coastal PNG	--- --- --- --- --- --- --- --- -C- - - - - - - - - T	54.0	7
Caucasian	--- --- --- --- --- --- --- --- --- - - - - - - - - -	11.0	3
	--- --- --- --- --- --- --- --- --T - - - - - - - - N		3
	--- T-- --- --- --- --- --- --- --- - L - - - - - - -		2
	--- --- --- --- --- --- --- --- -G- - - - - - - - - R		1
	--- T-- --- --- --- --- --- --- -G- - L - - - - - - R		1
B95.8	^{96625}GCG GTG TTT GAC CGA AAG TCA GAT GCA AAA^{96654} A V F D R K S D A K		
Highland PNG	T-- T-- --- --- --- --- --- --- --- --- S L - - - - - - - -		9
	T-A T-- --- --- --- --- --- --- --- --- S L - - - - - - - -		1
Coastal PNG	T-- T-- --- --- --- --- --- --- --- --- S L - - - - - - - -		9
Caucasian	--- --- --- --- --- --- --- --- --- --- - - - - - - - - - -		6
	T-- T-- --- --- --- --- --- --- --- --- S L - - - - - - - -		3

[a] Dashes indicate identity.

HLA A11-positive PHA blasts to CTL lysis (Fig. 1a). Sequencing of the AVFDRKSDAK epitope region in the Caucasian isolates revealed three isolates with amino acid changes identical to those found in PNG isolates (Table 2).

Sequence analysis of multiple CTL epitopes in EBV isolates from PNG populations. Since the results described above strongly suggest that epitope loss is not related to the frequency of HLA A11 in the different geographical regions, we extended our analysis to include five other CTL epitopes restricted through either HLA B8, B35, B44, or B7 (Table 1). It is important to mention here that most of these HLAs are either absent or present at a very low frequency within PNG

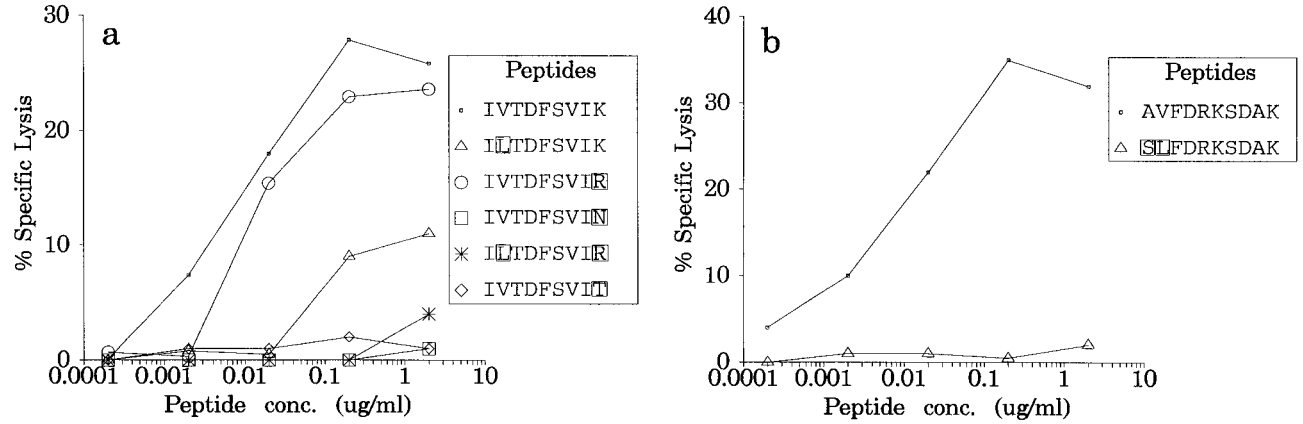

FIG. 1. CTL recognition of variant and prototypic HLA A11-restricted EBV epitopes. PHA blasts were sensitized with serial dilutions of each of the peptides and then exposed to either the IVTDFSVIK-specific CTL clone CM9 (a) or the AVFDRKSDAK-specific CTL clone CM29 (b).

populations (9, 29, 31). Surprisingly, sequence analysis of the HLA B35-restricted epitope YPLHEQHGM and the HLA B8-restricted epitope FLRGRAYGL showed changes affecting anchor residues in both highland and coastal PNG isolates (Table 3). In the case of the HLA B35-restricted epitope, all PNG isolates carried a substitution from methionine to arginine at position 9. The FLRGRAYGL epitope region at positions 1 and 9 was altered from phenylalanine and leucine to leucine and glutamine, respectively (Table 3). Interestingly, these changes in the HLA B8-restricted epitope matched the amino acid sequence (but not the nucleotide sequence) present in type 2 isolates of EBV (26) (Table 3). Moreover, of the seven nucleotide substitutions identified in this study within all the CTL epitope regions in these type 1 PNG isolates (Tables 2 and 3), three matched those present in type 2 EBV (26). In addition, comparison of nucleotide sequences flanking these epitope regions revealed two identical substitutions (A→C at position 93746 and T→G at position 96568) in all PNG isolates. The latter substitution matched the type 2 EBV sequence.

We then tested the ability of variant peptides to sensitize HLA B35-positive and HLA B8-positive PHA blasts to recognition by CTL clones raised against the YPLHEQHGM and FLRGRAYGL epitopes, respectively. Synthetic peptides with the substitutions identified in PNG isolates displayed significantly reduced CTL recognition compared with that of the cognate wild-type peptides (Fig. 2). Moreover, a synthetic peptide with the variant sequence YPLHEQHGR was unable to rescue MHC expression on T2.B35 cells in a peptide stabilization assay and therefore does not bind efficiently to HLA B35 (Fig. 3). This variant has also been reported in EBV isolates from Southeast Asian populations, among whom B35 is not common (21).

Although no sequence variation was seen within the FLR GRAYGL epitope in any Caucasian isolate, alterations in the YPLHEQHGM epitope were common (Table 3). They included changes of proline to threonine at position 2 in three isolates, glutamic acid to lysine at position 5 in three isolates, and histidine to arginine at position 7 in one isolate. Synthetic peptides with these substitutions were significantly less efficient than the prototypic wild-type YPLHEQHGM peptide at sensitizing autologous PHA blasts to lysis by specific CTL clones. Data for one such clone are shown in Fig. 2a. Furthermore, incubation of T2.B35 cells with the variant peptide YTLHE QHGM was unable to rescue HLA B35 expression. Other variant peptides (YPLHKQHGM and YPLHEQRGM), however, significantly increased MHC expression on these cells (Fig. 3), suggesting that the loss of antigenicity of these variants is due to inappropriate T-cell receptor interaction with the MHC-peptide complex rather than loss of MHC binding. Three other EBV CTL epitopes, restricted through either HLA B8 (QAKWRLQTL), HLA B44 (EENLLDFVRF), or

TABLE 3. Sequences of HLA B35 and HLA B8-restricted epitopes in EBV isolates from PNG and Caucasian individuals

Virus or origin	Epitope sequence[a]	HLA restriction and frequency (%)[b]	No. of isolates
B95.8	^{93702}TAC CCA TTA CAT GAA CAA CAC GGC ATG^{93728} Y P L H E Q H G M	B35	
Highland PNG	--- --- --- --- --- --- --- --- -G- - - - - - - - - R	0.0	9
Coastal PNG	--- --- --- --- --- --- --- --- -G- - - - - - - - - R	0.0	8
Caucasian	--- --- --- --- --- --- --- --- --- - - - - - - - - -	7.1	2
	--- A-- --- --- --- --- --- --- --- - T - - - - - - -		3
	--- --- --- --- A-- --- --- --- --- - - - - K - - - -		3
	--- --- --- --- --- --- -G- --- --- - - - - - - R - -		1
IARC-BL74	^{93303}TTT CTC CGG GGT CGT GCG TAT GGG CTA^{93329} F L R G R A Y G L	B8	
Ag876[c]	--G --- --- --G --- --- --C --- -A- L - - - - - - - Q		
Highland PNG	--G --- --- --- --- --- --- --- -A- L - - - - - - - Q	0.0	10
Coastal PNG	--G --- --- --- --- --- --- --- -A- L - - - - - - - Q	0.0	9
Caucasian	--- --- --- --- --- --- --- --- --- - - - - - - - - -	12.8	8

[a] Dashes indicate identity.
[b] Frequency for HLA B35 and B8 in different populations.
[c] Type 2 EBV.

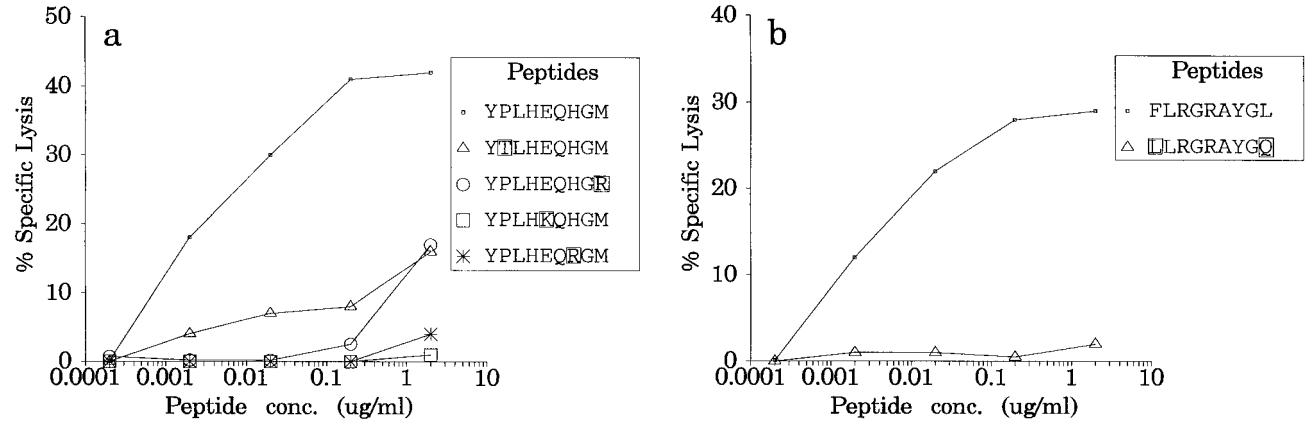

FIG. 2. CTL recognition of variant and prototypic HLA B35- and B8-restricted EBV epitopes. PHA blasts were sensitized with serial dilutions of each of the peptides and then exposed to either the YPLHEQHGM-specific CTL clone NB26 (a) or the FLRGRAYGL-specific CTL clone LC13 (b).

HLA B7 (QPRAPIRPIPT), were unchanged in the PNG isolates in comparison with the prototypic B95.8 isolate (Table 4). It is interesting that CTL epitopes that are conserved in the B95.8 (type 1) and Ag876 (type 2) strains (26, 27) are also invariant in PNG isolates. Although the precise reason for this observation is unclear, it is possible that amino acid substitutions in these conserved regions are incompatible with protein function and viral replication.

DISCUSSION

This study clearly demonstrates that EBV isolates from the coastal and highland regions of PNG carry identical substitutions within CTL epitope sequences irrespective of HLA distribution. In addition, substitutions were also seen in the regions flanking the CTL epitope sequences. Consequently, it is difficult to sustain the argument that CTL-mediated immune pressure and HLA frequencies in PNG have influenced the long-term evolution of the EBV CTL epitopes examined in this study. The importance of the host CTL response in controlling the proliferation of EBV-infected B cells is well established. In clinical conditions in which the CTL response is compromised, the potentially fatal EBV-induced lymphoproliferative disease occurs commonly (10, 32). However, there is no evidence to suggest that PNG highlanders, carrying the epitope loss variants, show an unusually high incidence of EBV-associated diseases compared with other communities of the world. It is likely that the host-virus balance in these individuals is maintained by directing the CTL response to other, yet-undefined epitopes. Since long-term viral persistence is integral to the life cycle of EBV, the evolutionary pressure on the CTL epitopes of the virus may be towards their conservation rather than their inactivation.

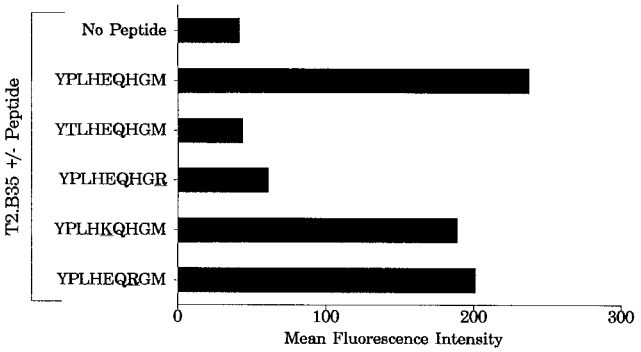

FIG. 3. MHC stabilization analysis with T2.B35 cells using variant and prototypic HLA B35-restricted epitopes. T2.B35 cells were initially incubated with 200 μl of each of the peptides (200 μg/ml) for 14 to 16 h at 26°C and then incubated at 37°C for 2 to 3 h. HLA B35 expression on these cells was analyzed by FACS analysis using SFR8 Bw6 antibody.

An interesting feature of these results is that virus isolates from two different regions of PNG carry identical substitutions, suggesting the wide distribution of a single dominant strain of EBV. The nucleotide substitutions in these type 1 isolates often match those present in type 2 EBV, suggesting that the dominant PNG EBV strain may have evolved along a lineage separate from that of the strains present in Caucasian populations. This is also supported by earlier studies of DNA sequence heterogeneity within the EBNA2 gene of EBV isolates from PNG (1). It is important to extend this analysis to other regions of the EBV genome to determine whether these PNG isolates represent a genetically distinct virus type. In any case, these observations have important implications for the development of a vaccine against EBV in this region.

The presence of identical sequences in virus isolates from the highland and coastal populations of PNG is difficult to accommodate within the framework of current theories on the colonization of and migration into PNG (2, 30). It is now agreed that the PNG population originated from two distinct founder groups arriving in small numbers over time (29, 34). The evidence for this is mainly linguistic but is now well supported by genetic and archaeological evidence. Along with Australia, PNG was first settled by non-Austronesian speakers more than 40,000 years ago, and subsequent migrations occurred over several millennia. Approximately 3,000 to 5,000 years ago, speakers of the Austronesian languages spread from Asia across the Pacific, including the coastal areas of PNG (2). There are two alternative explanations for the presence of a single dominant strain of EBV in PNG. First, it is possible that virus has spread from one of the founder groups into other nonimmune individuals within PNG. However, it is well established EBV infects all human populations, and in traditional societies, lifelong EBV infection and immunity are acquired soon after birth (20). Second, the limited sequence polymorphism is compatible with the notion that the virus variants were introduced by the early Australoids who originally migrated from the Southeast Asian landmass to settle the then-single continent of Australia and New Guinea (called Sahul). Any

TABLE 4. Sequence analysis of CTL epitopes in EBV isolates from PNG individuals

Virus or origin	Epitope sequence[a]	HLA restriction and frequency (%)[b]	No. of isolates
B95.8	[92802]CAG GCC AAA TGG AGA CTG CAA ACC CTG[92828] Q A K W R L Q T L	B8	
Highland PNG	--- --- --- --- --- --- --- --- --- - - - - - - - - -	0.0	9
Coastal PNG	--- --- --- --- --- --- --- --- --- - - - - - - - - -	0.0	10
B95.8	[99285]GAG GAG AAC CTT TTA GAT TTC GTG CGT TTC[99314] E E N L L D F V R F	B44	
Highland PNG	--- --- --- --- --- --- --- --- --- --- - - - - - - - - - -	1.0	9
Coastal PNG	--- --- --- --- --- --- --- --- --- --- - - - - - - - - - -	0.0	10
B95.8	[101085]CAG CCC CGA GCC CCC ATA CGC CCC ATT CCA ACA[101117] Q P R A P I R P I P T	B7	
Highland PNG	--- --- --- --- --- --- --- --- --- --- --- - - - - - - - - - - -	0.0	10
Coastal PNG	--- --- --- --- --- --- --- --- --- --- --- - - - - - - - - - - -	0.0	8

[a] Dashes indicate identity.
[b] HLA allele frequency.

subsequent migrations into PNG were either minor or originated from the same region and carried a similar strain of EBV. Thus, it is possible that the human genetic diversity of the present PNG population has been shaped primarily by the combined effects of genetic drift and natural selection (31). Further epidemiological studies of EBV isolates from other native Pacific communities will be required to distinguish these possibilities.

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