Abstract

Tissues from patients thought to have Epstein-Barr virus (EBV)-induced lymphoproliferative diseases were probed for EBV genomes using 2 independent hybridization techniques. Tissues from 6 patients with the X-linked lymphoproliferative syndrome, 5 with renal allograft recipients with immunoblastic sarcoma, and 8 patients with diverse types of immunodeficiency and lymphoproliferative diseases such as fatal infectious mononucleosis or malignant lymphoma associated with antecedent immunodeficiency contained significant numbers of EBV genome equivalents per cell. The use of 2 hybridization probes is recommended to confirm the presence of EBV genomes. The finding of significant numbers of EBV genomes in tissues from patients with immunodeficiency suggests that EBV is the etiological agent of the associated lymphoproliferative diseases.

Elsewhere in this issue, Henle and Henle (15) have summarized that an estimated 90% of adults carry neutralizing antibodies against EBV. Primary infections in early childhood are mostly silent; hence, seroconversion goes unnoticed (12). The primary target cells of EBV are probably epithelial cells in the oropharynx (32). B-Lymphocytes (bone marrow-derived lymphocytes), which have been consistently shown to contain EBV receptors (16), soon become infected, and hematogenous spread of virus occurs throughout the body. During acute EBV infection, the infected B-lymphocytes are destroyed by the cell-mediated immune system, notably the T-lymphocytes (thymus-derived lymphocytes) (52). Moreover, seropositive individuals contain "memory" T-lymphocytes that are able to inhibit the growth of EBV-transformed cells (33, 42). Details of these mechanisms are described by Rickinson et al. (43). In part, due to pressures from the cell-mediated immune system, EBV is forced into latency (20). Viral genetic information is present in the B-lymphocytes. Establishment of immortalized lymphoblastoid cell lines from seropositive individuals occurs with ease despite no sign of overt diseases (34). These cell lines contain multiple copies of the EBV genome (17), and all express the EBV-determined nuclear antigen (41).

EBV causes IM (13) and is strongly implicated as being etiological in BL (7) and nasopharyngeal carcinoma (18). Although EBV has hitherto only been etiologically associated with the above 3 disorders, high anti-viral capsid antigen titers have been observed in other malignant and nonmalignant disorders (14). The high antibody titers against EBV-specific antigens observed in these disorders probably result from reactivation of latent EBV. Henle and Henle (15) describe the highly variable EBV-specific antibody titers elsewhere in this issue.

Purtilo (39) has proposed that various lymphoproliferative diseases that occur in patients with inherited or acquired immunodeficiencies are due to EBV-triggered proliferation of infected B-lymphocytes, because normal immune mechanisms, especially cell-mediated immunity, are defective. Owing to the lack of antibody responses to EBV in such patients (46), special techniques are required to detect the virus or viral products in biopsy- or autopsy-derived tissues. Since virus is not readily isolated from tissues, probing for viral genetic material within tumor or lymphoid cells by molecular hybridization techniques is mandatory.

Here, we have used 2 independent methods, cRNA/DNA filter hybridization and vDNA/DNA reassociation kinetic analysis, to search for EBV genomes in tissues from patients with various lymphoproliferative diseases, which arise as a consequence of inherited or acquired immunodeficiency.

Materials and Methods

Isolation of Cellular and vDNA. Cellular DNA was isolated according to Petterson and Sambrook (38). vDNA from the B95-8 strain of EBV was either isolated by the method of Adams (1) or obtained from Dr. Meihan Nonoyama, Life Sciences, Inc., St. Petersburg, Fla., under a contract from the Division of Cancer Cause and Prevention, National Cancer Institute.

Nucleic Acid Hybridization. [32P]-labeled EBV cRNA was prepared in vitro by transcription of EBV DNA (22). The specific activity of the cRNA ranged between 1 to 2 × 10⁶ cpm/μg. cRNA produced by this method has been shown to contain sequences representing most of the EBV DNA (22). Nucleic
acid filter hybridization was carried out as described previously (22). Briefly, 10 μg of each DNA sample to be tested were adsorbed to nitrocellulose filters (Schleicher & Schuell). The filters were dried and baked, and 1 to 2 ng of cRNA were added per filter in 6× standard saline citrate (1× standard saline citrate = 0.15 M NaCl:0.015 M sodium citrate):50% formamide. Incubation was carried out for 4 days at 46°. After repeated washing and RNase treatment to remove unhybridized RNA, the filters were dried and counted by liquid scintillation.

The DNA content on each filter was determined by the diphenylamine method, and the counts were corrected for a DNA content of 10 μg and radioactive decay of 32P. DNA from the EBV-positive Raji line served as a positive control and DNA from the EBV-negative U698-M cell line and/or commercial CT DNA (Sigma Chemical Co.) served as negative controls. The genome content of the unknowns was determined by comparing the counts retained by the corresponding filters to the counts retained by the filters containing Raji DNA (Table 1). The genome number of each new batch of Raji DNA was determined separately. All samples were done in duplicate.

3H- and 32P-labeled EBV DNA (vDNA) was made by nick translation in vitro according to Rymo (44). The specific activities were 5 × 10⁶ and 1 × 10⁷ cpm/μg, respectively. More than 90% of the probes hybridized to excess DNA from the EBV-positive P3HR-1 line. About 95% of denatured vDNA was digestible with S1 nuclease. The vDNA/DNA hybridization conditions and the monitoring for hybrid formation by S1 nuclease have been described in detail elsewhere (45). Briefly, the samples to be tested were diluted to the same final concentration with CT DNA (50), and 5 to 10 ng of 3H or 1 to 2 ng of 32P were added. Both probes gave identical results when tested on the same samples. The samples were sheared by sonication and denatured by heat. Aliquots of 20 μl were sealed in micropipets. Hybridization was carried out in 10 mM Tris-HCl, pH 7.5:1 mM EDTA:0.1% sodium dodecyl sulfate:1 M NaCl at 86°. At given time intervals, micropipets were removed and frozen at −20°. All samples from the same experiment were treated with S1 nuclease at the same time. Each 20-μl aliquot was first diluted to 100 μl with 10 mM Tris-HCl, pH 7.5, containing 50 μg of partially denatured CT DNA and then to 1 ml with S1 buffer (0.1 M NaCl:0.03 M sodium acetate:2 mM ZnCl₂, pH 4.4). Incubation with 500 units of S1 nuclease (Boehringer/Mannheim) was carried out at 42° for 90 min. The DNA remaining double stranded was precipitated with 10% trichloroacetic acid on Whatman GF/C filters. The filters were then dried and counted. The data were plotted as \( (Co/C)^{2.3} \) against time (31, 48), and the slopes were determined by linear regression analysis as described by Sugden et al. (50). Linear regression is not only a convenient method for calculating the slopes but also gives a statistical evaluation of how well the points fit to a straight line (the correlation coefficient).

Four to 5 points were used to calculate the slopes of the various lines. In all experiments presented here, \( r \geq 0.9 \) and \( b \leq 1.1 \). By comparing the slopes of the positive controls to those of the unknowns, the genome numbers were determined. The amount of EBV DNA added as a probe was subtracted (50). The Raji DNA used in the vDNA/DNA reassociation experiments, as a positive control, was isolated from a different batch of Raji cells from that used in the filter hybridizations. The genome content was determined by reconstruction experiments as shown in Chart 1. DNA from the EBV-negative U698-M cell line and/or CT DNA were used as negative controls.

**Results**

Initially, we used cRNA/DNA filter hybridization to probe for EBV genomes in various tissues. However, high-molecular-weight DNA from some tissue specimens was lacking, and thus, we also used vDNA/DNA reassociation kinetic analysis. When sufficient material was available, both techniques were used to determine the number of EBV genome copies. Due to the varying amounts of cellular DNA obtained from the different tissues, dilution of samples with CT DNA became necessary to obtain the same final concentration of DNA for all samples used.

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Tissue</th>
<th>cRNA bound/10 μg DNA (cpm)</th>
<th>No. of EBV genomes/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>U698-M</td>
<td></td>
<td>357</td>
<td>0</td>
</tr>
<tr>
<td>Raji</td>
<td></td>
<td>7656</td>
<td>80</td>
</tr>
<tr>
<td>A. M. a</td>
<td>Cerebral cortex</td>
<td>1861</td>
<td>16</td>
</tr>
</tbody>
</table>

a From autopsy. The leptomeninges were extensively infiltrated by plasmacytoid lymphocytes in this patient with XLP.
in the reassociation experiments (50). The dilution of the samples with CT DNA was taken into account when EBV genome numbers were calculated.

Chart 2 shows representative results from a renaturation kinetic analysis of DNA from a lymphoma biopsy specimen of renal allograft recipient. Clinical, serological, and pathological findings of the patients are reported elsewhere in this issue (11). By comparing the slope of the line to that of the positive Raji control (50), which was diluted with CT DNA to yield 2 genome equivalents/cell, the lymphoma biopsy specimen was determined to contain 7 EBV genome equivalents/cell. Tables 2 to 4 show results of studies of tissues from a variety of lymphoproliferative disorders which are discussed elsewhere (11, 40).

Table 2 shows the results from EBV genome determinations obtained on tissue specimens from patients with XLP who had developed hypogammaglobulinemia following IM, fatal IM, or pseudolymphoma (40). No malignant lymphomas from XLP patients were available for study. All of the XLP patients showed significant numbers of EBV genomes in their tissues, except B. P. whose tissues had been stored at –70° for 3 years. Only 0.6 genome equivalents/cell were found in his spleen. Serological and other studies on the family indicate a diagnosis of XLP (40, 46). Good agreement was obtained between genome determinations with the 2 independent hybridization methods. Renal transplant recipients diagnosed to have immunoblastic sarcoma (11) had significant numbers of EBV genome copies in their tumors (Table 3). Patient D. P. (Table 4) had hypo-IgM and developed disseminated immunoblastic sarcoma (40). Involved lymph node and spleen contained many EBV genome copies.

Out of 9 cases of fatal IM, 7 (Tables 2 and 4, A. M., L. G., J. C., R. T., H. W., J. F., D. S.) contained significant numbers of EBV genome copies in the tissues tested and 2 (Tables 2 and 4, C. G., B. P.) contained less than one EBV genome/cell. Two cases of chronic or recurrent IM (R. M., J. W.) showed significant numbers of EBV genome copies (Tables 2 and 4).

One patient (O. A.) with ataxia telangiectasia had developed a malignant lymphoma which contained very high numbers of EBV genome equivalents by both methods (Table 4). Details of this case are summarized elsewhere (45).

**Discussion**

Etiological associations between EBV and African BL and nasopharyngeal carcinoma have been established by demon-

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**Table 2**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Diagnosis</th>
<th>Tissue</th>
<th>cRNA/DNA filter hybridization</th>
<th>vDNA/DNA reassociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. M.</td>
<td>16</td>
<td>Fatal IM</td>
<td>Spleen</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cerebral cortex</td>
<td>16</td>
<td>ND*</td>
</tr>
<tr>
<td>R. M.</td>
<td>15</td>
<td>Chronic EBV infection</td>
<td>Lymph node</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>V. G.</td>
<td>4</td>
<td>Malignant lymphoma*</td>
<td>Lymph node</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lung</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>K. W.</td>
<td>10</td>
<td>Pseudolymphoma</td>
<td>Lung</td>
<td>15</td>
<td>ND</td>
</tr>
<tr>
<td>L. G.</td>
<td>2</td>
<td>Fatal IM</td>
<td>Lymph node biopsy</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymph node (autopsy)</td>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>B. P.</td>
<td>4</td>
<td>Fatal IM</td>
<td>Spleen</td>
<td>&lt;1</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bone marrow</td>
<td>ND</td>
<td>0.6</td>
</tr>
<tr>
<td>J. C.</td>
<td>4</td>
<td>Fatal IM</td>
<td>Lung</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kidney</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not done.
* Neutropenia, hypogammaglobulinemia, malignant lymphoma, and aplastic anemia, sequentially.
stratifying EBV-determined nuclear antigen (23, 41) and EBV genomes by hybridization techniques in tumor biopsy specimens (3, 19, 23, 35, 37, 53). Also, EBV genomes have been demonstrated in non-African BL (2, 4, 5, 9, 10, 30).

In CTM, EBV causes malignant lymphomas in about one-third of experimentally inoculated animals (29, 47). The tumors resemble immunoblastic sarcoma of humans (25). Similar results have been obtained with other new world monkeys (8). Noteworthy is the lack of anti-EBV-specific antibodies in some inoculated CTM. The immunodeficiency of CTM is apparently similar to males with XLP (46).

Here, we have used 2 independent molecular hybridization methods to probe for EBV DNA in tissues from patients with inherited (XLP and ataxia telangiectasia) or acquired (renal allograft) immunodeficiencies and a group of patients suspected of having EBV-induced lymphoproliferative diseases. Where possible, both hybridization methods were used on the same tissue. Although both methods generally showed good agreement, some differences were observed. Others have made similar observations (37). This discrepancy is due chiefly to the higher specificity and sensitivity of the vDNA/DNA reassociation technique (35, 37), which often gives higher genome numbers than the less sensitive cRNA/RNA filter hybridization technique (37). The numbers presented here are only the average number of EBV genome copies in the tissues examined. It is probable that some specimens contained mixtures of EBV-infected and normal cells in unknown proportions. This seems to be the reason for the differences in genome numbers occasionally observed between different tissues from the same patients. That could also possibly explain why some tissues contained less than one EBV genome equivalent.

XLP patients have very impaired cellular (28, 51) and humoral (46) immunity. Following infection by EBV, males frequently die at an early age of IM or other lymphoproliferative diseases such as immunoblastic sarcoma (40). Results of this study indicate that patients with XLP carry significant numbers of EBV genomes in infected lymphoid organs. Of the 7 XLP patients in this study, 4 died of fatal IM, and 3 of these contained significant numbers of EBV genomes in their tissues (Table 2). Other cases of fatal IM and recurrent IM examined in this study were also shown to contain EBV genomes (Tables 2 and 4). These results corroborate findings of others who have found EBV DNA in tissues in IM, using reassociation kinetic analysis (36), cRNA/DNA filter hybridization (6), and in situ hybridization (21).

A rather frequent and serious complication of renal transplantation is the development of immunoblastic sarcoma (11). We have argued that their immunosuppressive treatment permits EBV reactivation, followed by polyclonal B-cell proliferation (11, 39). Many of these patients contain high antibody titers against EBV (27) and frequently shed the virus into the oropharynx (49). The high EBV antibody levels reflect immune competence (15). All 5 of the immunoblastic sarcomas from transplant recipients contained significant numbers of EBV genomes (Table 3). Clinical and pathological aspects are described elsewhere in this issue (11).

Table 3

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Tissue (biopsy)</th>
<th>cRNA/DNA filter hybridization</th>
<th>vDNA/DNA reassociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. S.</td>
<td>68</td>
<td>M</td>
<td>Liver</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymph node</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>M. R.</td>
<td>52</td>
<td>F</td>
<td>Palate lesions</td>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>C. S.</td>
<td>57</td>
<td>M</td>
<td>Lymphoma</td>
<td>ND</td>
<td>5</td>
</tr>
<tr>
<td>T. E.</td>
<td>15</td>
<td>M</td>
<td>Lymphoma (cervical)</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>J. A.</td>
<td>16</td>
<td>M</td>
<td>Parotid</td>
<td>9</td>
<td>14</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Hanto et al. (11) in this issue.
<sup>b</sup> ND, not done.

Table 4

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Tissue</th>
<th>cRNA/DNA filter hybridization</th>
<th>vDNA/DNA reassociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>J. F.</td>
<td>4</td>
<td>M</td>
<td>Fatal IM</td>
<td>Lymph node</td>
<td>11</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>R. T.</td>
<td>2½</td>
<td>M</td>
<td>Fatal IM</td>
<td>Spleen</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>C. G.</td>
<td>14</td>
<td>M</td>
<td>Fatal IM</td>
<td>Lymph node</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;1 ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>H. W.</td>
<td>10</td>
<td>M</td>
<td>Fatal IM</td>
<td>Spleen</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>D. P.</td>
<td>48</td>
<td>M</td>
<td>Immune mesothelial anemia</td>
<td>Lymph node</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T. T.</td>
<td>6</td>
<td>M</td>
<td>Lymph node</td>
<td>Liver</td>
<td>ND</td>
<td>0.7 ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>J. W.</td>
<td>16</td>
<td>M</td>
<td>Recurrent IM</td>
<td>Lymph node</td>
<td>14</td>
<td>ND</td>
</tr>
<tr>
<td>A. O.</td>
<td>9</td>
<td>M</td>
<td>Ataxia telangiectasia</td>
<td>Tumor</td>
<td>53</td>
<td>68</td>
</tr>
<tr>
<td>D. S.</td>
<td>4</td>
<td>M</td>
<td>Fatal IM</td>
<td>Lymph node</td>
<td>ND</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, not done.
<sup>b</sup> The 10-month-old brother of R. T. born of a different father has succumbed to IM. Hence, the brothers had XLP.
<sup>c</sup> Previous acute lymphocytic leukemia [see Look et al. (24) in this issue].
<sup>d</sup> See Purtilo et al. (40) in this issue.
<sup>e</sup> See Saemundsen et al. (45).
EBV-induced lymphoproliferative diseases suggests that immunodeficiency or abnormal immune responses to EBV can be responsible for life-threatening or fatal lymphoproliferative diseases.

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Documentation of Epstein-Barr Virus Infection in Immunodeficient Patients with Life-threatening Lymphoproliferative Diseases by Epstein-Barr Virus Complementary RNA/DNA and Viral DNA/DNA Hybridization

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