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Frequency of Multiple Epstein-Barr Virus Infections in T-Cell-Immunocompromised Individuals

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The Epstein-Barr virus (EBV) carrier state is characterized by latent infection of the general B-cell pool and by chronic virus replication at oropharyngeal sites. In Caucasian populations, most healthy carriers seem to harbor one dominant transforming virus strain, usually of type 1 rather than type 2, which persists over time and is detectable both in the blood and in the throat. This finding implies that once the virus carrier state is established, both viral reservoirs are largely if not completely protected from infection with additional strains. However, it is not known which facets of the immune response offer that protection. Here we address this question by a detailed study of EBV carriage in patients T-cell immunocompromised as a result of chronic human immunodeficiency virus (HIV) infection. Resident EBV strains were rescued from blood and from throat washings by using an in vitro transformation assay which aims to minimize bias toward faster-growing transformants; in this way, a mean of 16 independent isolations were made from each of 35 HIV-positive (predominantly male homosexual) patients. These virus isolates were characterized first at the DNA level by PCR amplification across type-specific polymorphisms in the EBNA2 and EBNA3C genes and across the 30-bp deletion and 33-bp repeat loci in the LMP1 gene and then at the protein level by immunoblotting for the strain-specific "EBNAprint" of EBNA1, -2, and -3C molecular weights. By these criteria, 18 of 35 patients harbored only one detectable EBV strain, usually of type 1, as do healthy carriers. However, the other 17 patients showed clear evidence of multiple infection with different EBV strains. In eight cases these strains were of the same type, again usually type 1, and were more often found coresident in throat washings than in the blood. By contrast, a further nine patients gave evidence of coinfection with type 1 and type 2 strains, and in these cases both virus types were detectable in the blood as well as in the throat. Immunological assays on these HIV-positive patients as a group showed a marked impairment of T-cell responses, reflected in reduced levels of EBV-specific cytotoxic T-cell memory, but an elevation of humoral responses, reflected in raised antibody titers to the EBV envelope glycoprotein gp340 and by the maintenance of virus neutralizing antibodies in serum. We infer that selective impairment of the T-cell system predisposes the host to infection with additional exogenously transmitted EBV strains.

Individual isolates of Epstein-Barr virus (EBV), a gammaherpesvirus of humans, are essentially homologous across large stretches of the genome but can be classified into two broad types, 1 and 2 (originally called A and B), based on polymorphisms in four latent genes encoding the nuclear antigens EBNA2, -3A, -3B, and -3C (2, 12, 38). All virus isolates can therefore be typed at the DNA level by PCR amplification across these polymorphic loci (38) and at the protein level, using selected human sera with type-specific reactivities to the EBNA2, -3B, and -3C antigens (36, 40). The bulk of evidence suggests that type 1 strains are the more prevalent in Caucasian and Oriental populations, whereas both types are relatively common in parts of equatorial Africa (1, 20, 24, 53, 57, 58). Within either of these two broad types, individual strains can be identified by a number of criteria. After isolates are rescued through their capacity to transform B cells into permanent lymphoblastoid cell lines (LCLs) in vitro, each viral strain is found to encode EBNA1, -2, -3A, -3B, and -3C proteins, which form a strain-specific "EBNAprint" when separated by gel electrophoresis and visualized by immunoblotting (19, 20, 36, 53). These size differences are particularly marked for EBNA1, -2, and -3C and in at least two of these cases appear to arise from interstrain variability in the size of a repeat region found within the protein coding sequence (14, 20). More recently, a number of other genomic polymorphisms which can help to identify individual strains have been noted. Many of these affect either restriction enzyme sites or T-cell epitope regions and tend to be most useful as markers of geographically separate strains, distinguishing in particular Oriental from Caucasian or African isolates (1, 13, 26, 27). However some polymorphisms can also be informative in distinguishing between strains derived from the same geographic area; in particular, the presence or absence of a 30-bp deletion and also the number of 33-bp repeats within the latent membrane protein LMP1 gene (30) can be useful markers for Caucasian strains (23, 30). Such polymorphisms therefore constitute useful adjuncts to the EBNAprint in strain identification.

These markers of heterogeneity among virus isolates have proved to be extremely valuable tools when it comes to studying the biology of the virus-host interaction. It is known that EBV, an orally transmitted agent, targets two main sites in vivo, the oropharynx, where mucosal epithelium (22, 42) and/or infiltrating lymphocytes (5) support full virus replication, and the general B-cell pool, where the infection is predominantly latent (31, 51). The virus can be detected at both

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sites not only during the acute primary infection, during which virus loads are relatively high (5, 42), but also at low levels throughout the life-long virus carrier state (52). However, the relationship between these two reservoirs of infection, and in particular the number of virus strains which can be found at each site, remain controversial issues. Thus, in two studies in which multiple independent EBV isolates were established in vitro from the blood and throat washings of more than 100 European donors, EBNAprint analysis strongly suggested that for each person, a single transforming strain (type 1 in >90% of cases and type 2 in <10% of cases) was dominant at both sites (20, 53) and remained dominant over several years (19, 53). By contrast, PCR analysis of viral DNA detectable directly in the throat washings of healthy donors from the southern United States not only suggested a higher incidence of type 2 viral infection in that community but also identified a small number of individuals carrying both type 1 and type 2 viral strains in the throat (43). Therefore, while an existing EBV infection elicits immune responses which in many individuals appear to protect them from additional exogenously transmitted viral strains, such protection may not always be complete, particularly with respect to viruses of different type. It is important to learn more about the virus-immune host's susceptibility to additional EBV infections, not least because of the implications that this could have for any future vaccine program. Specifically, we need to know what level of protection from exogenous EBV an existing virus carrier state provides, how broad that protection is against different EBV strains, and which facets of the virus-induced immune response are responsible.

In this context an interesting approach, complementing the earlier work on healthy carriers, is to study EBV infections in immunocompromised individuals. The most detailed work of this kind has been carried out on a group of European patients receiving immunosuppressive therapy following bone marrow or heart transplantation (20). In this study, virus isolation and EBNAprint analysis showed that a minority of such patients were indeed coinfected in the blood and/or throat with two or more independent strains, usually of type 1 and only rarely of types 1 and 2. However, the presence of more than one virus in these patients could not be ascribed to cumulative infections acquired by the natural route but, as earlier prospective studies have shown (18, 21), probably reflected the iatrogenic transmission of new strains via infected donor B cells in the transplant itself or in blood transfusions. Potentially more informative, therefore, is the situation in patients immunologically compromised as a result of chronic human immunodeficiency virus (HIV) infection. Interestingly, serological studies on HIV-positive male homosexuals (41) and also the analysis of EBV strains in the immunoblastic lymphomas to which such patients are prone (9) both suggested that type 2 EBV infection is more prevalent in this patient group than is apparent in the population at large. This trend has since been confirmed in three independent studies of the virus type present in LCLs derived from the blood or throat washings of such patients (10, 28, 39). However, though more than 50 individuals were sampled in these studies, only a very few cases gave evidence of mixed type 1 and type 2 infection, certainly fewer than might have been anticipated if the incidence of such coinfection is as high as direct PCR analysis would suggest (25). Much less attention has been given to the equally important question whether HIV-immunocompromised individuals become coinfected with EBV strains of the same type. Interestingly there was no direct evidence for this in any of the LCL isolation work (10, 28, 39), and the only indication that it might occur has come from the analysis of viral DNA in oral hairy leukoplakia biopsies (48, 49) and from prospective studies on two unusual patients selected from one of the above-described study cohorts (10).

The present work set out to analyze in greater depth the spectrum of EBV isolates present with HIV-positive patients. Using virus isolation protocols which sought to minimize any bias toward type 1-infected cells, whose in vitro growth tends to be more rapid than that of their type 2-infected counterparts (34), we elected to screen viral isolates both at the DNA level by PCR amplification across a range of polymorphic loci and at the protein level by EBNAprinting. At the same time, it was important to know the status of the patients with respect to those humoral and cell-mediated immune responses thought to be potentially protective against superinfection. Wherever possible, therefore, the patients' blood samples taken for virus isolation work were also used to assay serum antibody levels to the EBV envelope glycoprotein gp340 as an indication of the neutralizing antibody response (45), and in vitro regression of LCL outgrowth was used as a measure of EBV-specific cytotoxic T-cell control over latent infection in the B-cell system (33).

MATERIALS AND METHODS

Patients. The study group was made up of 40 HIV-infected patients who were attending outpatient clinics at Birmingham Heartlands Hospital and who gave informed consent for the study; they were predominantly Caucasian male homosexuals, none of whom had a history of intravenous drug abuse. Twenty-eight of these patients already had AIDS symptoms, including a history of oral hairy leukoplakia in six cases, three of which had resolved following treatment at least 1 year before the present study and three of which were being treated with acyclovir therapy at the time of sampling. All 40 patients were significantly immunocompromised in that $CD4^+$ T-cell counts were below 400/mm^3. Heparinized blood (20 to 30 ml) and throat washings were taken and processed as described previously (52), most patients being sampled on one occasion only. Blood samples provided plasma for serological studies and mononuclear cells for virus isolation and in vitro regression assays, while throat washings provided cell-free filtrates for virus isolation.

Immunological assays. Plasma preparations were first heat inactivated at 56°C for 30 min and then stored at −20°C until testing; note that an alternative method of HIV inactivation which we initially used, namely, treatment with β-propiolactone (8), significantly impaired antibody reactivity in the enzyme-linked immunosorbent assay (ELISA) (see below), and so such treatment was avoided thereafter. Patients' samples were tested for EBV antibody reactivities in parallel with control samples from healthy seropositive and seronegative adults. Immunoglobulin G antibody titers to EBV capsid antigen (VCA) and to the major EBV envelope glycoprotein gp340 were measured by standard immunofluorescence assay and by ELISA as described elsewhere (54). In selected cases, plasma samples were also screened for virus neutralizing activity as follows. Cell-free supernatant from the EBV-producing B95.8 cell line was diluted to a dose of 10^3 transforming units per ml, and then test plasma was added to a 10% (vol/vol) final concentration. After 1 h of incubation at 37°C, the mixture was added either neat or after further 10^{-1}, 10^{-2}, or 10^{-3} dilution in culture medium (see below) to cord blood indicator lymphocytes (1 ml/10^6 cells) for another hour at 37°C with frequent resuspension. The cells were then washed, resuspended in fresh culture medium, and seeded into replicate microtest plate wells (2×10^5 cells per well), and the culture was maintained for 6 weeks to score the outgrowth of transformed foci (52). When patients' blood mononuclear cell yields were sufficiently high, an aliquot of cells was screened for in vitro regression of B95.8 virus-induced transformation as described previously (52) to provide a measure of EBV-specific cytotoxic T-lymphocyte (CTL) memory function; cells from healthy seropositive donors were screened in parallel assays as a control.

Virus isolation. Virus isolations were carried out essentially as described previously (53), using as culture medium RPMI 1640 supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% (vol/vol) fetal calf serum. Blood mononuclear cells were assayed for spontaneous transformation across a range of cell seedings double diluted from 2×10^6 to 6×10^4 cells per 0.3-ml flat-bottom microtest well, with six replicate wells per seeding. Cultures were maintained in medium, initially containing cyclosporin A at 0.1 μg/ml to inhibit any T-cell regression of spontaneous outgrowth, and wells developing transformed foci within the initial 12-week assay period were then expanded to provide LCLs carrying the patient's endogenous EBV isolate(s). When spontaneous transformation was observed across the whole range of cell seedings, only cultures at the lower seedings were expanded to give lines. Throat washings (made by using 10 ml of RPMI 1640 and then clarified by bench centrifugation and filtered through a 0.45-μm-pore-size membrane) were sup-

plemented with 10% (vol/vol) fetal calf serum and added to parallel indicator pellets of cord blood and of seronegative donor mononuclear cells (2 ml of filtrate per 10^7 cell pellet); then the cells were cultured at 10^6/0.3-ml flat-bottom well, and cultures showing transformation within the 12-week assay period subsequently expanded to LCLs as described above. (Note that in all these blood and throat washing assays, care was taken to expand all wells with transformed foci irrespective of cell growth rate; hence, the time from initial culture to LCL cryostorage could be as long as 6 months.) Occasionally wells developed transformed foci which failed to expand, and in such cases the culture was used to provide a DNA preparation only.

In certain cases in which a blood- or throat washing-derived LCLs carried more than one EBV isolate, a second round of virus isolation was carried out. The LCLs in question were X irradiated (4,000 rad) and then cocultivated with seronegative donor indicator lymphocytes (2×10^4 LCLs and 2×10^5 indicator cells per replicate 0.3-ml well), and individual foci arising from such cocultures were subsequently expanded for analysis.

PCR analysis of viral polymorphism. The type 1/type 2 status of the EBV isolates was determined by PCR amplification across polymorphic regions of the EBNA2 and EBNA3C genes by using the type-specific primer-probe combinations first described by Sample et al. (38). In preliminary assays, we found that these particular combinations were type specific when tested across a panel of 30 different EBV reference isolates of known type and geographic origin; they were also extremely sensitive in that they could selectively detect LCLs carrying the relevant EBV type at a 10^{-4} to 10^{-5} cell dilution in an EBV-negative cell background and at a 10^{-3} to 10^{-4} dilution in a background of LCLs carrying the other EBV type. Because others have used different primer-probe combinations for virus typing at the EBNA 2 locus, we also screened the same reference cell panel using the protocol of Sixbey et al. (43) and using the 2A.1-2A.2 and 2B.1-2B.2 primer combinations and the protocol of Aitken et al. (3). In our hands, these alternative assays did not give the same level of specificity and/or sensitivity as that described above and were therefore not included in the main body of experiments.

Two other polymorphic markers used to characterize EBV isolates were the presence or absence of a 30-bp deletion and the number of 33-bp repeats within the 3′ region of the LMP1 open reading frame BNLF1 (7, 30). Screening for the 30-bp deletion was carried out under the PCR conditions described above, using the oligonucleotide sequences 5′-GCGACTCTGCTGGAAATGAT (B95.8 coordinates 168389 to 168370 [7]) and 5′-GACATGGTAATGCCTAGAAG (B95.8 coordinates 168130 to 168149) as 5′ and 3′ primers, respectively, and 5′-GGTTCCGGTGGAGATGATGA (B95.8 coordinates 168219 to 168200) as the probe; screening for the 33-bp repeats was carried out with the primer-probe combinations described by Miller et al. (30). Although it is possible that different numbers of repeats could be generated by recombination during replication of a single EBV strain (30), in practice we found that the great majority of LCL clones established by B-cell transformation with a reference EBV preparation in vitro retained the same 33-bp repeat number as shown by the cell line from which the virus was prepared (data not shown).

EBNAprint analysis of viral polymorphism. Extracts of 10^6 cells from each derived LCL were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted as described previously (36) alongside control extracts from the EBV-negative B-cell line BJAB, from the type 1 EBV-transformed LCL C2+OBA, and from the type 2 EBV-transformed LCL C2+BL16 (35). Antibody preparations used in immunoblotting included the polyclonal human sera Hu-MS (recognizing EBNA1 and type 1 EBNA3C), Hu-NZ (recognizing EBNA1 and type 2 EBNA3C), and Hu-AMo (recognizing EBNA1) (35, 36), the EBNA1-specific monoclonal antibody (MAb) IH4 (17), the EBNA2-specific MAb PE2 (56), and the type 1 EBNA3C-specific MAb E3CA10 (29). Immunoblots involving human sera were developed by using ^{125}I-labeled protein A; those involving MAbs were developed by using peroxidase-conjugated rabbit anti-mouse immunoglobulin G or anti-rat immunoglobulin G as appropriate for the second step, followed by chemiluminescence detection. Note that we prefer the term "EBNAprint" to its synonym "EBNOtype" (19) because the procedure identifies not only the virus type but also the individual virus strain.

RESULTS

EBV-immune status of HIV-positive patients. In a series of assays conducted in parallel with the virus isolations, we screened the cohort of HIV-positive patients for their prevailing levels of humoral and cell-mediated immunity to EBV. All 40 patients had serological evidence of prior EBV infection, their mean antibody titers to VCA (1:1,347) and to gp340 (1:1,336) being significantly higher than those in a control group of 30 healthy seropositive donors (mean anti-VCA titer, 1:428; mean anti-gp340 titer, 1:283). Because gp340-specific antibodies are thought to form the dominant component of the virus neutralizing response (45), this finding implied that the HIV-positive patients retain neutralizing capacity in serum. To formally check this point, we carried out virus neutralization assays on sera from five patients selected to cover a wide range of anti-gp340 titers; all gave evidence of detectable neutralization with relative efficacies broadly reflecting the anti-gp340 values. Detailed results of these serological assays are included for reference in Table 1 (see below). When blood mononuclear cell yields were sufficient, we also screened the patients for their EBV-specific memory CTL response as measured by the in vitro regression of B95.8 virus-transformed B-cell outgrowth. This analysis showed marked impairment of CTL reactivity in a substantial number of the patients. Individual results are recorded in Table 1 (see below) as the minimum cell seeding required to give a 50% incidence of regression in replicate wells; 12 of 16 patients analyzed showed either no detectable regression (50% endpoint, $>6 \times 10^5$ cells per well) or responses at the very edge of the normal range (50% endpoint, 6×10^5 cells per well).

Virus isolations from HIV-positive patients. EBV isolates were prepared from the blood and/or throat washings of all 40 patients, of whom 35 yielded sufficient numbers of independently established LCLs (mean of 16 LCLs per patient; range, 8 to 34) to warrant detailed analysis. As described below, three broad patterns of results were obtained with regard to the number of resident EBV strains.

(i) Patients with a single detectable EBV strain. Some 18 of 35 patients gave a pattern essentially similar to that observed earlier with healthy seropositive donors (20, 53) in that all EBV isolates from a single individual were of the same unique strain. In 17 patients, the resident strain was type 1; in 1 patient, it was type 2. To introduce the various screening assays and to illustrate this pattern of results, data from a representative patient, EBH1, are shown.

The DNA preparations from 12 independently isolated EBH1 LCLs (1 to 6 from the blood; 1* to 6* from the throat) were tested by PCR amplification with primer-probe combinations selective for type-specific sequences within the EBNA2 and EBNA3C genes. As illustrated in Fig. 1, all 12 LCLs gave a clear type 1 signal at both loci, with no evidence of detectable type 2 sequences. A similar uniformity among EBH1 isolates was observed across two polymorphic loci within the LMP1 gene. Thus, all resembled the B95.8 reference line in showing a full-length (i.e., undeleted) product when amplified across a region encompassing the 30-bp fragment that is deleted in the AG876 reference line (37) and in a number of other viral strains (1, 23, 30). Likewise, all gave the same size product when amplified across a region encompassing the 33-bp LMP1 repeats, in this case indicating a viral strain with exactly four such repeats; note that B95.8 and AG876 again serve as reference strains with, respectively, 4.5 and 4 copies of the 33-bp repeat (7, 37).

The analysis was then extended to protein extracts of the EBH1-derived LCLs, the sizes of the individual EBNA proteins encoded by the resident EBV isolate (i.e., the EBNAprint) being determined by immunoblotting. Figure 2 (upper panel) shows the results of an initial screening blot in which the LCL extracts were probed with a human serum, Hu-MS. As is clear from its reactivity against the reference control tracks (a type 1 LCL, a type 2 LCL, and an EBV-negative B-cell line), this serum has dominant reactivities against EBNA1 and against type 1 EBNA3C. While the reference virus strains clearly encode EBNA1 proteins of different apparent sizes, all EBH1 isolates show the same-size EBNA1 band. Likewise, all show the same-size EBNA3C species (one major band and two slightly smaller bands which represent breakdown products [14]). The data obtained from initial screening with human sera were then confirmed and extended

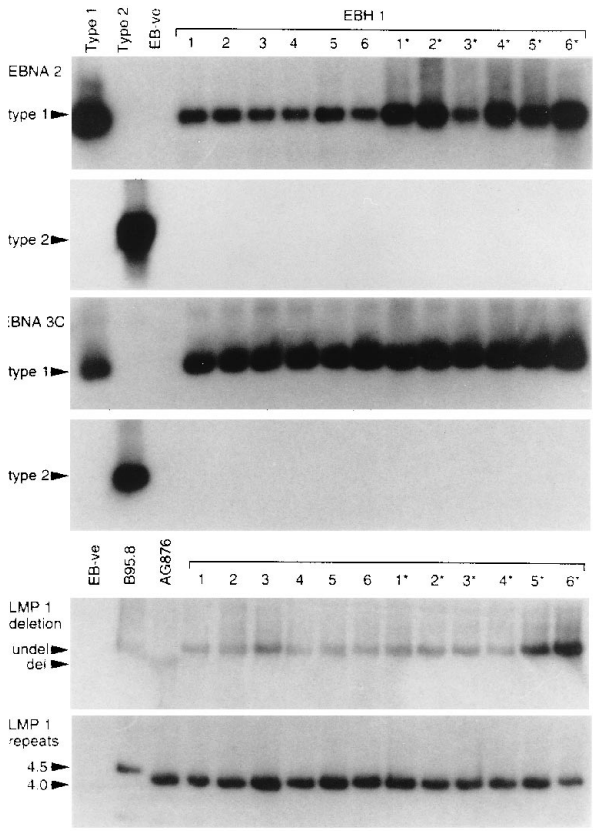

FIG. 1. PCR analysis of EBV strains carried by blood-derived LCLs 1 to 6 and throat washing-derived LCLs 1* to 6* from patient EBH1. For virus typing, EBNA2 gene amplification was carried out with a common 5′ primer and type-specific 3′ primers, and EBNA3C gene amplification was performed with common 5′ and 3′ primers; in each case, the products were probed separately with type-specific probes (38). For strain detection, LMP1 gene amplifications were carried out as described in Materials and Methods. All assays included reference DNA samples from the type 1 B95.8 cell line, the type 2 AG876 cell line, and the EBV-negative (EB-ve in all figures) BJAB cell line. Note that all EBH1-derived isolates are type 1 at the EBNA2 and EBNA3C loci and are indistinguishable at the LMP1 loci.

by blotting with EBNA-specific MAbs (Fig. 2, lower panels). The MAb IH4 again detects the same EBNA1 band in all isolates, while MAb PE2 shows that all virus isolates from this patient also encode the same-size EBNA2. It is known that type 1 and type 2 versions of the EBNA2 doublet characteristically occupy different size ranges in such gels, with type 2 always being smaller than type 1 (56, 57); hence, the size of the EBH1 EBNA2 protein in Fig. 2 is further confirmation of its type 1 nature. Identification of a type 1 EBNA3C reactivity can usually be checked by using MAb E3CA10, but interestingly, in the case of EBH1, the virus-coded EBNA3C protein was not detected by this MAb. Since only a small number of type 1 viruses lack the E3CA10 epitope (29), its absence from EBH1-derived isolates again suggests that these are all of the same unique strain. In all, we established 34 independent LCLs from patient EBH1, and all gave the same results by PCR analysis and by EBNAprinting (data not shown).

Among the other 17 patients in this same group, we very occasionally observed an LCL which was indistinguishable by PCR screening from all other isolates from the same donor but which showed a size change in just one of the proteins (usually EBNA1 or EBNA3C) constituting the EBNAprint. This type of variation has been observed before in the analysis of EBV isolates from immunosuppressed transplant recipients (20) and very probably arises from intrastrain recombination within repeat sequences in the EBNA1 and EBNA3C open reading frames (14). In the present study, such examples were therefore not taken as indicators of multiple infection with independent strains. The individual data from the 18 patients with only a single detectable virus strain are presented in Table 1 (see below).

(ii) Patients with multiple EBV strains of the same type. By contrast, 8 of 35 patients clearly showed multiple infection with two or more strains of the same type. In most cases, this involved coresident type 1 virus strains, exemplified here by data from two patients, EBH15 and EBH18. Figure 3 illustrates the results obtained when viral DNA from a panel of EBH15- and EBH18-derived LCLs was amplified across each of the genome polymorphisms introduced above. All of these viral isolates were exclusively type 1 at the EBNA2 and EBNA3C loci. However, evidence of diversity appeared when the analysis was extended to LMP1 sequence polymorphisms. Thus, by LMP1 repeat numbers, blood-derived isolate EBH15.2 was distinct from throat washing isolates 15.1* to -4*; likewise blood isolates EBH18.1 and -2 were distinct from throat washing isolates 18.1* to -3*. In fact, the 18.3* LCL appeared to carry two viral strains which were distinct both at the LMP1 repeat and at the LMP1 deletion locus.

Further diversity among these EBH15 and EBH18 isolates was revealed when the same panel of LCLs was analyzed at the protein level by immunoblotting. Figure 4 (upper panel) shows the initial screen using human serum Hu-MS, from which it is clear that EBH15 carries at least three different type 1 viruses represented by blood isolate 15.2, by throat washing isolates 15.1* and -2*, and by throat washing isolates 15.3* and -4*, respectively. The EBNAprint differences apparent in the

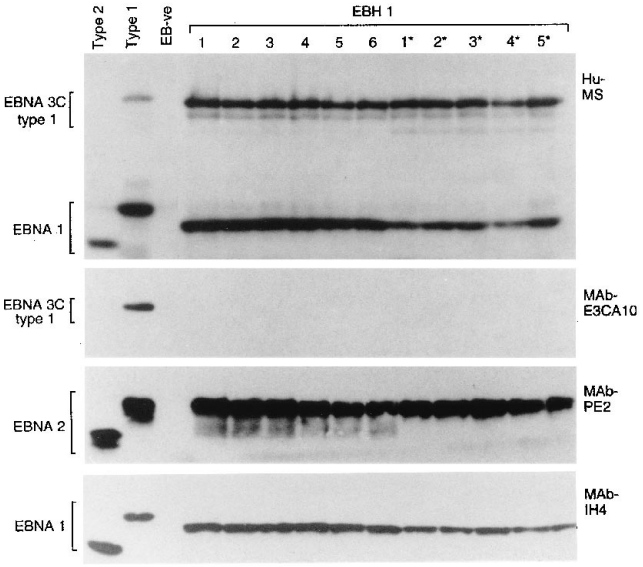

FIG. 2. EBNAprint analysis of EBV strains carried by blood-derived LCLs 1 to 6 and throat washing-derived LCLs 1* to 5* from patient EBH1. Protein extracts were separated by SDS-PAGE, and the immunoblots were probed with the human serum Hu-MS, principally recognizing EBNA1 and type 1 EBNA3C, with the EBNA1-specific MAb IH4, with the EBNA2-specific MAb PE2, and with the type 1 EBNA3C-specific MAb E3CA10. All immunoblots included reference protein samples from the type 1 LCL C2+OBA, from the type 2 LCL C2+BL16, and from the EBV-negative BJAB cell line. Note that all EBH1-derived isolates are type 1 and display identical EBNAprints, including a type 1 EBNA3C protein which is recognized by Hu-MS but which lacks the E3CA10 epitope.

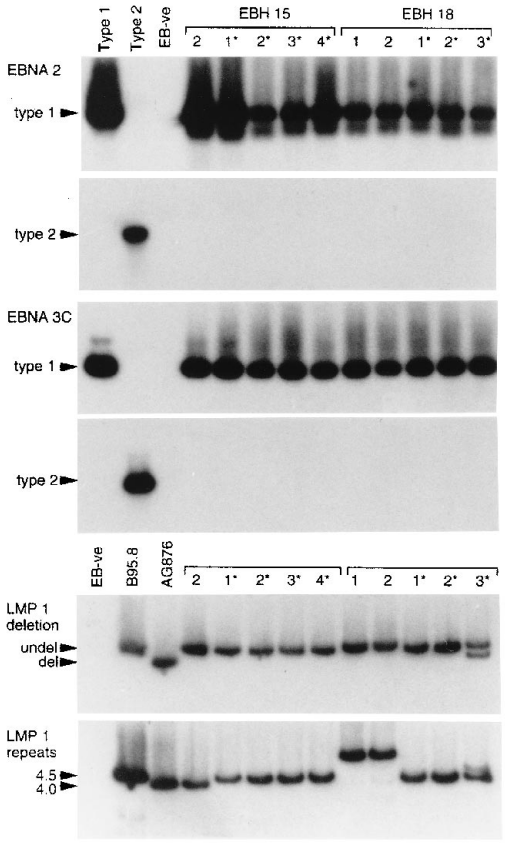

FIG. 3. PCR analysis of EBV strains carried by LCLs from patient EBH15 (blood-derived line 2 and throat washing-derived lines 1* to 4*) and from patient EBH18 (blood-derived lines 1 and 2 and throat washing-derived lines 1* to 3*). Virus typing assays were carried out at EBNA2 and EBNA3C gene loci and strain detection assays were carried out at LMP1 gene loci as for Fig. 1. Note that all EBH15 and EBH18 isolates are type 1 at the EBNA2 and EBNA3C loci. However, in both patients the virus strain carried in the blood is distinct from the virus strain(s) carried in the throat at the LMP1 repeat locus; there is also evidence both from LMP1 deletion and from LMP1 repeat analysis of more than one resident virus in the EBH18 LCL 3* cell line.

Hu-MS blot were confirmed at the EBNA3C locus by MAb E3CA10 (note that 15.1* and -2* again represent an unusual type 1 isolate lacking the E3CA10 epitope) and at the EBNA1 locus by MAb IH4; furthermore, the MAb PE2 blot reveals differences in the EBNA2 proteins of these distinct EBH15-derived strains (Fig. 4, lower panel). The same series of immunoblots also confirms that EBH18 carries a viral strain in the blood (18.2 and -3) different from that in the throat (18.1* to -3*). Note that the additional EBNA1 bands detectable in 18.3* may also reflect the presence of one or more additional virus strains within this polyclonal LCL, in accord with the earlier evidence from PCR analysis.

In all, 7 of 35 patients gave evidence of infection with multiple type 1 strains. The illustrated patients, EBH15 and EBH18, were interesting in that one viral strain was isolated from the blood whereas one or more quite different strains were isolated from the throat. Another four patients in this category (EBH13, -14, -40, -45) had one strain detectable both in the blood and in the throat, plus a second strain only detectable in the throat. The other patient (EBH28) had two distinct virus strains in the blood. These data are presented in Table 1 (see below).

Evidence of multiple infection was also seen in one type 2 virus-infected patient, EBH48. This patient yielded blood-derived LCLs (48.1 to -5) and throat washing-derived LCLs (48.1* to -5*) which gave exclusively type 2-specific signals on PCR amplification across the EBNA2 and EBNA3C loci (Fig. 5). All of these isolates also carried the LMP1 deletion, a feature which we have found to be unusually common among type 2 Caucasian virus strains (23). However, amplification across the LMP1 repeats showed quite complex repeat sequence signatures for the throat washing isolates, in contrast to the regular pattern shown by the blood isolates (Fig. 5). These differences were subsequently confirmed at the protein level. First, it was clear that all of the resident isolates were type 2 because the type 1-specific human serum Hu-MS did not detect any bands in the EBNA3C region of the gel (data not shown). By contrast, Fig. 6 (upper panel) shows the result of probing the EBH48 LCL extracts with human serum Hu-NZ from a type 2-infected individual. This serum predominantly recognizes EBNA1 and type 2 EBNA3C proteins with weaker recognition, detectable on long gel exposures, of both type 1 and type 2 EBNA3A (control tracks in Fig. 6). Because the type 2 EBNA3 proteins are less well characterized than their type 1 counterparts and are not yet defined by MAbs, it is difficult to identify unequivocally the individual bands in this region of the EBH48 tracks. However, the overall EBNAprints clearly show that the blood and throat washing isolates represent different type 2 virus strains (Fig. 6). Note also the presence of two distinct EBNA1 bands in some of the throat washing LCL tracks (subsequently confirmed by MAb IH4 [data not shown]) and the greater complexity of the type 2 EBNA2 species present in these LCLs as seen by MAb PE2. Both observations support the evidence from LMP1 repeat analysis that multiple type 2 viral strains were present in the throat of this patient.

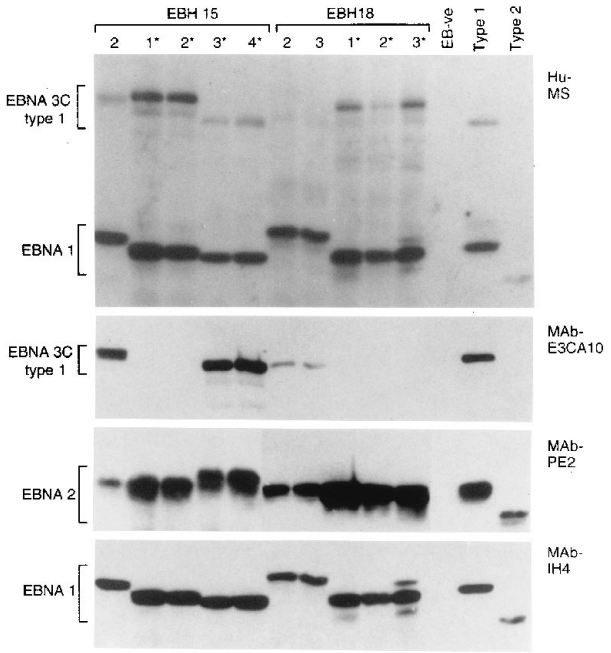

FIG. 4. EBNAprint analysis of EBV strains carried by LCLs from patient EBH15 (blood-derived line 2 and throat washing-derived lines 1* to 4*) and from patient EBH18 (blood-derived lines 2 and 3 and throat washing-derived lines 1* to 3*), using antibodies used for Fig. 2. Note that all EBH15 isolates were type 1, but three different EBNAprints were detected (for line 2, lines 1* and 2*, and lines 3* and 4*); likewise, all EBH18 isolates were type 1, but two different EBNAprints were detected (for lines 2 and 3 and for lines 1* to 3*).

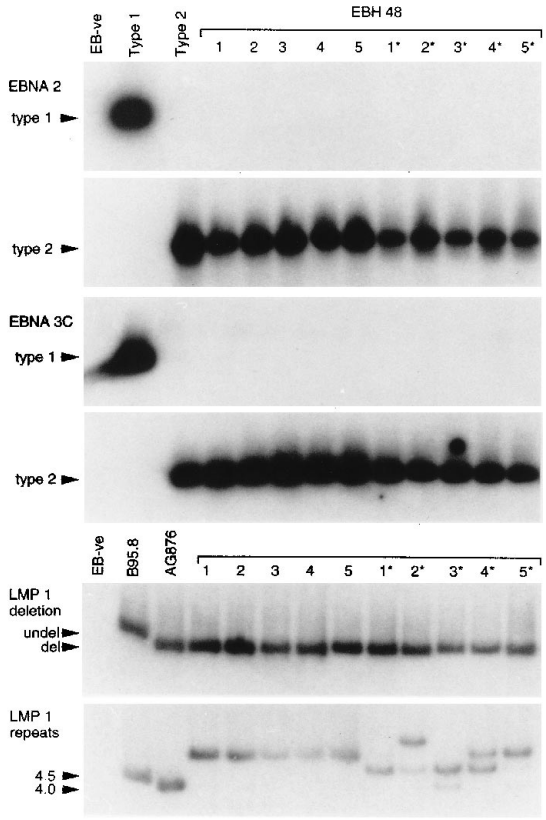

FIG. 5. PCR analysis of EBV strains carried by blood-derived LCLs 1 to 5 and throat washing-derived LCLs 1* to 5* from patient EBH48. Virus typing assays were carried out at EBNA2 and EBNA3C gene loci and strain detection assays were carried out at LMP1 gene loci as for Fig. 1. Note that all EBH48-derived isolates are type 2 at the EBNA2 and EBNA3C loci, but with evidence of LMP1 repeat diversity in some of the throat washing-derived lines.

(iii) Patients with multiple EBV infections of different types. A third group of patients gave evidence of coresident type 1 and type 2 virus strains. This is illustrated first by EBH43, a donor from whom almost every blood- and throat washing-derived LCL was dually positive for type 1 and type 2 signals in PCR assays. Figure 7 presents the results obtained after PCR amplification across the EBNA2 and EBNA3C polymorphic loci for a representative range of EBH43 isolates. Screening across the LMP1 repeat locus likewise gave evidence of multiple infection, whereas only a single product was obtained from amplification across the LMP1 deletion region; subsequent work in fact confirmed that both of the coresident EBH43 virus strains carried the LMP1 deletion (data not shown).

The tendency for type 1 virus transformants to grow out more quickly than type 2 transformants in vitro (34) introduces a potential bias in situations of mixed-type infection such as this. Indeed, a skewing toward type 1 LCL outgrowth was observed in this particular case, as reflected in the subsequent protein analysis carried out on cell pellets prepared 3 weeks after the initial aliquots of cells had been taken for DNA analysis. As shown in Fig. 8, all of the EBH43-derived LCLs express type 1-specific EBNA3C and EBNA2 proteins plus a consistent EBNA1 band characteristic of this particular type 1 strain (E3CA10, PE2, and IH4 blots, respectively). However, only the EBH43.8, -2*, and -5* LCLs express detectable levels of the type 2-specific EBNA3C and EBNA2 proteins and of a second distinct EBNA1 band (Hu-NZ, PE2, and IH4 blots). Note that these three particular LCL populations also gave arguably the strongest type 2-specific PCR signals in the original DNA analysis (Fig. 7). All of the later-passage LCL populations illustrated in Fig. 8 nevertheless still contained some type 2 viral genes detectable by PCR amplification. Furthermore, we were able to formally demonstrate the presence of distinct type 1 and type 2 virus strains in these EBH43-derived LCL populations by passaging the resident viruses into indicator cord blood lymphocytes, thereby establishing secondary transformants which carried either a type 1 or a type 2 virus (data not shown). A second patient (EBH41) was similar to EBH43 in that every blood- and throat washing-derived LCL proved to be a mixture of type 1-transformed and type 2-transformed cells, the coresident type 1 and type 2 viral strains being separable by secondary passage into cord blood lymphocytes (data not shown).

However, most of the other patients identified with mixed-type infections yielded individual LCL populations which were not mixed but which carried a single virus type from the outset. This is exemplified by EBH3, some of whose isolates were entirely type 1 by PCR amplification across the EBNA2 and EBNA3C loci, whereas other isolates were entirely type 2 (Fig. 9). Again these distinctions were confirmed at the protein level, as shown by the results of immunoblotting in Fig. 10. Thus, isolates 3.4 and 3.5 clearly carry one strain with a type 1 EBNAprint (E3CA10, PE2, and IH4 blots), whereas isolates 3.1* to -7* carry a different strain with a type 2 EBNAprint (Hu-NZ, PE2, and IH4 blots).

The individual results for all nine patients coinfected with type 1 and type 2 viruses are included in Table 1; note that two patients, EBH5 and -34, are special cases described in detail in the accompanying report (55). Considering this group as a whole, we did not observe any obvious skewing of isolates toward type 1 virus strains at the expense of type 2. Thus, while the great majority of isolates from some patients (e.g., EBH8 and -12) were type 1, in other cases (e.g., EBH21) the great majority were type 2. One notable feature shown by all nine patients with mixed-type infections, and apparently distinguishing them from most of the patients carrying multiple

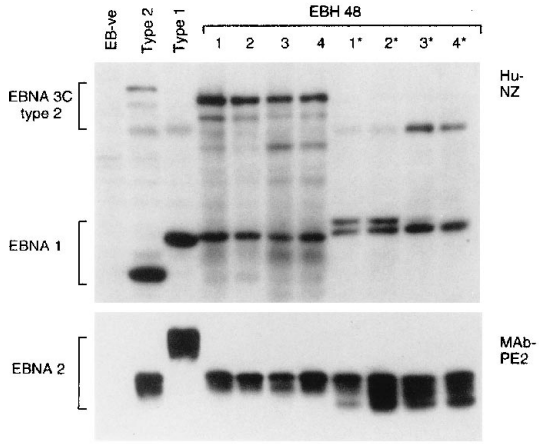

FIG. 6. EBNAprint analysis of EBV strains carried by blood-derived LCLs 1 to 4 and throat washing-derived LCLs 1* to 4* from patient EBH48. Protein samples were separated by SDS-PAGE, and immunoblots were probed with the human serum Hu-NZ, principally recognizing EBNA1 and type 2 EBNA3C, and with the EBNA2-specific MAb PE2. Note that blood-derived and throat washing-derived LCLs are all type 2 but display different EBNAprints, with evidence of more than one resident virus in some of the throat washing-derived lines.

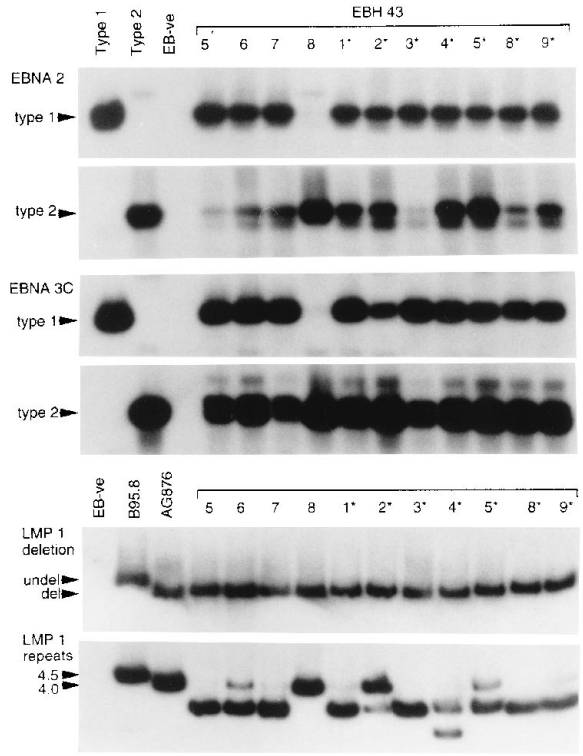

FIG. 7. PCR analysis of EBV strains carried by blood-derived LCLs 5 to 8 and throat washing-derived LCLs 1* to 5*, 8*, and 9* from patient EBH43. Virus typing assays were carried out at EBNA2 and EBNA3C gene loci strain detection assays were carried out at LMP1 gene loci as for Fig. 1. Very occasionally in the EBNA2 PCR typing assay, the type 1 probe shows weak hybridization to the type 2 PCR product if the latter is present in abundance (type 2 control track in the EBNA2 type 1 panel); this is easily recognized as a cross-reaction because of the characteristic size difference between the type 1 and type 2 PCR products. All test LCLs in this figure showed evidence of coresident type 1 and type 2 viral signals (type 1 signals from line 8 were detectable after longer exposures), and in many cases there was further evidence of dual infection from LMP1 repeat analysis.

strains of the same type, was the presence of coresident viruses in the blood as well as in the throat.

DISCUSSION

At the outset of this work, we sought to establish experimental protocols capable of detecting coresident EBV strains, whether of the same or of a different type and whether in the blood or in the throat. Coinfection with different virus types is the most easily discerned because polymorphisms in the EBNA2, -3A, -3B, and -3C genes can be exploited in PCR typing assays. Indeed, as we and others have shown, healthy donors' throat washings can be directly screened for the resident EBV type(s) by PCR assays on material immediately ex vivo (24, 43, 53). However, by comparison with the relatively large viral genome loads provided by replicative lesions in the oropharynx, those associated with latent infection in the B-cell pool are much lower and correspondingly more difficult to detect (6). More importantly, however, when it comes to detecting different Caucasian virus strains of the same type, there are currently not enough informative markers to attempt this by direct PCR analysis alone, and the only successful studies of this kind have involved extensive sequencing of the directly amplified products (47, 49). In the present work, therefore, we chose to screen resident viruses by in vitro isolation, thereby generating LCL foci which could be analyzed both by PCR assays and by EBNAprinting. While our in vitro isolation protocol is designed to minimize any bias toward faster-growing LCL clones, and therefore should detect all transforming EBV strains, it will by definition not detect transformation-defective variants. In this context, direct cloning has shown that defective genomes, particularly with deletions and/or rearrangements in the EBNA2 gene region, can be generated in the oropharynx as aberrant products of virus replication (44, 48, 50). Oropharyngeal persistence of a defective viral strain has been observed in replicative lesions (4, 47, 49) and in one case has also been detected in the blood (47). However, in most cases it seems to us unlikely that viruses without transforming function would efficiently colonize the B-cell pool (46).

The main conclusion from this work is that multiple EBV infection, either with different viruses of the same type or with viruses of different types, is much more common among immunocompromised AIDS patients than had been apparent from previous virus isolation studies in vitro (10, 28, 39). We attribute this increased detection rate to the much larger number of individual LCLs established per patient in the present work and to the larger number of polymorphic markers analyzed. In this context, our criteria for identifying two coresident viruses as being distinct strains were either (i) that one isolate was type 1 and the other was type 2 both by PCR analysis and by EBNAprinting or (ii) that they were of the same type but showed differences in at least three of the five polymorphic markers analyzed (LMP1 deletion, LMP1 repeats, and EBNA1, -2, and -3C molecular weights). It is of course possible that heterologous recombination between genomes of the same strain can generate variants with alterations in one or more of these loci, particularly when these involve repeats (14, 20, 30, 49, 50). However, when we detected what appeared to be such an intrastrain recombinant in the present work (usually

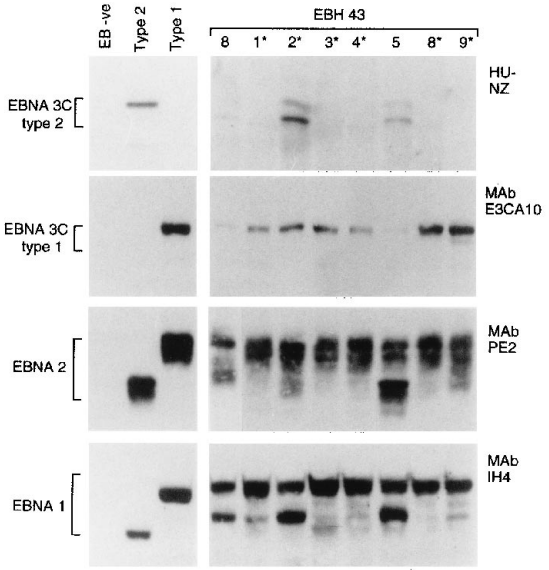

FIG. 8. EBNAprint analysis of EBV strains carried by blood-derived LCL 8 and throat washing-derived LCLs 1* to 5*, 8*, and 9* from patient EBH43, using human serum Hu-NZ (see Fig. 6) and MAbs E3CA10, PE2, and IH4 (see Fig. 2). All LCLs express detectable levels of an EBNA1 protein of constant size (upper band, IH4 blot), a type 1 EBNA2 protein (upper doublet, PE2 blot) and a type 1 EBNA3C protein (E3CA10 blot). In addition, at least three LCLs (8, 2*, and 5*) express detectable levels of a second EBNA1 protein (lower band, IH4 blot), a type 2 EBNA2 protein (lower doublet, PE2 blot), and a type 2 EBNA3C protein (Hu-NZ blot).

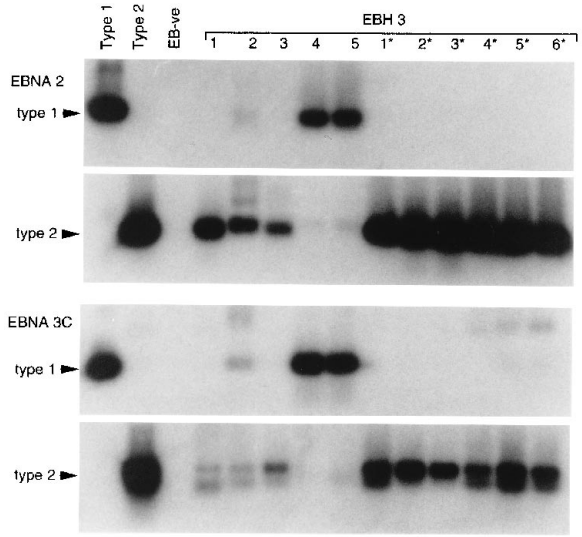

FIG. 9. PCR analysis of EBV strains carried by blood-derived LCLs 1 to 5 and throat washing-derived LCLs 1* to 6* from patient EBH3. Virus typing assays were carried out at EBNA2 and EBNA3C gene loci as for Fig. 1. Note that some lines (e.g., 1 and 3) display type 2 signals exclusively and other lines (e.g., 4 and 5) display type 1 signals exclusively.

with a single change involving either EBNA1 or EBNA3C), that particular pattern of variance was observed in only one isolate per patient. By contrast, what was judged by the criteria described above (i.e., by three or more changes) to be an independent strain was quite often represented in several of the LCLs derived from a particular patient. In light of these considerations, we would argue that most if not all of our assignations of independent viral strains are correct.

It is possible that a more exhaustive analysis of the patient cohort used would have revealed an even greater incidence of multiple infection. In this context, however, Table 1 shows that the mean number of LCLs analyzed per patient in the singly infected group was at least as high as the mean number analyzed per patient in the multiply infected group. We consider it unlikely, therefore, that the apparent differences in virus carriage between different individuals can be ascribed to sampling error alone. Rather, we infer that up to 50% of patients in the study cohort remain qualitatively similar to immunocompetent virus carriers in the European Caucasian population (20, 53) in that a single transforming strain of EBV, usually a type 1 strain, is dominant both in the blood and in the throat. By contrast, around 25% of the patients carry two or more coresident virus strains of the same type (again usually type 1), and a further 25% are coinfected with type 1 and type 2 strains. Such coinfections have rarely if ever been seen in virus isolations from healthy immunocompetent donors (20, 53). Previous studies of AIDS patients (10, 28, 39) have already suggested that the overall incidence of type 2 virus infection is around the level observed in the present work (12 of 40 patients in this study gave type 2 isolates), but these earlier studies underestimated the frequency with which type 2 carriers are also coinfected with type 1 strains. The present findings are, however, in accord with recent work on biopsies of oral hairy leukoplakia, an epithelial focus of exaggerated EBV replication seen on the tongues of AIDS patients, which reported frequent codetection of type 1 and type 2 viral sequences (48, 49). It is also possible that we have underestimated the level of type 2 infection in our patient cohort, since type 2-infected cells tend to grow out more slowly to LCLs in vitro than their type 1 counterparts (34). However, considerable efforts were made to expand all foci arising in culture wells in the present work, and the fact that we isolated type 2 LCLs from almost one-third of the patients studied, very often in the face of competition from a coresident type 1 strain, suggests to us that our in vitro protocol did not significantly prejudice type 2 virus detection.

The full biological significance of these observations remains to be determined. One view holds that the EBV carrier state in AIDS patients is qualitatively similar to that which exists in the general population, but that the true spectrum of resident viruses is easier to see in such patients because of their higher viral load. This view would imply that many healthy individuals are also infected with multiple viral strains, in particular viral strains of different types, but that this has gone undetected in conventional screening assays because their type 2 virus is the subdominant strain in vivo and/or is inefficiently rescued in vitro. An alternative view is that the multiple EBV infections seen in many AIDS patients are an opportunistic consequence of immune impairment and do not reflect the situation in healthy carriers. In this regard, the frequency with which we were able to isolate different coresident strains from individual patients in the present work contrasts with the complete absence of such findings in an earlier study of healthy donors (53). Given that the mean numbers of LCLs rescued per individual in this and in the earlier study were not dramatically different (16 and 9, respectively), it is again unlikely that the different patterns of results observed can be wholly ascribed to sampling error.

The precise situation regarding EBV carriage in immunocompetent individuals therefore remains to be resolved. One way forward will be to examine healthy donor blood and throat washing samples directly, using a larger panel of well-validated PCR assays than has been used in studies to date (6, 43).

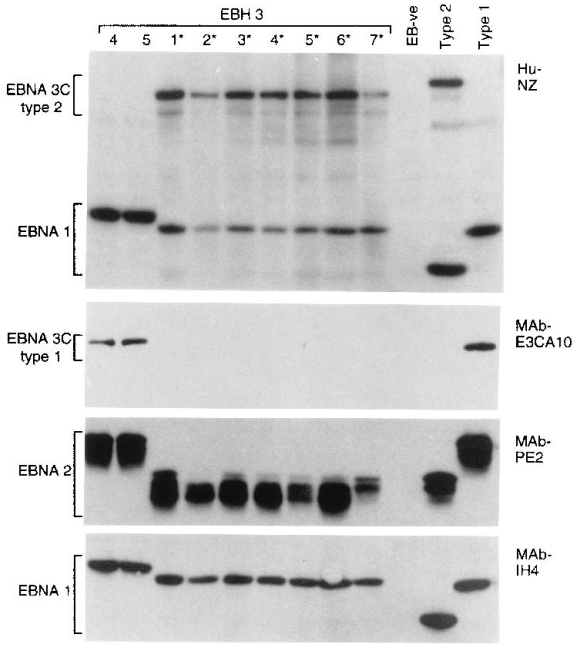

FIG. 10. EBNAprint analysis of EBV strains carried by blood-derived LCLs 4 and 5 and throat washing-derived LCLs 1* to 7* from patient EBH3, using human serum Hu-NZ (see Fig. 6) and MAbs E3CA10, PE2, and IH4 (see Fig. 2). Note that some LCLs (4 and 5) display a single type 1 EBNAprint and other LCLs (1* to 7*) display a single type 2 EBNAprint.

TABLE 1. Summary of data from HIV-positive patients

Patient[a]	Immunological status[b]			No. of virus isolates		Multiplicity of EBV infection
	Anti-VCA titer	Anti-gp340 titer	T-cell regression (10^5)	B-cell isolates	Throat isolates	
EBH1	1:1,280		>6	24	10	Single type 1 strain
EBH2	1:2,560		2.2	16	0	
EBH6	1:1,280	1:1,500		3	12	
EBH7	1:640	1:10,240		12	13	
EBH11	1:640		1.5	3	12	
EBH22	1:5,120	1:5,120		0	8	
EBH26	1:320	1:400	6	0	13	
EBH29	1:1,280			0	18	
EBH30	1:640	1:120	1.5	6	7	
EBH31	1:1,280		>6	8	17	
EBH35	1:1,280	1:240	>6	8	8	
EBH36	1:2,560			10	0[c]	
EBH37	1:640	1:120		6	6	
EBH38	1:5,120	1:1,280		10	8	
EBH39	1:640			10	0[c]	
EBH44	1:5,120	1:1,280		4	5	
EBH47	1:2,560	1:5,120		4	8	
EBH9	1:80	1:80	>6	0	8	Single type 2 strain
EBH13	1:640	1:640		8	10	Multiple type 1 strains
EBH14	1:160	1:400	4	10	10	
EBH15	1:80	1:40[d]		2	9	
EBH18	1:640	1:320[d]	>6	3	9	
EBH28	1:2,560		>6	8	0[c]	
EBH40	1:1,280			4	5	
EBH45	1:640			7	9	
EBH48	1:640			5	10	Multiple type 2 strains
EBH3	1:640	1:1,280		5	10	Mixed type 1 and type 2 strains
EBH4	1:160		6	12	0	
EBH8	1:160	1:80	>6	8	16	
EBH12	1:160	1:1,280		1	17	
EBH21	1:1,280	1:960	>6	2	19	
EBH41	1:1,280	1:480[d]		4	9	
EBH43	1:640			9	11	
EBH5[e]	1:2,560	1:2,560[d]	>6	10	6	
EBH34[e]	1:1,280	1:4,000[d]	6	12	19	

[a] Of the 40 patients in the initial study group, 5 (EBH16, -19, -23, -25, and -46) were excluded from the analysis because they each yielded only a small number of LCLs; LCLs of EBH16, -19, -23, and -25 carried a type 1 strain; LCLs of EBH46 carried a type 2 strain.

[b] Anti-VCA titers were determined for all 35 patients (mean, 1:1,367; range, 1:80 to 1:5,120) and for a control group of 30 healthy EBV-seropositive adults (mean, 1:428; range, 1:20 to 1:1,280). Anti-gp340 titers were determined for 22 of 35 patients (mean, 1:1,706; range, 1:20 to 1:10,240) and for the same control group of 30 healthy adults (mean, 1:283; range, 1:20 to 1:1,280); where no result is shown, the patient's anti-gp340 titer could not be measured because of prior β-propiolactone treatment of plasma. T-cell regression titers were determined for the 16 of 36 patients from whom blood mononuclear cell yields were high enough to allow some cells to be reserved for the assay. Results are expressed as the minimum initial cell seeding per 0.3-ml microtest plate well that was required to give a 50% incidence of regression among replicate cultures; note that healthy EBV-seropositive adults all show 50% regression endpoints in the range 4×10^4 to 6×10^5 (33), whereas 9 of 16 patients tested in the present work gave no detectable regression even at the highest cell seeding (endpoint designated as $>6 \times 10^5$).

[c] At the time of sampling, the patient was receiving acyclovir, a drug which blocks EBV replication in the oropharynx (51); this explains the absence of throat washing-derived isolates for EBH28, EBH36, and EBH39.

[d] The plasma sample was also tested for virus neutralizing activity with the following reductions in virus titers: EBH15, 50-fold; EBH18, 100-fold; EBH41, 1,000-fold; EBH5, >1,000-fold; and EBH34, >1,000-fold.

[e] Distinct in carrying type 1/type 2 recombinant virus strains, as described in the accompanying report (55); inclusion in the group of patients with mixed type 1 and type 2 infection is by inference from the presence of an intertypic recombinant rather than by the direct demonstration of coresident type 1 and type 2 strains.

Another approach will be to study virus carriage in other immunologically compromised patient groups. If the increased incidence of type 2 EBV seen in HIV-positive male homosexuals (references 9, 10, 25, 28, 39, 41, 48, and 49 and this study) is genuinely indicative of widespread type 2 infection in the general population, then a similar pattern of virus isolation should be obtained from immunosuppressed transplant patients (even though the situation in these individuals will be complicated by iatrogenic EBV transmissions [18, 21]). Typing of EBV isolates from transplant patients has indicated low type 2 prevalence in one study (20) and a much higher prevalence in another (43), perhaps reflecting the geographically different populations from which the patients were drawn. However, an interesting difference between HIV-positive homosexual patients and transplant patients from the same geographic area does seem to be emerging from work on the EBV-driven immunoblastic lymphomas to which both are prone; these tumors appear to be much more frequently associated with type 2 virus in the former group than in the latter (15).

Whatever the actual baseline of EBV carriage in healthy

individuals, the present findings and those from more limited prospective studies (10, 48) suggest to us that HIV-positive homosexuals are unusually prone to infection with additional exogenously transmitted EBV strains. We cannot yet say whether such transmission occurs by the natural oral route because in this particular type of patient, it is possible that these additional strains are acquired via blood transmission from homosexual contact. However, current work is beginning to detect multiple EBV infections in other types of HIV-positive patients also (50a), suggesting that HIV-mediated immune suppression may indeed predispose to cumulative EBV infections via the natural route. If this is so, what can this tell us about the immune mechanisms which normally defend against such infection? In this context, we found that anti-VCA antibody titers were significantly higher in our HIV-positive patient cohort than in control donors, as might be expected from many earlier serological studies (32). More importantly, however, anti-gp340 titers were also significantly higher than in controls; this finding implied the persistence of a strong EBV neutralizing antibody response (45), a fact which neutralization assays on a limited number of patient sera subsequently confirmed (Table 1). By contrast, EBV-specific cytotoxic T-cell function was clearly impaired in the same group of patients, with more than half of those analyzed showing no detectable reactivity in the in vitro regression assay (Table 1); although this is only a semiquantitative assay operating over a fairly narrow range, the results are indicative of the general impairment of T-cell function which underlies AIDS. The simplest interpretation of these findings is that humoral immunity alone, in particular the virus neutralizing antibody response, cannot protect from additional EBV infections and that cell-mediated immune controls must have a role to play. It remains to be seen what these controls might be, but the HLA class I-restricted cytotoxic T-cell response is one possible component. In that context, there was a suggestion in the present work that multiple infections in the circulating B-cell pool more commonly involve viral strains of different types than viral strains of the same type. If that trend is confirmed in subsequent work, it may be related to the fact that a significant fraction of the cytotoxic T-cell response to latently infected cells is against type-specific epitopes in the EBNA3A, -3B, -3C proteins (see, for example, references 11 and 16). Thus, in patients with, let us say, an existing type 1 EBV infection, as the cytotoxic T-cell response gradually wanes, it may be easier for an incoming type 2 virus to colonize the B lymphocyte pool than for a second type 1 viral strain.

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