Multiple Epstein-Barr Virus Strains in Patients with Infectious Mononucleosis: Comparison of Ex Vivo Samples with In Vitro Isolates by Use of Heteroduplex Tracking Assays

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Recent work using a heteroduplex tracking assay (HTA) to identify resident viral sequences has suggested that patients with infectious mononucleosis (IM) who are undergoing primary Epstein-Barr virus (EBV) infection frequently harbor different EBV strains. Here, we examine samples from patients with IM by use of a new Epstein-Barr nuclear antigen 2 HTA alongside the established latent membrane protein 1 HTA. Coresident allelic sequences were detected in ex vivo blood and throat wash samples from 13 of 14 patients with IM; most patients carried 2 or more type 1 strains, 1 patient carried 2 type 2 strains, and 1 patient carried both virus types. In contrast, coresident strains were detected in only 2 of 14 patients by in vitro B cell transformation, despite screening >20 isolates/patient. We infer that coacquisition of multiple strains is common in patients with IM, although only 1 strain tends to be rescued in vitro; whether nonrescued strains are present in low abundance or are transformation defective remains to be determined.

Epstein-Barr virus (EBV), a γ-herpesvirus that is widespread in all human populations, can be isolated in vitro via its ability to transform resting human B cells into permanent lymphoblastoid cell lines (LCLs) expressing the virus-coded antigens EBNA1, 2, 3A, 3B, 3C, and LP and the latent membrane proteins (LMPs) 1, 2A, and 2B. EBV isolates can be categorized as type 1 or type 2 on the basis of marked allelic polymorphisms within the EBNA2, 3A, 3B, and 3C genes [1, 2] and into distinct strains on the basis of more-subtle sequence variations within the EBNA1, EBNA2, and LMP1 genes and certain lytic cycle genes [3–9]. Studies in which multiple LCLs were established from blood and throat wash (TW) samples suggested that, in European populations, most people carried a single dominant strain—usually of type 1 but, in ~10% of cases, of type 2 [10, 11]. In contrast, immunocompromised patients were frequently found to carry multiple virus strains—again, usually of type 1 [10, 12]. The exceptions were male homosexual patients with AIDS who, like HIV-negative homosexual men [13], often harbored both virus types [14, 15].

Because LCL outgrowth may select for some but not all resident EBV strains [16], other studies have used direct amplification of viral DNA sequences from ex vivo samples, either to screen for type 1/type 2 coinfection [17, 18] or for the presence of coresident strains with different LMP1 alleles, distinguished either by the number of copies of a 33-bp repeat sequence or by the
Table 1. Oligonucleotide primer/probe combinations for standard polymerase chain reaction (PCR) and heteroduplex tracking assay (HTA) analysis.

<table>
<thead>
<tr>
<th>Assay, primer/probe</th>
<th>Sequence (5′→3′)</th>
<th>Coordinates</th>
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<tr>
<td>EBNA2 typing PCR analysis</td>
<td></td>
<td></td>
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<tr>
<td>E2C</td>
<td>AGGGATGCCTGGACACAAGA</td>
<td>48810–48829</td>
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<td>E2SEQ4</td>
<td>GTAATG6CATAAGTGGAATG</td>
<td>49374–49355</td>
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<tr>
<td>2A.2</td>
<td>TCTCGGACTATCTGATCAT</td>
<td>49328–49309</td>
</tr>
<tr>
<td>E2 type 1 probe</td>
<td>TCCAGCCACATGTCCTCCCCCTCTACGCACGA</td>
<td>48997–49028</td>
</tr>
<tr>
<td>E2 type 2 probe</td>
<td>AAGCTCAACCTGTCCAAACCTCGGCCAGGAG</td>
<td></td>
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<td>EBNA2 HTA</td>
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<tr>
<td>E2SEQ7.1</td>
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<td>48959–48978</td>
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<tr>
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<td></td>
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<tr>
<td>E2SEQ8.1</td>
<td>acgtagatctAGGCCTTTGTAGTACCGTGA</td>
<td>49208–49189</td>
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<tr>
<td>E2SEQ8.2</td>
<td>acgtagatctAGGCCTTTGTAGTACCGTGA</td>
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<tr>
<td>LMP1 HTA</td>
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<tr>
<td>LMP3UT</td>
<td>at cacgaaggaattcAATGTGGCTTTTCAGCCTAG</td>
<td>168017–168036</td>
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<td>FUC-HINDIII</td>
<td>at cagagaagcttTGACAATGGCCCACATGACC</td>
<td>168427–168408</td>
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<td>FUE ECO</td>
<td>at cagagaaggaattcGTCATAGTAGCTTAGCACC</td>
<td>168163–168183</td>
</tr>
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</table>

**NOTE.** Lowercase letters indicate sequences containing restriction enzyme sites that facilitate cloning. LMP, latent membrane protein.

presence or absence of a 30-bp deletion [19, 20]. Some of these studies indicated that use of in vitro isolation may have led to an underestimation of the incidence of multiple infection in long-term virus carriers. More surprisingly, however, use of a heteroduplex tracking assay (HTA) to map coresident LMP1 gene sequences has suggested that most patients with infectious mononucleosis (IM) who are undergoing primary EBV infection frequently harbor different EBV strains [21, 22]. This was unexpected because, apart from 1 unusual case [23], previous in vitro studies had given no indication of the presence of coresident strains. Here, we further examine the situation in patients with IM by use of both the LMP1 HTA and a newly developed HTA that detects polymorphisms at the EBNA2 gene locus, comparing the results for ex vivo samples with those for in vitro isolates.

**PATIENTS, MATERIALS, AND METHODS**

**Patients.** Patients with acute IM were identified on clinical grounds, and diagnoses were confirmed by a positive heterophile antibody (monospot) test and by the presence of EBV-specific CD8+ T cell expansion in the blood [24]. Unfractionated blood mononuclear (UM) cells and TW samples were obtained during acute IM. These were analyzed directly ex vivo in polymerase chain reaction (PCR) assays and were also used (as described elsewhere [15]) to rescue resident virus strains as UM cell–derived spontaneous LCLs (sp-LCLs) or as TW–derived LCLs (TW-LCLs).

**Standard PCR analysis for EBNA2 type and LMP1 repeat and deletion status.** All LCLs were screened using the EBNA2 type–specific primer/probe combination described by Sample et al. [2]. The type 1/type 2 status of the ex vivo UM cell and TW samples was determined using a more sensitive nested PCR approach with first-round E2C and E2SEQ4 primers and second-round E2C and 2A.2 primers; products were then Southern blotted and hybridized with type-specific radiolabeled probes (table 1). Standard PCR assays directed against strain-specific polymorphic markers in the LMP1 gene were also used to identify the presence or absence of a 30-bp deletion and to determine the number of copies of the 33-bp repeat present [5, 15].

**HTA at the EBNA2 and LMP1 gene loci.** The LMP1 HTA was performed as described elsewhere, using probes specific for 2 selected variants, Ch2 and Med+ [25]. An EBNA2 HTA was developed that was based on a polymorphic region (B95.8 coordinates 48959–49208) in the EBNA2 gene. First, EBNA2 type-specific HTA probes were generated by PCR amplification of the prototypic type 1 strain B95.8 with the E2SEQ7.1/E2SEQ8.1 primers and from the prototypic type 2 strain Ag876 with the E2SEQ7.2/E2SEQ8.2 primers (table 1). The resulting PCR products were then digested with EcoRI and BglII, gel purified, and ligated into EcoRI/BglII-digested pSG5, to generate the E2.1HTA-pSG5 and E2.2HTA-pSG5 plasmids, respectively. Radiolabeled HTA probes were generated from these plasmids as described elsewhere [25]. For EBNA2 HTA analysis, aliquots of DNA from ex vivo samples or LCLs were PCR amplified with first-round E2C and E2SEQ4 primers, and then separate aliquots of the first-round products were reamplified with type-specific second-round combinations—namely, E2SEQ7.1 and E2SEQ8.1, which are type 1 specific, and E2SEQ7.2 and E2SEQ8.2, which are type 2 specific. The subsequent product/probe binding reactions and heteroduplex analysis were performed as described elsewhere [25].
RESULTS

Development of an EBNA2 HTA

Sequencing >50 geographically diverse EBV isolates within a region of the EBNA2 gene (B95.8 coordinates 48810–49374) identified 5 major variants of the type 1 sequence. To maintain consistency with a previously published nomenclature [6], these are referred to as EBNA2 alleles 1.1, 1.2, 1.3A, 1.3B, and 1.3E; allele-specific changes relative to the B95.8 sequence (defined as allele 1.1) within the most polymorphic region (B95.8 coordinates 48959–49208) are shown in figure 1A. In contrast, all type 2–carrying LCLs analyzed were identical to the Ag876 prototype sequence in this region. To distinguish between these different EBNA2 gene sequences, we developed an HTA based on PCR amplification of this most-polymorphic region followed by heteroduplex formation with the corresponding 1.1 allele–specific (B95.8) and type 2–specific (Ag876) probes (figure 1B). Mobility of the complexes depends on the degree of sequence homology with the probe, with homoduplexes having the fastest mobility. Results of HTA analysis on 10 representative type 1 LCL isolates (A–K) and on 2 type 2 LCL isolates (L and M) are illustrated in figure 2A. The 2 virus types could be clearly distinguished from one another, because there was no detectable complex formation between the resulting PCR products and probes of the wrong type. More importantly, by heteroduplex mobility the type 1 isolates could be placed in 1 of the 5 subsets (1.1, 1.2, 1.3A, 1.3B, or 1.3E) on the basis of the identity of their EBNA2 allelic sequence.

The same representative panel of LCL isolates were examined for LMP1 sequence identity using the previously described LMP1 HTA [25]. This assay spans the 30-bp deletion locus and can distinguish the 7 different LMP1 variants shown in figure 1C—B95.8, Ch2, AL, NC, and Med—, none of which have the 30-bp deletion, and Med+ and Ch1, both of which have the deletion. Analysis of PCR products with a combination of Ch2 and Med+ probes allowed each of the LCLs to be identified as carrying a particular LMP1 sequence (figure 2B). Importantly, this showed that individual LMP1 alleles were not linked to particular EBNA2 alleles. Thus, combining the 2 HTAs produced an EBNA2/LMP1 allele signature for each LCL (figure 2C) that provided much better discrimination between individual strains than did either assay alone.

Characterization of Virus Strains Carried by Patients with IM

With these assays in place, we then turned to the analysis of the EBV strains present in 14 patients with IM. The ex vivo UM cell and TW samples and the in vitro LCL isolates derived from them (a mean of 13 sp-LCLs and 10 TW-LCLs per patient) were analyzed (1) at the EBNA2 gene locus by standard DNA typing PCR analysis and then by allele-specific HTA and (2) at the LMP1 gene locus by standard PCRs spanning the 33-bp repeats and the 30-bp deletion and then by allele-specific HTA. Four different patterns of results were obtained.

Detection of a single allelic sequence in a type 1 virus–infected patient. Assays for one patient, IM87, detected a single EBNA2 allele (1.3B) and a single LMP1 allele (Ch1) in the ex vivo UM cell and TW samples as well as in all 12 sp-LCLs and 9 TW-LCLs rescued in vitro. This suggested that this patient carried only a single type 1 strain.

Detection of multiple allelic sequences in type 1 virus–infected patients. A further 11 of the 14 patients were carrying type 1 sequences only (in the absence of type 2) at the EBNA2 gene locus; however, in all 11 patients there was evidence of the presence of multiple EBNA2 and/or LMP1 allelic sequences in ex vivo samples. Figure 3 shows data from the ex vivo samples and from some LCL isolates (representative of 16 sp-LCLs and 10 TW-LCLs) from patient IM75. Standard EBNA2 typing PCR analysis (figure 3A) detected type 1 signals throughout. However, the more discriminatory EBNA2 HTA (figure 3B) detected both 1.1 and 1.3B allelic sequences in the ex vivo UM cell sample, whereas only the 1.3B sequence was detected in the ex vivo TW sample. Interestingly, only the 1.3B allele was rescued in vitro—and not just in the TW-LCLs but also in every sp-LCL, even though the 1.1 allele had given the stronger HTA signal in the ex vivo UM cell sample. This was the first indication that not all resident viral sequences were represented in the in vitro–isolated LCLs.

When the same samples were analyzed at the LMP1 gene locus, the standard LMP1 30-bp deletion PCR was uninformative, because all of the samples were found to contain a nondeleted LMP1 sequence. However, amplification across the LMP1 33-bp repeats detected 2 different products, with 4.5 and 5 copies of the repeat in the ex vivo UM cell and TW samples, respectively, whereas only the sequence with 5 copies of the repeat had been rescued in vitro (figure 3C). LMP1 HTA analysis detected the presence of 2 LMP1 variant sequences, Med— and Ch1, in the blood but only 1 sequence, Med—, in the throat; again, only 1 of these sequences, Med—, had been rescued in the LCLs (figure 3D). The results suggest that this patient was infected with 2 type 1 strains. One strain, preferentially rescued in vitro, had a 1.3B EBNA2 allele and a Med— LMP1 allele with 5 copies of the 33-bp repeat; the nonrescued strain is predicted to have a 1.1 EBNA2 allele and a Ch1 LMP1 allele with 4.5 copies of the repeat.

In a second such patient (IM81) found to be carrying only type 1 virus sequences by standard EBNA2 typing PCR analysis (figure 4A), the EBNA2 HTA detected strong 1.1 and weak 1.3B allelic signals in the ex vivo UM cell sample but only 1.3B sequences in the throat. Again, all 12 sp-LCLs and 12 TW-LCLs from this patient carried only 1 of the sequences, 1.3B (figure 4B and data not shown). Standard PCR assays at the LMP1 gene locus reinforced the evidence indicating that
Figure 1. Classification of EBNA2 and latent membrane protein (LMP) 1 variant sequences. Panel A shows the position of nucleotide substitutions within the 1.2, 1.3A, 1.3B, and 1.3E EBNA2 alleles, relative to the 1.1 (B95.8) allelic sequence between coordinates 48959 and 49208. Note that the 1.3 family of EBNA2 alleles contain a CTC insertion not present in the 1.1 and 1.2 alleles (shaded squares).

Panel B shows a sequence alignment of the B95.8-derived (EBNA2 1.1 allelic) probe and the corresponding Ag876-derived (EBNA2 type 2 allelic) probe used in the heteroduplex tracking assay (HTA). The genome coordinates of the nucleotide changes found in the type 1 allelic variants are shown above the B95.8 sequence. The arrow between coordinates 49136 and 49137 indicates the site of the CTC insertion found in the 1.3 family of EBNA2 alleles.

Panel C shows the positions of nucleotide substitutions in the 7 LMP1 allelic sequence variants, relative to the B95.8 LMP1 allele; the shaded squares indicate nucleotides that are removed by the 30-bp deletion.

Panel D shows the sequence of the B95.8 LMP1 allele from coordinate 169163 to 169427, the region analyzed by the LMP1 HTA. The genome coordinates for each of the potential nucleotide changes found in the LMP1 allelic variants are shown above the sequence. The position of the 30-bp deletion is indicated by the shaded box.
different virus strains were dominant in the blood (4.5 copies of the repeat, nondeleted) versus the throat (5 copies of the repeat, deleted) and that only the latter virus had been isolated in vitro (figure 4C). However, the more discriminatory LMP1 HTA revealed 3 different LMP1 allelic sequences in the blood (Ch1, B95.8, and Ch2), of which 2 (Ch1 and B95.8) were also detectable in the throat. Again only 1 of these, the Ch1 allele, had been rescued in vitro (figure 4D). This suggested that patient IM81 carried 3 type 1 virus strains. One strain, preferentially rescued in vitro, carried a 1.3B EBNA2 allele and a Ch1 LMP1 allele. The other 2 predicted strains appear to have different alleles at the LMP1 gene locus (B95.8 and Ch2); their identity at the EBNA2 gene locus could not be determined unequivocally, but at least 1 must carry a 1.1 EBNA2 allele.

Of the 11 patients with IM who were apparently coinfected with 2 or more type 1 viruses, different allelic sequences were detected in both the ex vivo UM cell samples and the TW samples from 7 patients (IM72, IM75, IM79, IM80, IM81, IM82, and IM84); in the TW samples but not in the UM cell samples from 2 patients (IM85 and IM86); and in the UM cell samples but not (where tested) in the TW samples from 2 patients (IM73 and IM78). Most importantly, from all but 1 of these 11 patients only a single virus strain was ever rescued in vitro, despite the analysis of multiple LCLs in each case.

**Detection of type 1 and type 2 allelic sequences.** One patient, IM76, showed evidence of both type 1 and type 2 EBNA2 allelic sequences. These were initially detected in the ex vivo TW sample by standard EBNA2 typing PCR analysis, although the same analysis detected only type 2 sequences in the blood (figure 5A). EBNA2 HTA screening of the same ex vivo samples confirmed these results, with the TW sample carrying both a 1.3A allele and a type 2 allele and the UM cell sample carrying only a type 2 allele (figure 5B). However, a type 1 strain was preferentially rescued in vitro, with only 1 of 10 sp-LCLs (sp-LCL7) and 1 of 10 TW-LCLs (TW-LCL8) with detectable type 2 as well as type 1 signals in standard EBNA2 typing PCR analysis (figure 5A). Surprisingly, however, EBNA2 HTA analysis showed that the type 1 virus being rescued in vitro carried a 1.3B allelic sequence, not the 1.3A allele that had been detected ex vivo (figure 5B). Furthermore, assays at the LMP1 gene locus (data not shown) showed that a virus strain with a Med− allele and 5 copies of the 33-bp repeat was preferentially rescued in vitro, whereas a Ch1 allele with 6 copies of the 33-bp repeat was the dominant sequence in the ex vivo samples. This suggests that this patient harbored 3 virus strains. One type 1 strain carrying a 1.3B EBNA2 allele and a Med− LMP1 allele was preferentially rescued in vitro, and another predicted type 1 strain apparently carrying a 1.3A allele was not rescued. The coresident type 2 strain, carrying a type 2 EBNA2 allele and a Ch1 LMP1 allele, was rescued in vitro, but with low efficiency.

**Detection of multiple allelic sequences in a type 2 virus–infected patient.** A final patient, IM 43, was found to be carrying only type 2 sequences (in the absence of type 1) at the EBNA2 gene locus, both by standard EBNA2 typing PCR analysis and by HTA. Results for the ex vivo UM cell sample and for 8 representative LCLs are shown in figure 6A and 6B. However, the corresponding assays at the LMP1 gene locus detected in the ex vivo TW sample the presence of a dominant B95.8 allelic sequence, nondeleted and with 4.5 copies of the repeat, and of an additional sequence that had the 30-bp deletion. Parallel assays of the ex vivo UM cell sample identified a dominant Ch1 allelic sequence, deleted and with 6 copies of the repeat, and an additional B95.8 allelic sequence. The type 2
Figure 3. Analysis of Epstein-Barr virus sequences present in ex vivo unfractionated blood mononuclear (UM) cell and throat wash (TW) samples, in up to 4 (representative of 16) UM cell–derived spontaneous lymphoblastoid cell lines (sp-LCLs), and in up to 4 (representative of 10) TW–derived LCLs (TW-LCLs) from patient IM75. Panel A shows the results of an EBNA2 typing polymerase chain reaction (PCR) analysis, with B95.8 and Ag876 serving as type 1 and type 2 reference controls, respectively. Only EBNA2 type 1 sequences are detectable in the ex vivo UM cell and TW samples and in all of the sp-LCLs and TW-LCLs. Panel B shows the results of the EBNA2 heteroduplex tracking assay (HTA), which indicates the presence of a 1.3B allele and a 1.1 allele (both indicated by an arrow) in the ex vivo UM cell sample and the presence of a 1.3B allele only in the ex vivo TW sample and in all 4 representative LCL isolates. The gel also shows results obtained in the same experiment from reference control isolates known to carry a 1.1, 1.2, 1.3A, 1.3B, or 1.3E allele. The faint band running below the 1.1 heteroduplex represents probe homoduplex. Panel C shows the results of the latent membrane protein (LMP) 1 repeat and deletion PCRs performed on the same samples and reference controls as for panel A. LMP1 sequences with 4.5 and 5 copies of the repeat (both indicated by an arrow) are detectable in both the ex vivo UM cell and the TW sample, whereas all in vitro isolates have 5 copies of the repeat. Screening at the LMP1 deletion locus showed that all ex vivo samples and LCL isolates contained sequences that were nondeleted (in the figure, “WT” indicates nondeletion, and “DEL” indicates deletion). The B95.8 and Ag876 virus strains are known to have a 4.5-repeat, nondeleted LMP1 allele and a 4-repeat, deleted LMP1 allele, respectively. Panel D shows the results of the LMP1 HTA; in this case, only the data for the ex vivo samples are shown, alongside reference controls containing B95.8 and Ch2 allelic sequences in one track; Med+, Med−, and AL allelic sequences in a second track; and Ch1 and NC allelic sequences in a third track. The left panel shows an HTA performed with a Med+ allelic probe, and the right panel shows an HTA performed with a Ch2 allelic probe. The results show that the ex vivo UM cell sample from IM75 contains detectable Ch1 and Med− sequences (both indicated by an arrow) and that the ex vivo TW sample from IM75 contains Med− sequences only.

strain carrying the Ch1 LMP1 allele was preferentially rescued in vitro not just from the blood, where it was dominant, but also from the throat, where, ex vivo assays suggested, it was the minor strain (figure 6C and 6D).

The detailed results for all 14 patients with IM analyzed are summarized in figure 7. This figure shows, for each patient, the EBNA2 and LMP1 sequences detected in ex vivo samples and in their derived in vitro isolates. The patients are arranged by shading into the 4 categories described above, and in each case we give our interpretation of the number of resident virus strains present.

DISCUSSION

Recent evidence suggesting that patients with IM are often infected with multiple EBV strains [21, 22] was entirely based on HTA analysis of ex vivo samples at a single locus, the LMP1 gene locus. Given the apparent discordance between these findings and the results of earlier in vitro isolation studies, we extended the HTA analysis to a second polymorphic locus, the EBNA2 gene locus [3, 6, 26], and to ex vivo samples from patients from whom a large number of in vitro LCL isolates had already been recovered. The type and strain specificity of
Figure 4. Analysis of Epstein-Barr virus sequences present in ex vivo unfractionated blood mononuclear (UM) cell and throat wash (TW) samples, in up to 4 (representative of 12) UM cell–derived spontaneous lymphoblastoid cell lines (sp-LCLs), and in up to 4 (representative of 12) TW–derived LCLs (TW-LCLs) from patient IM81. Results are presented essentially as described in figure 3. Panel A (EBNA2 typing polymerase chain reaction [PCR] analysis) shows that only EBNA2 type 1 sequences are detectable in the ex vivo UM cell and TW samples and in all sp-LCLs and TW-LCLs. Panel B (EBNA2 heteroduplex tracking assay [HTA]) shows that 1.1 and 1.3B EBNA2 allelic sequences (both indicated by an arrow) are present in the ex vivo UM cell sample but that only the 1.3B allelic sequence is detectable in the TW sample and in all of the sp-LCLs and TW-LCLs. Panel C (latent membrane protein [LMP] 1 repeat and deletion PCRs) shows the presence in the ex vivo UM cell sample of an LMP1 allele that has 4.5 copies of the repeat and that is nondeleted (in the figure, “WT” indicates nondeletion, and “DEL” indicates deletion) at the 30-bp deletion locus, whereas the ex vivo TW sample and all of the LCLs carried an LMP1 allele with 5 repeats and a 30-bp deletion. Panel D (LMP1 HTA) shows the presence of 3 LMP1 allelic sequences (Ch1, B95.8, and Ch2) (all indicated by an arrow) in the ex vivo UM cell sample and of 2 alleles (Ch1 and B95.8) in the ex vivo TW sample; all sp-LCLs and TW-LCLs carried only the Ch1 allele.

Figure 5. Analysis of Epstein-Barr virus sequences present in ex vivo unfractionated blood mononuclear (UM) cell and throat wash (TW) samples, in up to 4 (representative of 10) UM cell–derived spontaneous lymphoblastoid cell lines (sp-LCLs), and in up to 4 (representative of 10) TW–derived LCLs (TW-LCLs) from patient IM76. Panel A (EBNA2 typing polymerase chain reaction [PCR] analysis) shows that only type 2 EBNA2 allelic sequences are detectable in the ex vivo UM cell sample, whereas both type 1 and type 2 sequences are detectable in the ex vivo TW sample. All of the LCLs carried type 1 alleles, but type 2 allelic sequences were also weakly detected in certain LCLs—namely, sp-LCL7 and TW-LCL8. Panel B (EBNA2 heteroduplex tracking assay [HTA]) shows data for ex vivo samples and some of the above LCLs, alongside reference control isolates known to carry a type 1 EBNA2 allele or a type 2 allele. The upper panel shows an HTA performed with a type 1 probe and the lower panel shows an HTA performed with a type 2 probe. The ex vivo UM cell sample contains only detectable type 2 sequences, whereas the ex vivo TW sample contains both type 2 and 1.3A allelic sequences (indicated by an arrow). However, all of the LCLs carried a 1.3B allelic sequence (indicated by an arrow) either alone or, in the case of sp-LCL7 and TW-LCL8, together with a type 2 allele.

The newly developed EBNA2 HTA was validated using a panel of type 1 and type 2 EBV reference isolates. These experiments showed that EBNA2 and LMP1 allelic polymorphisms are not linked. This strengthened a conclusion from earlier work with standard PCR assays, in which the EBNA2 type of virus strains did not correlate with the 30-bp deletion status of the LMP1 allele [27]. It was therefore clear that combining the 2 HTAs
Figure 6. Analysis of Epstein-Barr virus sequences present in ex vivo unfractionated blood mononuclear (UM) cell and throat wash (TW) samples, in up to 4 (representative of 12) UM cell–derived spontaneous lymphoblastoid cell lines (sp-LCLs), and in up to all 4 available TW–derived LCLs (TW-LCLs) from patient IM43. Results are presented essentially as described in figure 3. Panel A (EBNA2 typing polymerase chain reaction [PCR] analysis) shows that only type 2 EBNA2 allelic sequences are detectable in the ex vivo UM cell and TW samples and in all sp-LCLs and TW-LCLs. Panel B (EBNA2 heteroduplex tracking assay [HTA]) confirms the presence of type 2 sequences (indicated by an arrow) in all samples, with the exception of the ex vivo TW sample, for which no signal was obtained; note that no type 1 allelic sequences were detected in any sample by EBNA2 HTA with the 1.1 probe (data not shown). Panel C (latent membrane protein [LMP] 1 repeat and deletion PCRs) shows the presence of an LMP1 allele with 6 repeats and a 30-bp deletion in the ex vivo UM cell sample as well as weak signals (indicated by arrows) for a 4.5-repeat sequence and for both deleted and nondeleted (in the figure, “WT” indicates nondeletion, and “DEL” indicates deletion) sequences in the ex vivo TW sample. All of the sp-LCLs and TW-LCLs carried the 6-repeat, 30-bp deleted LMP1 allele. Panel D (LMP1 HTA) revealed the presence of a dominant Ch1 allele and a weak B95.8 allele (seen on longer gel exposures) in the ex vivo UM cell sample and the presence of a B95.8 allele only in the ex vivo TW sample. However, all of the sp-LCLs and TW-LCLs carried a Ch1 LMP1 allele.

would provide greater discriminatory power than would either the EBNA2 or the LMP1 HTA alone.

The relative prevalences of type 1 versus type 2 EBV strains in different human populations has remained contentious. Some studies have suggested that the majority of European and Southeast Asian donors carry type 1 strains, with low incidences of detectable type 2 infection or type 1/type 2 coinfection [10, 11, 28–30], whereas studies of other white populations have reported a higher prevalence of type 2 strains [17, 18]. To some extent, these anomalies may reflect subtle differences in the composition of study cohorts, such as differences in precise geographic origin or even in social group. For example, male homosexuals in Western societies have a much higher incidence of type 2 virus infection than does the general population [12, 13, 15, 17, 18], thereby explaining the unexpectedly high frequency of type 2 virus infection in early AIDS cohorts [14, 31, 32]. The present study focused on classic IM as it presents in young adults in the United Kingdom population. On the basis of EBNA2 gene amplification, it was found that, of our 14 patients, only type 1 sequences were detectable in 12 patients, only type 2 sequences were detectable in 1 patient, and both type 1 and type 2 sequences were detectable in 1 patient. Such a distribution is broadly in line with previous estimates of type 1 and type 2 virus infection prevalences in the general United Kingdom population [11, 12]. Our data leads us to question earlier suggestions that type 1/type 2 coinfection is common in patients with IM [17].

More importantly, the combination of EBNA2 and LMP1 HTA analysis detected different coreident EBV sequences in all but 1 of the patients with IM studied, results that are in line with recent findings obtained using only the LMP1 assay [21, 22]. In 7 of 12 informative patients in the present study, the same sequences were present in both the blood and the throat. Of the other patients, 2 had an additional sequence in the blood that was not detected in the throat, and 3 had an additional sequence in the throat that was not detected in the blood; such differences in the range of sequences found at different sites may reflect limitations in assay sensitivity rather than a genuine compartmentalization of infection in vivo. The reproducibility of the HTA data for the ex vivo samples (all were tested at least twice), as well as the fact that standard PCR amplification assays at the LMP1 repeat and deletion loci frequently detected coreident sequences in the same samples, strongly suggest that the present results are not artifactual but genuinely reflect the presence of 2 or more independent virus strains in patients with IM.

It was nevertheless surprising that, in many cases, only 1 virus strain was rescued in vitro despite the screening of multiple independent LCL isolates. Furthermore, the rescued strain’s EBNA2 and LMP1 alleles were not necessarily those that gave the strongest signals in ex vivo assays. In that regard, the results
### Figure 7.

Summary of results for all 14 patients with infectious mononucleosis (IM) analyzed, showing the outcome of standard polymerase chain reaction (PCR) and heteroduplex tracking assay (HTA) analysis at the EBNA2 and latent membrane protein (LMP) 1 gene loci on ex vivo unfractionated blood mononuclear (UM) cell and throat wash (TW) samples and on UM cell–derived spontaneous lymphoblastoid cell lines (sp-LCLs) and TW–derived LCLs (TW-LCLs) (no. of available LCLs are shown in brackets). The right-hand column shows our interpretation of the data in terms of the no. of resident Epstein-Barr virus strains in the blood and the throat. Patients are arranged into 4 main categories, identified by shading, to match the 4 patterns described in Results. DEL, deletion; WT, nondeletion.

<table>
<thead>
<tr>
<th>Sample</th>
<th>EBNA2 sequences</th>
<th>LMP1 sequences</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM 87  UM</td>
<td>PCR type</td>
<td>HTA allele</td>
<td>Repeats</td>
</tr>
<tr>
<td>IM 87  UM</td>
<td>PCR type</td>
<td>HTA allele</td>
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<tr>
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<tr>
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<tr>
<td>IM 43  UM</td>
<td>PCR type</td>
<td>HTA allele</td>
<td>Repeats</td>
</tr>
</tbody>
</table>

Two or more type 1 strains in the blood and the throat, only 1 rescued in vitro
Two type 1 strains in the throat, only 1 rescued in vitro
Two type 1 strains in the blood, only 1 rescued in vitro
Two type 1 strains in the blood and both rescued in vitro
One type 1 strain in the blood and the throat, same strain rescued in vitro
One type 1 and 1 type 2 strain in the blood and 2 type 1 and 1 type 2 strains in the throat, 1 strain of each type rescued in vitro
Two type 2 strains in the blood and the throat, only 1 rescued in vitro
of such assays, because they involve 2 rounds of PCR, may not accurately represent the relative allele loads in ex vivo samples. It is therefore still possible that in vitro isolation does, in fact, identify the dominant strain, at least when the competition for in vitro outgrowth involves coresident type 1 viruses. In contrast, type 1 strains will tend to be rescued preferentially above any coresident type 2 virus [16]. However, another possible explanation for the differences in results between the ex vivo samples and the in vitro isolates is that some viruses are not rescued in vitro because they are transformation defective. It will be important to study this possibility further, because the presence of transformation-defective strains in the blood of patients with IM would imply that transforming ability is not required for colonization of the B cell system.

We believe that the presence of multiple strains in patients with IM can be explained only by coacquisition of these strains during primary infection. Although it has been postulated that EBV quasispeciation within the host occurs during virus persistence, leading to EBV-positive malignancies [33, 34], there is no firm documentary evidence of such rapid evolution in vivo, nor is there any evidence indicating that EBV DNA polymerase is particularly error prone [35]. Furthermore, it seems implausible that such quasispeciation would always produce the particular range of EBNA2 and LMP1 allelic sequences described here. The most likely scenario is that the multiple strains detected in many of the patients with IM in the present study were coacquired from a single source—namely, a virus carrier who was shedding multiple strains in saliva [22]. It is clear that infection with multiple strains is not unique to patients with IM, because many healthy carriers with no history of the disease show evidence of coinfection when analyzed by LMP1 HTA [21] and by other assays [18, 28]. It is therefore possible that many subclinical primary infections, as well as cases of IM, involve the coacquisition of multiple strains. If so, it could explain the apparent high frequency of multiple infection in the general population. However, it does not rule out the possibility that at least some instances of multiple infection are the result of serial acquisition over time from independent sources. Prospective studies are required to resolve this important point. If serial acquisition does occur, it would mean that infection with 1 or more primary strains does not render the host immune to subsequent virus challenge. This would severely damage the prospects of designing an EBV vaccine that provided sterile immunity against natural virus infection.

References


32. Luxton JC, Williams I, Weller I, Crawford DH. Epstein-Barr virus infection of HIV-seropositive individuals is transiently suppressed by high-dose acyclovir treatment. AIDS 1993; 7:1337–43.

