Changes in the dominant Epstein-Barr virus type during human immunodeficiency virus infection

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Two types of Epstein-Barr virus (EBV), EBV-1 and EBV-2, were identified on the basis of DNA sequence divergence in the genes for nuclear proteins EBNA 2, 3a, 3b and 3c. In the present study, we conducted an immunological and genomic analysis in a human immunodeficiency virus (HIV)-infected population to determine the prevalence of the two types, and whether the identified type was stable over years. The EBNA-2 serotyping and genotyping showed that HIV-infected patients were highly infected by EBV-2, and that the dominant strain was mostly retained. However, during a follow-up study, a change in the dominant viral strain was observed in two patients. A first HIV-positive patient (patient A), although having a stable level of anti-EBNA-2A and -2B antibodies, showed a change in the genotype and antigen produced in spontaneously established lymphoblastoid cell lines (LCL). The sequence analysis of LCLs confirmed the emergence of the EBV-2 type population. A strain from a second HIV-positive patient (patient B) was clearly identified as EBV-2: the genotype from a saliva sample and from sequential LCLs belonged to EBV-2, as well as the antigen produced from LCLs, and serum antibodies. After a 5-year continuous EBV-2 infection, a reactivation of the EBV-1 strain was observed. In both cases, sequence analysis of the EBNA-2 gene showed, only with EBV-1, the presence of EBV variants related to the B95-8 prototype. Two mutations (at nucleotides 49212 and 49304) were found in both patients A and B, whereas an additional mutation (at nucleotide 49237) was characteristic of the patient A. No mutation relative to the prototype B95-8 strain was observed in a subsequent analysis of this EBNA-2 region from LCLs obtained from two HIV-negative patients predominantly infected by EBV-1. Therefore, we speculate that these mutations may be EBV-1 mutations specifically occurring during HIV infection.

In Epstein-Barr virus (EBV)-immortalized lymphoblastoid cell lines (LCLs), six nuclear proteins (EBNA-1, -2, -3a, -3b, -3c and -LP) and three latent membrane proteins (LMP-1, -2A and -2B) (Kieff & Liebowitz, 1990) are expressed. The EBNA-2 gene-encoded protein shows a genetic polymorphism. In this way, two types of EBV were identified, EBV-1 (expressing the EBNA-2A protein) and EBV-2 (expressing the EBNA-2B protein) (Dambaugh et al., 1984; Addinger et al., 1985; Mueller-Lantzsch et al., 1985). It is now evident that significant differences between EBV-1 and EBV-2 include the EBNA-3a, -3b and -3c genes (Rowe et al., 1989; Sculley et al., 1989; Sample et al., 1990). Not all the biological differences between the two EBV types are known but, compared with type 1, type 2 has a lower transforming efficiency, a poorer initial outgrowth, and a higher cell density dependence for cell viability (Rickinson et al., 1987). Seroprevalence and molecular studies indicated that both types occur worldwide, but EBV-2 is less ubiquitous (Zimber et al., 1986; Kunimoto et al., 1992; Chen et al., 1992; Shu et al., 1992).

The analysis of human immunodeficiency virus (HIV)-seropositive individuals showed that this population was highly infected by EBV-2 as compared with immunocompetent controls (Sculley et al., 1988, 1990). Because of the lack of information about persistence of the two different viral types during the course of HIV infection, we studied the prevalence of EBV types 1 and 2 in an HIV-infected population during a long-term follow-up.

In a first study, 146 HIV-seropositive individuals were tested for their immunological response against the EBNA-2A or EBNA-2B antigens. The EBNA-2 serotyping was performed on Rat-1 epithelial cells, cotransfected with the plasmid pSV2neo, and either the plasmid p729-15 containing the region coding for the EBNA-2B protein (data not shown) or the plasmid p780-28 containing the region coding for the EBNA-2A protein (Mueller-Lantzsch et al., 1985).

We observed (Table 1) that the prevalence of antibodies to EBV-2 (EBNA-2B) in HIV-positive individuals was 10-fold higher than that found in the general population (50 blood donors) and correlated with an
Table 1. Analysis of the anti-EBNA-2 response by immunofluorescence assay

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of anti-EBNA-2-negative individuals</th>
<th>Prevalence of EBV type*</th>
<th>GMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serotype 1</td>
<td>Serotype 2</td>
</tr>
<tr>
<td>HIV-seronegative (n = 49)†</td>
<td>13 (26.5%)</td>
<td>34 (94.4%)</td>
<td>1 (2.7%)</td>
</tr>
<tr>
<td>HIV-seropositive (n = 146)</td>
<td>38 (26.0%)</td>
<td>63 (58.3%)</td>
<td>30 (27.8%)</td>
</tr>
</tbody>
</table>

* After titration of the test sera by anti-complement immunofluorescence using twofold dilutions (1:5 to 1:5120), the individuals were classified into serotype groups on the basis of preferential antibody reactivity to either EBNA-2A, corresponding to serotype 1, or EBNA-2B corresponding to serotype 2. A titre difference greater than at least two dilutions was considered to be significant.
† One donor had not been infected by EBV.

Table 2. EBV gene typing from 33 EBNA-2 serotyped individuals (HIV-seropositive)*

<table>
<thead>
<tr>
<th>Genotypes of LCLs rescued from</th>
<th>Genotype from PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>Saliva</td>
</tr>
<tr>
<td>Serotype</td>
<td>Type 1</td>
</tr>
<tr>
<td>Serotype 1</td>
<td>12</td>
</tr>
<tr>
<td>Serotype 2</td>
<td>3</td>
</tr>
<tr>
<td>Non-serotypable</td>
<td>6</td>
</tr>
</tbody>
</table>

* For PCR analysis, primers and probes were designed, based on the published sequences of B95-8 (EBV type 1) and Jijoye (EBV type 2) cell lines, corresponding to the long divergent domain of the EBNA-2 gene (see Fig. 1 for sequences). DNA (1 μg) was denatured at 94 °C for 10 min and amplification was carried out by 30 cycles of denaturation at 94 °C for 90 sec, annealing at 50 °C for 90 sec and extension at 72 °C for 2 min in a Thermal Cycler (Perkin-Elmer Cetus).

This difficulty in identifying the EBV type was overcome by PCR analysis. Samples from 33 individuals, randomly selected among the 146 HIV-infected patients, were tested. Because of the small number of EBV-infected cells in peripheral blood mononuclear cells (PBMC) from healthy individuals, and the limited sensitivity of our assay to amplify the EBNA-2 gene (data not shown), DNA amplification was performed in lymphoblastoid cell lines (LCLs) spontaneously established from PBMC and saliva rather than directly from PBMC. Saliva, inoculated into umbilical cord lymphocytes after centrifugation and filtration, as well as unfractionated mononuclear cells isolated from peripheral blood using Ficoll–Hypaque, were cultured, until spontaneous outgrowth, in a 25 cm² flask (Falcon) containing 4 × 10⁶ cells in 4 ml of complete culture medium, supplemented with 10% fetal bovine serum containing 1 μg of cyclosporin A per 3 × 10⁶ cells.

EBNA-2 genotyping (Table 2) by PCR analysis of LCLs established from patients previously classified by antibody screening was entirely consistent with the results of serotyping. Therefore, we suggest that the immunofluorescence assay of anti-EBNA-2 antibodies could be used as a first approach to study the prevalence of EBNA-2A or EBNA-2B. In our series, all the individuals lacking antibodies against the EBNA-2 protein as well as five out of six patients with equal IgG
mutations: Patient A
Patient B
common primer (BM1)
EBNA 2 type 1
5' CACAGAGGGCTACCCTGCTCTCCACTCTGCACCCACACACACTCTCACAGTACT
EBNA 2 type 2
5' CACACAGGGCTACCCTGCTCTCCACTCTGCACCCACACACACTCTCACAGTACT

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T

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type 1 probe (BM3)
ACAAAGGCCCTACCCGACCTCAACCAATCACCACTACCCACACACAGCTACCT

ACAAAGGCCCTACCCGACCTCAACCAATCACCACTACCCACACACAGCTACCT


GCATGTGCCCCAACCATCAATGACCCCTTTAATCATACGCCACCCCAATGATCCA

GCATGTGCCCCAACCATCAATGACCCCTTTAATCATACGCCACCCCAATGATCCA


ACAAAGGCCCTACCCGACCTCAACCAATCACCACTACCCACACACAGCTACCT

ACAAAGGCCCTACCCGACCTCAACCAATCACCACTACCCACACACAGCTACCT


CACCACCACCAGGGCAC

CACCACCACCAGGGCAC

3' 262 bp
3' 259 bp
common primer (BM2)

Table 3. EBNA-2 follow-up analysis of patients A and B

<table>
<thead>
<tr>
<th>Serotyping</th>
<th>EBV-1</th>
<th>EBV-2</th>
<th>Antigen typing</th>
<th>Gene typing</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCA</td>
<td>EA</td>
<td>EBNA</td>
<td>IF+</td>
<td>WB+</td>
</tr>
<tr>
<td>A</td>
<td>4/5/90</td>
<td>LCL-A1</td>
<td>1280 20 80</td>
<td>40 +</td>
</tr>
<tr>
<td>1/7/90</td>
<td>LCL-A2</td>
<td>1280 20 80</td>
<td>40 +</td>
<td>80 +</td>
</tr>
<tr>
<td>30/7/90</td>
<td>LCL-A3</td>
<td>1280 20 80</td>
<td>40 +</td>
<td>80 +</td>
</tr>
<tr>
<td>B</td>
<td>24/7/87</td>
<td>LCL-B1</td>
<td>2560 320 160</td>
<td>80 +</td>
</tr>
<tr>
<td>25/9/87</td>
<td>LCL-B2</td>
<td>2560 320 160</td>
<td>80 +</td>
<td>640 +</td>
</tr>
<tr>
<td>2/3/89</td>
<td>LCL-B3</td>
<td>2560 320 160</td>
<td>80 +</td>
<td>640 +</td>
</tr>
<tr>
<td>21/2/91</td>
<td>saliva</td>
<td>2560 320 160</td>
<td>640 +</td>
<td>640 +</td>
</tr>
<tr>
<td>5/3/91</td>
<td>LCL-B4</td>
<td>2560 320 160</td>
<td>640 +</td>
<td>640 +</td>
</tr>
<tr>
<td>5/3/91</td>
<td>PBMC</td>
<td>2560 320 160</td>
<td>640 +</td>
<td>640 +</td>
</tr>
</tbody>
</table>

* Day on which sample was obtained.
† Serology corresponding to this day.
‡ IF, Immunofluorescence; WB, Western blotting.
§ ND, Not done.

antibody titres to EBNA-2A and EBNA-2B, harbored EBV-1. The fact that 21% of the generated LCLs were identified by PCR as EBV-2-positive confirms the high prevalence of this type among a HIV-positive population. Kyaw et al. (1992) showed that 19% of LCLs and 27% of PBMCs obtained from HIV-positive patients belonged to type 2. Once again these results are in agreement with those of Sculley et al. (1990): 19% of LCLs from HIV-infected individuals harbored EBV-2 compared to 3% from a healthy population.

To determine whether temporal changes of EBV type could occur during a long-term follow-up, multiple anti-EBNA-2 serologies were performed on 68 of the 146 HIV-infected patients. In most patients, the serological
Fig. 2. EBNA-2 typing by Western blot analysis of spontaneously established cell lines from patients A and B. Extracts of cell lines from both patients (patient A: lanes 5, LCL-A2; lanes 6, LCL-A3 and patient B: lanes 7, LCL-B3; lanes 8, LCL-B4) and of four reference cell lines B95-8, type 1 EBV (lanes 2); Rat-2A, type 1 EBV (lanes 3) and Rat-2B, type 2 EBV (lanes 4) were probed with (a) human serum reactive against EBNA-2B and (b) human serum reactive against EBNA-1 and EBNA-2A. For EBNA-2 typing total proteins from 2 x 10^7 cells of each cell line were dispersed in 2% (w/v) SDS, 2% 2-mercaptoethanol, 2 mM-PMSF, 2 mM-EDTA, 32.5 mM-Tris-HCl and 10% sucrose. Protein extracts were separated in a 10% polyacrylamide gel, and after a passive transfer onto nitrocellulose for 72 h at room temperature were probed with human EBNA-2A- and EBNA-2B-specific antibodies selected in our laboratory. Specifically bound antibody was detected using a peroxidase-conjugated goat antibody to human IgG and 3,3'-diaminobenzidine substrate.

response was stable. We selected a subgroup of 10 patients for detailed virological analysis, this subgroup including the few patients with interesting serological changes. In fact, multiple isolations from eight out of 10 patients gave consistent results for EBV type (five type 1, three type 2). Detailed results are presented for the two unusual patients only and appear in Table 3 and Fig. 2 and Fig. 3.

Patient A, a French homosexual man with anti-HIV antibodies since 1985, who had never received transfusion, had been classified until now as CDC II; he had been treated with AZT. Sera from this patient were regularly tested between 1985 and 1991. For each serum, specific antibody titres directed against lytic antigens were consistent with viral reactivation (anti-VCA 1:1280, anti-EA 1:120 and anti-EBNA 1:80). EBNA-2 serotyping by immunofluorescence was difficult because anti-EBNA-2A and EBNA-2B antibodies had a very similar titre and were stable over 6 years. Although immunoblotting showed that the anti-EBNA-2 response was stronger with EBNA-2B than with EBNA-2A, the patient was considered as non-serotypable. Three LCLs (LCL-A1, LCL-A2 and LCL-A3) were established from PBMC (Table 3). In a 3-month period, different EBNA-2 proteins were produced by these spontaneously established cell lines: EBNA-2B protein was identified in LCL-A1 and LCL-A3, whereas EBNA-2A was identified in LCL-A2 (Fig. 2).

DNA samples were then subjected to DNA amplification. In LCL-A1 and LCL-A2 the PCR products gave a single band of 262 bp specific to the EBNA-2A gene as confirmed by hybridization. LCL-A3 after DNA amplification showed a single band of 259 bp that did not hybridize with the EBNA-2A probe, whereas a specific hybridization was obtained with the EBNA-2B probe. The results with LCL-A1, -A2 and -A3 favour the hypothesis of dual infection and occasional reactivation of one type. A sequence analysis of the EBNA-2 region of LCL-A2 and LCL-A3 was then performed. Four clones obtained from the PCR product of LCL-A2 (262 bp) were studied. The sequences were nearly identical to the corresponding sequence of the prototype B95-8 strain (EBV-1), except that three mutations were observed at nucleotides 49212, 49237 and 49304 (Fig. 3). Two of these mutations induced amino acid changes: a proline was substituted by a serine at position 49237, and a threonine by an isoleucine at position 49304. For LCL-A3, three clones were obtained from the PCR product
Fig. 3. Nucleotide sequence (3' → 5') of EBNA-2 PCR product in type 1 LCLs from an HIV-positive individual (patient A; a) and a HIV-negative individual (b). Arrowheads 1, 2 and 3 indicate the nucleotide positions 49212, 49237 and 49304 respectively, and 1*, 2* and 3* indicate the base mutations at these positions. For EBNA-2 gene sequencing, fragments were purified following PCR, from an ethidium bromide-stained agarose gel on an ultra-free filter (Millipore). After precipitation in ethanol, the DNA redissolved in water was estimated for the blunting/kinase treatment reaction (SureClone ligation Kit, Pharmacia). PCR products were then cloned into pUC19 plasmid at the SmaI site and sequenced with a T7 polymerase system (Sequenase Kit, USB). The sequencing reaction was carried out by the chain-termination method of Sanger et al. (1977).

(259 bp). The sequence of these clones was exactly identical to the corresponding sequence in the Jijoye strain (EBV-2).

The second patient (patient B) was a drug addict, HIV-seropositive since 1986, never transfused, and classified as CDC IVe. He had been treated with AZT since 1987 until his accidental death in September 1992. He was splenectomized in 1989. The CD4 cell count remained stable at 200/mm³ over 6 years. Eighteen sera from this patient were tested between 1986 and 1991. The anti-VCA and anti-EA antibody titres were high and stable, suggesting a viral reactivation (VCA 1:2560, EA 1:1280). The anti-EBNA antibody titre was also stable (1:160) and indicated a past infection. The serological response against EBNA-2 protein showed hyper-reactivity against the EBNA-2B antigen (titre ≥ 1:640), whereas anti-EBNA-2A antibody titres were weak at the beginning of the study (1:80), and gradually increased to 1:640. This increase in anti-EBNA-2A antibody titre was confirmed by Western blotting. Four LCLs were established: one from saliva (LCL-B1) and three from PBMC (LCL-B2, LCL-B3 and LCL-B4) (Table 3). Western blot analysis of LCL-B3 and LCL-B4 revealed a change in the EBNA-2 protein; LCL-B3 exhibited an EBNA-2B protein whereas an EBNA-2A antigen was observed in LCL-B4 (Fig. 2).

Six samples were studied by PCR: the previous four LCLs, a saliva sample obtained in 1991 and PBMC from which LCL-B4 was established. After amplification, using EBNA-2 primers, LCL-B1, LCL-B2, LCL-B3 and the saliva sample gave a single band of 259 bp, specific for the EBNA-2B gene. Hybridization with specific probes gave no signal with the EBNA-2A probe whereas the EBNA-2B probe gave positive results with the four samples.

Six clones from LCL-B1 were sequenced and all of them were identical to the prototype Jijoye strain sequence. The clones obtained from the PCR product from LCL-B4 hybridized with the EBNA-2A probe and not with the EBNA-2B probe. The sequencing of these seven clones confirmed the EBV-1 genotype. All clones showed mutations at nucleotide positions 49304 and 49212, the same as for patient A. We did not detect the mutation at nucleotide 49237. A single clone harbored a mutation at nucleotide 49340.

So, in both individuals, the oligonucleotide sequence from EBV-2 was identical to the reference Jijoye strain, whereas EBV-1 showed mutations relative to the B95-8 cell isolate. Mutations at nucleotides 49212 and 49304 were common to patients A and B; however, patient A was characterized by an additional mutation at nucleotide 49237. It is interesting to note that the mutations always involved a cytosine substituted by a thymine. It was not possible to correlate these changes with the progression of the disease; in fact, no clinical manifestations were noted at that time. Each of the patients examined seemed to have been infected by different strains since specific mutations were observed.

To determine whether such mutations exist in a HIV-negative population, we sequenced this EBNA-2 region from LCLs obtained from two healthy individuals (not infected by HIV). In these two subjects, an infection by EBV-1 was found to be predominant, since they were classified into serotype 1, and the EBV-1 genotype was
identified in LCLs obtained from each patient. Sequence analysis showed that the clones containing the EBNA-2 fragment did not harbour any of the three mutations, since the sequences were identical to those of the corresponding B95-8 strain (Fig. 3). LCLs obtained from these two HIV-negative individuals were spontaneously established during the same period as those from HIV-positive individuals.

Our longitudinal data from HIV-infected patients have shown that a change in the EBV dominant types may be observed during the course of the disease. We are not sure, as yet, whether the type identified was a reactivating virus, the other type remaining undetectable, or whether it represented an exogenous superinfection. In the latter case, however, it is most likely that both strains should have been identified, unless the immune response had selected one strain only. Indeed, type-specific T cell epitopes have been described with the EBNA-2 (Moss et al., 1988; Rowe & Clarke, 1989) or the EBNA-3a proteins (Apolloni et al., 1992).

The possibility of a dual EBV infection in an HIV-infected population as well as in the general population remains, however, unclear. The follow-up study of healthy individuals, carried out by Yao et al. (1991), demonstrated that a single type and strain were observed after sequential isolations from blood and throat washings. However, Sixbey et al. (1989) reported one healthy individual shedding the type 2 virus in throat washings and the type 1 virus 10 weeks later. By contrast, many authors have shown that co-infection may be more common in immunocompromised patients. Sixbey et al. (1989, 1991) for throat washings, Sculley et al. (1990) for LCLs and Kyaw et al. (1992) for PBMCs and LCLs suggested that HIV-positive subjects might be infected by both types.

One of our patients, who was not serotyplable, provided strong evidence for co-infection with the two EBV strains since PCR product hybridized with both specific probes. Moreover, Walling et al. (1992) recently observed that both types 1 and 2 could be present within an oral hairy leukoplakia lesion and suggested the presence of multiple viral strains or variants arising from mutations in the EBNA-2 gene.

The sequence analysis of the EBNA-2 gene showed the presence of EBV variants occurring only with EBV-1. To our knowledge, no systematic sequencing studies have analysed the EBNA-2 gene so far. The mutations we observed have not been reported as yet. Further studies on the prevalence of these mutations in EBV isolates from HIV-infected individuals could be of interest.

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References


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