Multiple Epstein-Barr Virus Infections in Healthy Individuals

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We employed a newly developed genotyping technique with direct representational detection of LMP-1 gene
sequences to study the molecular epidemiology of Epstein-Barr virus (EBV) infection in healthy individuals. Infections
with up to five different EBV genotypes were found in two of nine individuals studied. These results provide evidence
that multiple EBV infections of healthy individuals are common. The implications for the development of an EBV vaccine
are discussed.

Multiple Epstein-Barr virus (EBV) infections are common among immunocompromised individuals (21, 29, 31, 39, 41, 43, 44, 46), but the origin of the multiple EBV strains remains a mystery. Multiple EBV strains could accumulate as superinfections in individuals who have lost previous protective immunity to EBV. Alternatively, they could represent the reactivation of latent EBV strains that were acquired prior to the onset of immunodeficiency. The reported prevalence of multiple EBV infections in healthy individuals ranges broadly between 0 and 100% (Table 1) (4, 7, 13, 16, 19, 20, 23, 30, 32, 35, 38, 45; M. L. Lung and R. S. Chang, Letter, J. Infect. Dis. 162:994-995, 1990), but differences among these studies in the molecular detection and definition of an EBV strain confound the interpretation of their results.

Molecular epidemiologic studies requiring EBV isolation by B-lymphocyte transformation (16, 19, 23, 45; Lung and Chang, letter) suffer from selection bias toward transformation-competent EBV isolates (10, 27, 33). PCR amplification directly detects the EBV genome and avoids transformation selection bias, but the genetic definition of an EBV strain has been inconsistent across studies. Restriction fragment length polymorphisms detect either point mutations within restriction enzyme cleavage sites or variations of large repetitive regions within genome fragments (19, 23, 35; Lung and Chang, letter). Similarly, size variation in EBNA proteins (“EBNotype” or “EBNAPrint”) (16, 45) and size variation in specific gene PCR products (LMP-1, BZLF1, EBNA-6) (13, 35) reflect variations in repetitive and other genome sequences. However, many EBV genome sequences are susceptible to intrastrain homologous and nonhomologous recombination during productive replication. The number of repeat units present may vary in different isolates of the same EBV strain (12, 38, 41–43). Studies examining the major sequence divergence between EBV types 1 and 2 have reported EBV coinfection rates ranging from 0 to 53% (4, 13, 20, 34, 35, 45). However, EBV types 1 and 2 can both be further subdivided into different strains (1, 24, 41) and only three studies to date have utilized EBV gene

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Saliva and peripheral blood mononuclear cells from nine individuals were nonrandomly selected (based on detection of EBV in saliva for eight of the nine subjects and on the absence of EBV in the saliva for the remaining subject) from a cohort of 30 healthy human research subject volunteers enrolled in a long-term, prospective study of virus reactivation and shedding (22). Quantitative measurement of the EBV in each of 28 pairs of saliva and blood specimens was accomplished by real-time quantitative PCR of the EBER gene (Table 3) as previously described (28). EBV was detected in 25 of 28 saliva specimens by EBER PCR, and detectable quantities ranged from 6 to 2,220,000 EBV genome copies per 0.5 μg of DNA (Table 3). For subjects 2 and 4, the quantity of EBV detected in the saliva was remarkably high, approaching 30 EBV genomes per cell equivalent, a range similar to levels of productive EBV replication in oral hairy leukoplakia (15, 40).

EBV was detected in 3 of 28 blood specimens by EBER PCR, and detectable quantities ranged from 13 to 80 EBV genome copies per 0.5 μg of DNA (Table 3). Previous studies have indicated that healthy individuals carry EBV in the peripheral blood at 1 to 63 EBV genome copies per 10^6 B lymphocytes (25, 37). This quantity is at or below the limit of detection for this assay using up to 0.75 × 10^3 blood mononuclear cell genome equivalents of DNA per reaction.

**Multiple EBV infections in healthy individuals.** In this pilot study, we tested the hypothesis that healthy individuals harbor infections with multiple LMP-1-defined EBV genotypes, representative of multiple independent EBV infections. We determined that an individual study subject harbored multiple EBV infections when one or more of the following three criteria were met: (i) two or more EBV genotypes are present in a single saliva or blood specimen; (ii) different EBV genotypes are present among simultaneously collected saliva and blood specimens from the same individual; (iii) temporal changes in the EBV genotype are present in sequentially collected saliva or blood specimens from the same individual.

Single-genotype EBV infection was identified in seven of the nine subjects, including five subjects that had a single EBV genotype repeatedly detected in saliva at multiple time points over periods of up to 4 months (Table 3). Two subjects were found to harbor multiple EBV infections (Table 3). Subject 3 harbored different EBV genotypes among simultaneously collected saliva and blood specimens at two different time points. Additionally, subject 3 exhibited temporal changes in the EBV genotypes present in sequentially collected saliva and blood specimens. In total, four different EBV genotypes were detected for subject 3 over a period of 2 months, with at least two detectable genotypes simultaneously infecting this subject at two separate time points. Subject 9 harbored two or more EBV genotypes present in four different saliva and blood specimens. Additionally, subject 9 harbored different EBV genotypes between simultaneously collected saliva and blood specimens at two different time points. Finally, subject 9 also exhibited temporal changes in the EBV genotypes present in sequentially collected saliva and blood specimens. In total, five different EBV genotypes were identified for subject 9 over a period of 8 months, with up to four detectable genotypes simultaneously infecting this subject at any single point in time.

Our data demonstrated multiple EBV infections in two of nine subjects. This prevalence rate of 22% is very close to the mean prevalence rate of 23% calculated from the pooled data (Table 1). However, the limitations of this study (small sample size, nonrandom selection, short duration, and low success rate for blood specimens) could tend to either underestimate or overestimate the true prevalence of multiple EBV infections. A large, well-designed, EBV LMP-1 genotyping study is warranted in order to accurately determine the prevalence of multiple EBV infection in healthy individuals.
Immunocompromised individuals routinely exhibit high-level oral EBV shedding (2, 14), coinfection with multiple EBV genotypes (39, 41, 43), and temporal changes in EBV populations (26, 31). Yet, even among this small sample of nine healthy individuals, we found examples of high salivary levels of EBV, infection with up to five different EBV genotypes, and temporal changes in the EBV populations in saliva and blood. The different magnitude, yet similar nature, of the EBV behavior in immunocompromised and in healthy individuals suggests that acquired immunodeficiency simply unmasks or exaggerates intrinsic aspects of the normal EBV-host relationship. More-frequent reactivations and higher levels of replication in immunocompromised individuals allow preexisting multiple EBV infections to be detected more easily.

If multiple EBV infections are common in healthy individuals, then the temporal nature of acquisition of these multiple infections remains to be determined. It is possible that multiple EBV genotypes are simultaneously acquired during primary EBV infection, through exposure to an individual (such as subject 9) who is orally shedding multiple EBV genotypes. In this single-event hypothesis, all of the coinfected genotypes would simultaneously establish persistent latent infection prior to the development of EBV-specific immunity in the host. Once developed, host immunity may prevent further EBV superinfection.

Alternatively, it is possible that multiple EBV genotypes are sequentially acquired as successive superinfections from multiple exposures over the lifetime of the host. In this accumulation hypothesis, immunity to EBV that developed after primary infection may prevent further EBV superinfection.

![Image](http://jvi.asm.org/)

**TABLE 2.** LMP-1 genotype sequence ratios before and after PCR and cloning

<table>
<thead>
<tr>
<th>A:B ratio before PCR</th>
<th>No. of resultant clones A</th>
<th>No. of resultant clones B</th>
<th>A:B ratio after PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>15</td>
<td>26</td>
<td>1:1.7</td>
</tr>
<tr>
<td>2:1</td>
<td>24</td>
<td>13</td>
<td>3:1</td>
</tr>
<tr>
<td>4:1</td>
<td>45</td>
<td>6</td>
<td>7:1</td>
</tr>
<tr>
<td>10:1</td>
<td>18</td>
<td>2</td>
<td>9:1</td>
</tr>
<tr>
<td>30:1</td>
<td>28</td>
<td>2</td>
<td>14:1</td>
</tr>
<tr>
<td>100:1</td>
<td>14</td>
<td>0</td>
<td>Not applicable</td>
</tr>
<tr>
<td>300:1</td>
<td>40</td>
<td>0</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>
Subject and specimen

1

Saliva 0 ND – 0
Blood 0 ND – 0
Saliva 2 ND 3c-C 10
Blood 2 ND – 0

2

Saliva 0 10,400 3c-D 10
Blood 0 ND – 0
Saliva 2 2,220,000 3c-D 10
Blood 2 ND – 0
Saliva 4 328,000 3c-D 9
Blood 4 ND – 0

3

Saliva 0 6 2a-G 8
Blood 0 ND B958a-B958 10
Saliva 2 365 B958a-B958 10
Blood 2 ND – 0
Saliva 4 4,240 B958a-B958 10
Blood 4 ND – 0

4

Saliva 0 81,500 2a-G 10
Blood 0 ND – 0
Saliva 2 66,700 2a-G 10
Blood 2 ND – 0
Saliva 4 158,000 2a-G 22
Blood 4 ND – 0

5

Saliva 0 2,920 B958a-F2 9
Blood 0 ND – 0
Saliva 2 365 B958a-F2 10
Blood 2 ND – 0
Saliva 4 4,240 B958a-F2 10
Blood 4 ND – 0

6

Saliva 0 42 – 0
Blood 0 13 – 0
Saliva 2 17 2a-G 10
Blood 2 ND – 0
Saliva 4 161 – 0
Blood 4 ND – 0

7

Saliva 0 14,100 B958a-F2 10
Blood 0 ND – 0
Saliva 2 15 – 0
Blood 2 ND – 0
Saliva 4 566 B958a-F2 10
Blood 4 ND – 0

8

Saliva 0 428 – 0
Blood 0 ND – 0
Saliva 2 690 3c-D 10
Blood 2 ND – 0
Saliva 4 4,210 3c-D 10
Blood 4 ND – 0

9

Saliva 0 350 2a-F2 + 3c-D 8 + 2
Blood 0 80 B958a-B958 + B958b-F2 6 + 4
Saliva 2 276 2a-F2 + 3c-D 3 + 7
Blood 2 ND – 0
Saliva 8 320 1-G + 2a-F2 + 3c-D 1 + 2 + 6
Blood 8 ND 2a-F2 10

a In 0.5 μg of DNA extracted from salivary cells or peripheral blood mononuclear cells. ND, not detectable.

b PCR and cloning unsuccessful.

mmary infection may not protect the host against exogenous EBV superinfection. Each successively encountered genotype may establish persistent latent infection despite preexisting host immunity to EBV. This hypothesis is supported by molecular epidemiologic data from another human herpesvirus. Healthy individuals previously infected with cytomegalovirus are susceptible to superinfection with additional, genetically different cytomegalovirus strains (5, 6, 8).

The relationship between EBV infection and host immune response must be understood before designing an EBV vaccine. If natural EBV infection does not protect against subsequent EBV superinfection with a different genotype, then the goal of an EBV vaccine to prevent wild-type EBV infection may not be achievable. However, natural infection with EBV early in life appears to protect against later developing the infectious mononucleosis syndrome (17, 18, 36). A vaccination that induces an immunity similar to that obtained by wild-type infection may protect against developing the infectious mononucleosis syndrome if wild-type EBV infection does subsequently occur. The precedent for this concept has been established with another human herpesvirus. Vaccination with live, attenuated varicella-zoster virus does not prevent wild-type varicella-zoster virus superinfection but does prevent or greatly attenuate the clinical syndrome of chickenpox (3, 9).

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REFERENCES


