Epstein-Barr Virus-Specific DNase Activity in Nonproducer Raji Cells After Treatment with 12-O-Tetradecanoylphorbol-13-Acetate and Sodium Butyrate

T. OOKA,* M. DE TURENNE, G. DE THE, AND J. DAILLIE
Laboratoire d’Epidémiologie et d’Immunovirologie des Tumeurs, Faculté de Médecine Alexis Carrel, 69372 Lyon Cedex 2, France

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An Epstein-Barr virus (EBV)-specific DNase was induced in EBV nonproducer Raji cells after treatment with 12-O-tetradecanoylphorbol-13-acetate and sodium butyrate. The increase in EBV DNase activity was related to the appearance of early antigen-positive cells. The enzyme had a sedimentation coefficient of 4S and was resistant to 300 mM KCl, and its induction did not depend on viral DNA synthesis. The EBV-specific DNase activity was specifically inhibited by sera from patients who had nasopharyngeal carcinoma with high early antigen activities but not by sera from normal, healthy individuals. There was a correlation between the degree of anti-EBV DNase activity and the titers of early antigen antibody.

Epstein-Barr virus (EBV), a member of the human herpesvirus family, is associated with infectious mononucleosis (IM), African Burkitt’s lymphoma (BL), and undifferentiated nasopharyngeal carcinoma (NPC) (5). The virus is able to immortalize normal human B lymphocytes into continuously proliferating cell lines carrying the EBV genome, and it is produced in only 1 to 10% of the cells, in contrast with all other human herpesviruses, such as herpes simplex virus, which infects cells lytically and produces a number of virus-specific DNA enzymes such as DNA polymerases (9, 13), DNases (7), and thymidine kinases (TK) (8).

The presence of EBV-specific DNase activity in EBV-carrying lymphoid cell lines in which viral DNA synthesis and subsequent viral production are stimulated has been reported. This is the case for Raji cells superinfected with EBV (2), for EBV producer D98/HR-1 hybrid cells treated with iododeoxyuridine (2), and for EBV producer P3HR-1 (3, 4). Biochemical studies have demonstrated that the viral enzymes induced during viral replication differ from cellular enzymes and can be neutralized by sera from NPC patients (1). This enzyme is absent in EBV nonproducer Raji cells and other virus-negative lymphoid cell lines (2, 3). Whether the induction of the viral DNase occurs at an early or late stage of the viral cycle and is related to EBV antigen induction has not been determined, and we decided to examine this question. Virus nonproducer Raji cells were used and treated with a tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), and sodium butyrate (SB), leading to the synthesis of early antigens (EAs) in only ca. 30 to 50% of the cells (11; C. Lenoir, T. Ooka, M. Tovey, M. de Turenne, and J. Daillle, Abstr. Cold Spring Harbor Meeting on Herpesviruses, 4th, New York, N.Y., 1979).

The BL-derived cell line, the Raji cell line with its TK+ and TK− variants, P3HR-1 with its TK+ and TK− variants, BJAB, and RAMOS were maintained at 4 × 104 to 4 × 106 cells per ml in RPMI 1640 medium containing 10% heat-inactivated fetal serum supplemented with 100 U of penicillin and 250 μg of streptomycin per ml (12).

Cells were induced with TPA and SB as previously described (10). Briefly, all cells were grown to a density of at least 2 × 108 cells per ml and diluted to a final concentration of 5 × 106 cells per ml. TPA and SB were added at final concentrations of 20 ng/ml and 2 mM, respectively.

In the preliminary experiments, we characterized the nuclease activity from EBV-negative, EBV producer or nonproducer, and induced or noninduced lymphoid cells. The enzyme activity of the BJAB cells, Raji cells, and P3HR-1 cells was inhibited by KCl to almost 80 to 90% inhibition at 200 mM and 98 to 100% inhibition at 300 mM. In contrast, the activity that was induced after chemical induction of both Raji and P3HR-1 cells was more resistant to KCl, and 20 to 60% of this activity was found to be resistant to 300 mM KCl. The high-salt-resistant nuclease activity, as identified only in induced-cell extracts, had a sedimentation coefficient of 4S, different from the EBV-specific DNA polymerase with a sedimentation coefficient of 8.3S (12). The optimum condition for the induced activity was a pH of ca. 8.0 in the presence of 3 mM magnesium (data not shown).

In these regards, the chemically induced DNase activity has characteristics similar to the EBV-associated DNase activity described by Cheng et al. (2) and Clough (3) in superinfected Raji cells and in producer P3HR-1 cells, respectively.

Studies have shown that EBV-specific DNA enzymes such as DNA polymerase (12) and TK (11) can be induced at an early stage of the Epstein-Barr virus cycle. The synthesis of these enzymes correlates with EA production, suggesting that these enzymes belong to a group of virus-induced early proteins. To know whether viral DNase activity is induced at an early stage of viral replication, the induction of viral DNase activity in different human lymphoid cell lines was studied. The activity, tested in the presence of 300 mM KCl, was only present in P3HR-1 and Raji cells treated with TPA and SB (Table 1). In addition, the EBV-specific DNase activity could be induced in P3HR-1 cells treated with TPA-SB plus 1-β-D-arabinofuranosylthymine, inhibiting completely viral DNA synthesis (10, 11). Similar observations were made with EBV-specific DNA polymerase. EBV-negative cell lines (BJAB and RAMOS) did not exhibit EBV-specific DNase activity after treatment with chemical inducers. Thus, the DNase activity observed in EBV-carrying cells after treatment with TPA-SB cannot be a nonrelated EBV artifact due to chemical treatment. The induction of EBV-specific DNase activity was limited to EA-induced...
TABLE 1. Induction of EBV antigens and activity of DNase and DNA polymerase in human lymphoblastoid cell lines after treatment with TPA and SB

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Chemical induction</th>
<th>% of EA- or VCA-positive cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EBV-specific DNA polymerase activity&lt;sup&gt;b&lt;/sup&gt; (pmol of dTTP incorporated/mg of protein in 30 min)</th>
<th>EBV-specific DNase activity&lt;sup&gt;c&lt;/sup&gt; (U/mg of protein in 60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raji TK&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Control</td>
<td>0</td>
<td>8</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>TPA + SB</td>
<td>35.9</td>
<td>1.115</td>
<td>126.4</td>
</tr>
<tr>
<td>P3HR-1 TK&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Control</td>
<td>34.6</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>TPA + SB</td>
<td>33.4</td>
<td>570</td>
<td>96.3</td>
</tr>
<tr>
<td>BJAB TK&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Control</td>
<td>0</td>
<td>4</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>TPA + SB</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RAMOS TK&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Control</td>
<td>0</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>TPA + SB</td>
<td>0</td>
<td>0</td>
<td>1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lymphoblastoid cell lines were grown at 37°C with or without TPA-SB for 3 days. The evaluation of the percentage of EA- and VCA-positive cells was carried out on smears by an indirect immunofluorescence test (6).

<sup>b</sup> EBV-specific DNA polymerase activity was measured in the presence of 150 mM (NH₄)₂SO₄ in enzyme assays.

<sup>c</sup> EBV-specific DNase activity was measured in the presence of 300 mM KCl in enzyme assays. The cells, at a concentration of 2 × 10⁶ cells per ml in TKMD buffer (50 mM Tris-hydrochloride [pH 8.0], 10 mM KCl, 1 mM dithiothreitol), were sonically disrupted for four 30-s periods with a Branson sonicator at the level 4 setting. The sonically disrupted suspension was centrifuged at 10,500 × g for 60 min. After centrifugation, the supernatant fluid was collected and used as enzyme. A 60-µl final assay volume consisted of 20 µl ofenzyme, a final concentration of 3 mM MgCl₂, 10 µg of native lymphocyte DNA radioactively labeled in vivo with [³H]thymidine, 1 mM dithiothreitol, and 50 mM Tris-hydrochloride (pH 8.0). All assays were incubated at 37°C for 60 min. The incubation was stopped by placing the tube in ice water and adding 10 µl of salmon testis DNA and 20 mM EDTA. The reaction mixtures (40 to 50 µl) were transferred on Whatman GF/C glass-fiber disks, and the acid-insoluble fraction was precipitated with a 5% cold trichloroacetic acid solution. The disks were dried with ethanol and ether, and cells were counted with a Packard counter. The quantity of digested DNA was calculated. One unit is defined as the amount of enzyme which digests 1 µg of salmon testis DNA and 20 mM EDTA.

<sup>d</sup> 1-b-D-Arabinofuranosylthymine (AraT) was added (50 µg/ml) in culture medium to inhibit viral DNA synthesis (11).

cultures. This suggests that the EBV DNase is possibly part of the early protein complex, appearing before viral DNA synthesis and related to EA synthesis.

Cheng et al. (2) demonstrated that DNase activity from EBV-superinfected Raji cells can be neutralized by EBV-positive serum, mainly by sera from NPC patients. We examined whether the EA activity in EBV-superinfected Raji cells was also neutralized by EBV-positive sera. Table 2 summarizes the results obtained with sera from healthy donors, with viral capsid antigen (VCA)-positive sera (from normal adults), with EBV-negative sera, and with sera from patients with EBV-related diseases (NPC, 1M, or BL). Striking similarities were observed between the results from our two experiments and those obtained with EBV-superinfected Raji cells (2). A total of 29 EBV-negative and normal adult sera neutralized only 0 to 7 U of DNase activity. Only 2 of 13 1M sera and 0 of 2 BL sera neutralized more than 7 U of viral DNase. In contrast, 44 of 53 sera from NPC patients neutralized between 8 and 47 U, representing 83% of sera tested in this study.

We examined the neutralization by NPC sera of cellular extracts prepared from EBV-negative BJAB cells. None of these sera neutralized this activity. In this respect, the induced activity in Raji cells seems to be virus specific.

Anti-EBV DNAse activity plotted against various EBV-specific antibody titers showed a striking correlation with EA antibody titers (Fig. 1). The correlation coefficients were 0.75 for EA and 0.6 for VCA. However, the EBV-related antibodies of NPC sera being polyclonal, the high correlation coefficient between EAs and EBV-specific DNase activity does not imply identification of these polypeptides.

This finding raises an important question as to whether viral DNase is one of the constituents of the EA complex. In fact, viral DNase activity and EA-positive cells increased almost in parallel with time after the addition of chemical inducers, and moreover, EBV DNase activity was found only in EA-expressing cultures.

In conclusion, the EBV-induced DNase in Raji cells appears to be a virus-associated enzyme, as supported by the following data: (i) the biochemical properties of this

<table>
<thead>
<tr>
<th>Source of sera</th>
<th>No. of sera</th>
<th>No. of sera neutralizing the following U of anti-EBV DNase activity&lt;sup&gt;c&lt;/sup&gt;</th>
<th>% of sera neutralizing &gt;7 U</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV-negative normal adults</td>
<td>17</td>
<td>17 (0-7)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>EBV-positive normal adults&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12</td>
<td>12 (0-5)</td>
<td>0</td>
</tr>
<tr>
<td>Patients with BL</td>
<td>2</td>
<td>2 (0-1)</td>
<td>0</td>
</tr>
<tr>
<td>Patients with 1M</td>
<td>13</td>
<td>11 (0-2)</td>
<td>2 (10-17)</td>
</tr>
<tr>
<td>Patients with NPC</td>
<td>53</td>
<td>39 (0-7)</td>
<td>44 (8-47)</td>
</tr>
</tbody>
</table>

<sup>a</sup> For determination of anti-DNase activity, 10 µl of DNase (5.2 U) from crude extracts of TPA-SB-treated Raji cells was preincubated with 10 µl of serum at a 1:10 dilution with phosphate-buffered saline for 20 min at room temperature, after which the DNase activity was determined. The difference between activities in the presence and absence of serum was calculated, and the anti-DNase antibody activity was expressed as the units of DNase activity neutralized by 10 µl of undiluted serum.

<sup>c</sup> Numbers in parentheses indicate the range of units neutralized by 10 µl of serum.

<sup>c</sup> All subjects were anti-VCA positive.
enzyme differ from those of the cellular enzymes found in noninduced cells; (ii) the immunological properties of the enzyme are specifically neutralized by NPC sera; (iii) this type of enzyme activity is absent in EBV-negative lymphoid cells whether treated or not treated by chemical inducers; and (iv) the correlation between the increase in enzyme activity and in the number of EA-positive cells is high.

The next step will be to purify and characterize this DNase activity to determine the role within the EBV replicative cycle.

We are grateful for the excellent assistance of G. Decaussin in performing enzyme assays, A. Calender for statistical studies, C. Legrand for testing the EBV antibodies in the sera, and C. Weller for secretarial work.

LITERATURE CITED
Page 741, column 1, line 5: "(3, 47)" should read "(3, 47, 50)."
Page 741, column 1, line 50: "M. Cho" should read "M.-S. Cho."
Page 741, column 2, line 3: "Kieff" should read "Kieff."
Page 741, column 2, Acknowledgments, line 3: "Mol Gen" should read "MOLGEN."
Page 742, column 1, reference 21: The article title should read as follows: DNA of Epstein-Barr virus. VI. Mapping of the internal tandem reiteration.
Page 742, column 1: Reference 25 should be deleted.

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Volume 49, no. 2, p. 626, column 1, line 36: "4 \times 10^5 to 4 \times 10^6" should read "4 \times 10^5 to 1 \times 10^6."
Page 628, column 1, legend to Fig. 1, line 5: "footnote a" should read "footnote c."
Page 628, column 2, Literature Cited, reference 7, line 2: "injection" should read "infection."
Page 628, column 2, Literature Cited, reference 13, line 3: "6270–6271" should read "6270–6277."