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Evidence for trafficking of Epstein–Barr virus strains between hairy leukoplakia and peripheral blood lymphocytes

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Hairy leukoplakia (HL), an epithelial lesion found on the side of the tongue in immunocompromised individuals, is characterized by high-level replication of Epstein–Barr virus (EBV) and multiple EBV strains. The source of these strains and their relationship to peripheral blood lymphocyte (PBL) strains has not previously been characterized. Using matched pairs of HL scrapings and PBL from 16 HIV-positive men, variation in EBV strain identity was characterized by detection of a 30 nucleotide deletion of the EBV latent membrane protein (LMP)-1 gene, variation in the LMP-1 repeat region and typing for Epstein–Barr nuclear antigen (EBNA)-2. Multiple EBV strains were found in both the HL and PBL specimens, but 13 of 16 (81%) patients showed evidence of strain identity for at least one strain and analysis of two patients suggested that EBV strains from HL could infect the PBL. Our data are consistent with active trafficking of EBV between these two compartments.

Hairy leukoplakia (HL) is a white lesion found on the lateral margin of the tongue. HL is the only epithelial lesion characterized by active virus replication associated with Epstein–Barr virus (EBV), and many of the superficial cells in the lesion contain large numbers of EBV particles in fully replicating form (Greenspan *et al.*, 1985). Multiple EBV strains co-exist within the lesion and there is evidence for both inter- and intra-strain recombination (Palefsky *et al.*, 1996; Walling *et al.*, 1992, 1995; Walling & Raab-Traub, 1994).

The source of EBV strains in HL is unknown. EBV strains in HL may derive from reactivation of latent strains in the tongue epithelium (Brandwein *et al.*, 1996) or they may be acquired *de novo* through contact with saliva containing EBV-positive oropharyngeal cells. EBV strains may also be acquired from the lymphocyte compartment, either through EBV-positive lymphocytes in saliva or through circulating EBV-positive peripheral blood lymphocytes (PBLs). Studies of bone marrow transplant recipients who shed donor EBV strains in their oropharynx have shown that lymphocyte strains may infect the epithelium (Gratama *et al.*, 1988). In contrast, transmission of EBV strains from epithelium to PBLs has not been shown. Earlier studies suggested that primary oropharyngeal EBV infection occurs during infectious mononucleosis with presumed spread to lymphocytes (Sixbey *et al.*, 1984; Allday & Crawford, 1988; Rickinson, 1990). More recently a study failed to show evidence for either latent or replicative EBV infection of the epithelium (Niedobitek *et al.*, 2000).

HL offers a unique opportunity to study the relationship between EBV in the epithelium and PBL compartments because of well-documented long-standing infection at both sites. The aim of this study was to examine the relationship between EBV strains in HL and PBLs of the same subject using different measures of EBV strain polymorphism. EBV strain polymorphism may be examined in a number of ways. We analysed strains for a 30 bp deletion at the 3′ end of the latent membrane protein (LMP)-1 gene, variation in the size of the LMP-1 repeat region and analysed the Epstein–Barr nuclear antigen (EBNA)-2 type. The 30 bp deletion at the 3′ end of LMP has been described in a number of different EBV-associated lesions including HL (Hu *et al.*, 1991; Knecht *et al.*, 1993; Miller *et al.*, 1994; Palefsky *et al.*, 1996). The LMP-1 repeat region consists of a series of 33 nucleotide repeats, with or without a 15 nucleotide insertion, that together constitute a 'repeat region unit' (RRU). An EBV strain may contain different numbers of repeats, resulting in a unique RRU length as detected by Southern blot hybridization. Detection of multiple RRUs in a given sample may reflect infection with multiple EBV strains or intra-strain recombination (Miller *et al.*, 1994; Palefsky *et al.*, 1996; Walling *et al.*, 1995). EBNA-2 polymorphism has been used as a measure of strain identity and classifies EBV types as type 1 or type 2 (Sixbey *et al.*, 1989).

All subjects were HIV-positive men diagnosed with HL by clinical criteria. HL tongue lesions were scraped with the blunt edge of a scalpel, collected in 500 µl of Sample Transport Medium (STM) (Digene, Inc.) and treated with proteinase K (Boehringer Mannheim) at a final concentration of 200 µg/ml.

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Table 1. Results of PCR amplification of the LMP-1 deletion and repeat regions and the EBNA-2 region

Patient no.*	Time (t) months	LMP-1 deletion	LMP-1 repeats	EBNA-2 type
SC 0349	t 0	Yes + no	2†	1 + 2
SC 0349	t 10	Yes + no	1	1 + 2
LC 0349	t 10	Yes	1	1 + 2
LC 0349	t 14	–	2	–
LC 0349	t 18	Yes	1	2
LC 0349	t 20	Yes	2	1 + 2
SC 2207	t 0	No	1‡	1
LC 2207	t 0	Yes + no	2	1 + 2
SC 2207	t 2	No	1	1
LC 2207	t 2	Yes + no	3	1 + 2
SC 1957	t 0	Yes	1‡	1 + 2
LC 1957	t 0	Yes	3	1
SC 1957	t 4	Yes	1	1
LC 1957	t 4	–	–	–
SC 2060		Yes	3‡	1
LC 2060		Yes	1	1
SC 2101		Yes	2‡	1
LC 2101		Yes	1	1 + 2
SC 2151		Yes	2‡	1
LC 2151		Yes	1	1 + 2
SC 2161		No	1‡	1
LC 2161		Yes	2	1
SC 2217		Yes	1‡	1
LC 2217		Yes + no	1	1 + 2
SC 2244		Yes	1‡	1
LC 2244		Yes	3	1
SC 2247		Yes	2‡	1 + 2
LC 2247		Yes	1	2‖
SC 2272		Yes	2‡	1
LC 2272		Yes + no	2	1 + 2
SC 2275		Yes	3§	1 + 2
LC 2275		Yes	3	2
SC 2309		No	1‡	1
LC 2309		Yes + no	3	1
SC 2258		No	1‖	1 + 2
LC 2258		Yes + no	3	1
SC 2266		Yes + no	2‖	1 + 2
LC 2266		Yes + no	2	1 + 2
SC 2306		No	2‖	1
LC 2306		Yes	1	1

* SC, Epithelial scraping; LC, peripheral blood lymphocyte specimen.
† One band of same size throughout, second larger band also of same size.
‡ One band of same size.
§ Three bands of same size.
‖ No bands of same size.

Blood was drawn at the same time. Lymphocytes were separated by standard Histopaque methods (Sigma), lysed in STM ($\sim 2 \times 10^3$ cells/μl) and digested with proteinase K. The DNA was concentrated by ethanol precipitation. PCR of the lymphocyte and HL DNA digests was performed using the same primer sets and conditions.

The 3′ end of the LMP-1 gene and the repeat region were amplified with 30 cycles of PCR as described previously (Palefsky *et al.*, 1996). The region spanning the 3′ 30 bp deletion was amplified using primers spanning nucleotides 168392–168372 and 168105–168124 (sequence numbering based on the published B95-8 sequence) (Baer *et al.*, 1984). To

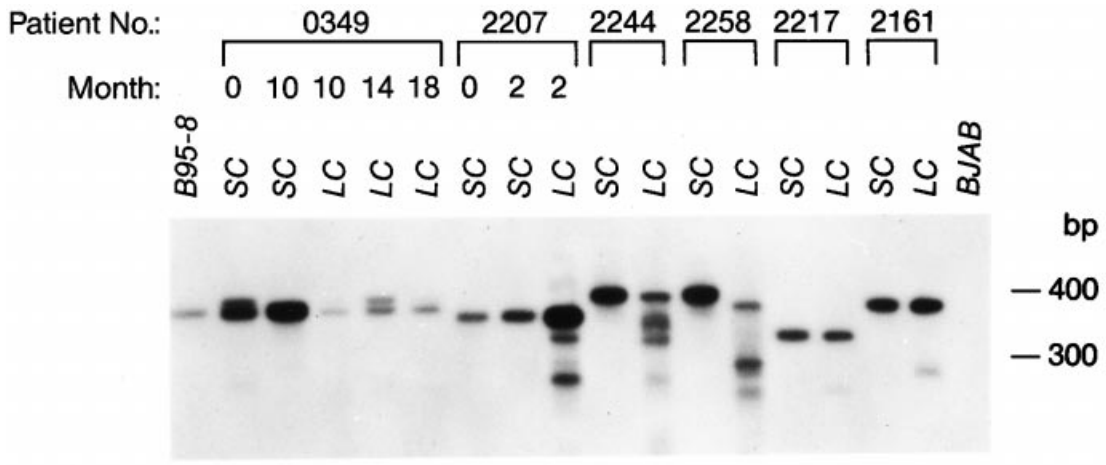

Fig. 1. Southern blot hybridization of LMP-1 repeat region PCR products in HL scrapings (SC) and peripheral blood lymphocyte (LC) specimens. Positive control B95-8, EBV-positive lymphocyte cell line, shows a single LMP-1 repeat region band. Patient 0349 specimens obtained over time show the same repeat region band in the HL SC and LC specimens. At 0 and 14 months, respectively, the SC and LC specimens also show a second band of similar size to each other. Patients 2207, 2244 and 2161 specimens show one band in common between SC and LC and extra variations in the latter. Patient 2258 specimens show no bands in common between SC and LC. Patient 2217 specimens show a single band of the same size in SC and LC. Negative control BJAB, an EBV-negative lymphocyte cell line, shows no repeat region bands.

characterize variation in the size and number of RRUs, the specimens were amplified and studied by Southern blot hybridization as described previously (Palefsky *et al.*, 1996). PCR-based typing of the EBNA-2 region was done as described previously using EBNA 2-type-specific primers and probes (Yao *et al.*, 1991). Positive controls for EBNA 2 type 1 amplification consisted of B95-8 cells, positive controls for EBNA 2 type 2 amplification consisted of Jijoye cells and the BJAB EBV-negative cell line was used for the negative control. The sensitivity of PCR detection using these methods was 10 EBV particles. PCR products of selected samples were sequenced to confirm their identity as described previously (Palefsky *et al.*, 1996).

Data were analysed from the first matched pair of HL scrapings (denoted 'SC') and lymphocytes (denoted 'LC') obtained from 16 subjects. Three subjects donated samples at more than one visit. In 10 other subjects, EBV was detected in SC but not LC specimens and no data on these subjects are presented. The results of matched pairs from each subject are shown in Table 1.

Of the 16 matched pairs, two (13%) SC specimens and six (37%) LC specimens contained strains both with and without the 3′ 30 bp LMP-1 deletion. In five of the 16 pairs, the LC specimen contained strains both with and without the deletion, while the SC specimen contained strains with one or the other but not both.

The number and size of LMP-1 RRUs were analysed in the SC and LC specimens. A representative Southern blot hybridization is shown in Fig. 1. The identity of the PCR product and number of RRUs were confirmed by sequencing of samples from patients SC2207 and SC2217 (Table 1), and from three other samples of EBV DNA as described previously (Palefsky *et al.*, 1996). In six of the 16 (37%) pairs, more RRUs were seen in the LC specimen than the SC specimen, while the opposite was true in five (31%) pairs. Four (25%) pairs showed the same number of RRUs. Thirteen of 16 (81%) pairs had one or more RRUs of the same size, indicating a similar combination of repeat elements and suggesting that the same EBV strain had infected both PBLs and epithelium. Consistent with strain identity, each of these 13 pairs also had at least one strain of the same EBNA 2 type. Eleven of the 13 pairs also showed identity with respect to the presence or absence of the 3′ 30 bp deletion. However, in two of these 13 pairs, the deletion was present in the LC specimen but not in the SC specimen. Three pairs had no bands of the same size.

Ten (63%) of 16 SC specimens contained EBNA-2 type 1 only, and six (37%) contained both EBNA-2 types 1 and 2. Seven of 16 (44%) LC samples contained EBNA-2 type 1 only, seven (44%) samples had both EBNA-2 types and 2 (12%) specimens had EBNA-2 type 2 only. In five specimens, EBNA-2 types 1 and 2 were both detected despite the presence of only one RRU, suggesting that recombination had occurred between these strains. Five pairs contained only EBNA-2 type 1, and in five pairs the SC specimen contained type 1 while the LC specimen contained both type 1 and 2. Of four pairs the SC specimen had both type 1 and 2 while the LC specimen only contained type 1. Two pairs had both type 1 and 2 in the SC and LC specimens.

In all three subjects who donated multiple specimens, the results with the SC specimen remained the same over time with respect to size of RRUs and presence or absence of the 3′ 30 bp LMP-1 deletion. There was no change in the size of the RRUs detected in any of the specimens, although the number of RRUs varied from one to two in one subject (patient 0349,

Table 1) who donated specimens over a 20 month period. This patient had EBNA types 1 and 2 detected at 10 and 20 months in his SC specimen, but only type EBNA type 2 at 18 months, and the EBNA 2 typing results did not correlate consistently in this patient with the number of LMP-1 repeats.

The relationship between EBV infection of the PBL and epithelial compartments is poorly understood. The high levels of EBV DNA in HL provide a unique opportunity to study the relationship between epithelial and PBL infection with EBV. To date, information comparing EBV strains in HL and PBLs has been presented for only one patient (Walling *et al.*, 1995). In that patient, the HL specimen contained one EBV strain and the lymphocytes contained three different strains of EBV, one of which was identical to the HL strain. Using EBNA-2 typing and the LMP-1 gene as markers of EBV strain identity, we analysed EBV DNA in 16 matched sets of HL epithelial scrapings and lymphocytes. Consistent with previous reports (Palefsky *et al.*, 1996; Walling *et al.*, 1992, 1995; Walling & Raab-Traub, 1994) we found evidence for multiple EBV strains in HL, as determined by the proportion of specimens with EBNA-2 type 1 and 2. The large number of specimens with more than one LMP-1 RRU is also consistent with multiple strains, but could also represent intra-strain recombination. Differences in EBNA typing and RRU number over time such as those observed in patient 0349 are consistent with complex patterns of recombination or variation in levels of replication of individual EBV strains leading to variability in PCR detection. Our data also suggest that, as in HL, infection with multiple EBV strains and possibly intra-strain recombination are common in PBLs in HIV-positive men.

Given the observation of multiple EBV strains in both the epithelial and PBL compartments, the frequency with which the compartments shared identity with at least one strain was striking. These data indicate that there is active exchange of EBV strains between HL and the PBL compartment. Differences observed in the size of the LMP-1 RRU in the epithelial and lymphocyte specimens, exemplified by patient 2258, may represent divergence from a single strain in the two compartments or establishment of different strains in these compartments from the outset. However, data from the three subjects who were studied longitudinally suggest that the LMP-1 gene remains relatively stable over a period ranging from 2 to 20 months with respect to number and size of RRUs.

Because of the frequency of multiple strains, the direction of flow of EBV infection between the compartments was difficult to ascertain in most cases. However, in two subjects who had one LMP-1 RRU of the same size and the same EBNA-2 type, the LMP-1 gene in the HL scraping did not have the 3′ deletion, whereas the LMP-1 gene in the PBLs did have the deletion. Since a deletion is a more probable event than an insertion, these data suggest that the EBV strain from the epithelium infected the PBLs in these two cases.

In summary, our data suggest that there is active movement of EBV strains between the epithelial and PBL compartments in patients with HL. Infection with multiple EBV strains and/or active intra-strain recombination are common in both compartments. Although our data do not exclude infection of HL with EBV strains from PBLs, they do suggest that, at least in some cases, EBV strains of HL may infect the PBL compartment.

The implication for ongoing exposure of PBLs to new epithelial EBV strains is not known. However, those at risk for HL such as those HIV-positive individuals, are also at increased risk of EBV-associated lymphoma. Although there is as yet no direct evidence, it is possible that exposure of the PBL compartment to new strains may permit the emergence of more pathogenic EBV strains or EBV strains that evade host immune defences, and may play a role in the development of lymphopoietic malignancies in this population. Likewise, continued exposure of the tongue epithelium to new strains of EBV may contribute to the pathogenesis of HL.

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