New Method for Detecting Epstein-Barr Virus Association in Nonproducer Lymphoblastoid Cell Lines

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ABSTRACT

Epstein-Barr virus association in nonproducer human lymphoblastoid cell lines can be demonstrated by the presence of the virus genome (nucleic acid hybridization studies) or by the detection of the virus-coded complement-fixing antigen (complement fixation and/or anti-complement immunofluorescent test).

This paper describes an enzyme immunoassay for the detection of Epstein-Barr virus complement-fixing antigen and its application to the demonstration of Epstein-Barr virus association in nonproducer lymphoblastoid cell lines. The assay is based on competition for complement between Epstein-Barr complement-fixing antigen and its specific antibody and a probe complex composed of Escherichia coli $\beta$-galactosidase and specific anti-EBV $\beta$-galactosidase antibody. This competitive enzyme immunoassay is a specific and sensitive procedure for detecting Epstein-Barr virus association in nonproducer cell lines, allowing also quantitative estimation of the amount of antigen produced.

INTRODUCTION

A growing body of evidence indicates that a relationship exists between some herpesviruses and several forms of cancer (2, 4, 6, 7, 16, 18). In particular, simian herpesviruses are able to induce malignant lymphoproliferative diseases in several species of nonhuman primates (5). EBV is a human herpesvirus associated with lymphoproliferative diseases (7, 13). B-lymphocytes are the target cells of EBV infection (1); the virus can enter a nonpermissive interaction with these cells which may lead to transformation, and EBV can persist in lymphoblastoid cells in a repressed state. Human EBV-transformed cell lines can be divided into "producers" and "nonproducers." Virus activation leading to abortive or productive infection occurs spontaneously in producer cells in which a small fraction of the cell population expresses EBV antigens: VCA (12), EA (14), and membrane antigen (15). Nonproducer cell lines contain the EBV genome in a repressed state, and none of the previously mentioned antigens is expressed in them, but activation of the viral genome can be induced by treatment with halogenated pyrimidines (8, 11, 23).

The demonstration that nonproducer cell lines contained the EBV genome was formerly achieved by the detection of a specific complement-fixing antigen in these cells (20) and later was confirmed by nucleic acid hybridization experiments (19, 24). A substantial practical improvement in detection of the presence of the EBV genome came from the discovery of Reedman and Klein (21) of EBNA. This antigen was present in all EBV-transformed cell lines, both producers or nonproducers, while it was absent in lines lacking the viral genome. EBNA was localized in the cell nucleus as revealed by ACIF. The results of comparative cultural and serological studies clearly suggested that EBNA was the morphological counterpart of the previously detected complement-fixing antigen (21). Of the 3 procedures available for detecting EBV marker in nonproducer lymphoblastoid cell lines, i.e., nucleic acid hybridization, CF, and ACIF, the latter procedure seems to be highly sensitive and certainly convenient for the rapidity and ease of execution. However, all these techniques constitute important tools for different kinds of research concerning EBV, and certainly one of their main functions is the detection of EBV association in nonproducer cell lines.

We have recently reported a CEIA for detecting CF antibodies in diagnostic virology (10), but the assay also allows for the detection and quantitative evaluation of antigens. The method utilized conglutinin-covered plastic beads as the solid phase to detect specific antibody-antigen complexes that compete for complement with a probe complex composed of Escherichia coli $\beta$-galactosidase and its specific antibody. The binding to the solid phase is $C_{3b}$ mediated, and when specific antibody antigen complexes are not present, the probe is bound and an enzymatic reaction ensues following addition of substrate. Competition happens also at the level of the solid phase where conglutinin presents some degree of undersaturation. This report concerns CEIA detection of EBV-associated CF antigen in some nonproducer human lymphoblastoid cell lines.

MATERIALS AND METHODS

Cell Lines

Six lymphoblastoid cell lines were selected for this study; HeLa cells and peripheral blood lymphocytes were also investigated. All the cell cultures were of human origin. Raji and Molt-4 cells were originally obtained from Flow Laboratories, Ltd., Paisley, Scotland; the other 4 EBV-carrying nonproducer lymphoblastoid cell lines (A.P., R.G., T.A., IM3) were established in our laboratories from leukocyte cultures of normal human subjects and from a patient with heterophile antibody-positive infectious mononucleosis by methods described previously (1, 9). All the EBV-carrying lymphoblastoid cell lines were nonproducers, but A.P. cells occasionally showed a very small number of EA-positive cells (0.1% or fewer). All the cell lines, with the exception of HeLa cells, were propagated in Roswell Park Memorial Institute Medium 1640 with 10% fetal calf serum; HeLa cells were cultured in Eagle's minimal essential medium with 10% fetal calf serum.

Antigens

EBV complement-fixing antigen was prepared from each EBV-carrying lymphoblastoid line as described by Pope et al. (20) with minor modifications, and the negative CF antigens were prepared from EBV-free cell lines by the same method.
Sera

An EBV antibody-positive human serum from a convalescent infectious mononucleosis patient showing high EBV antibody titers was used as positive serum (VCA, 1:320; EA, 1:16; CF, 1:540; EBNA positive). An EBV-negative serum from an 18-year-old healthy male subject was used as negative control. Six EBV antibody-positive and 2 negative human sera were also screened. All the sera used came from male donors who did not receive blood transfusion or suffer from immunological disorders; these sera were found to be negative for antinuclear antibodies and circulating immune complexes.

CF Test

The microcomplement-fixation technique was used as described by Sever (22).

EBV VCA and EA

These antigens were investigated following the indirect immunofluorescent methods described by Henle et al. (12, 14).

ACIF Test

The procedure was similar to that of Reedman and Klein (21) with minor modifications. Cells smeared from suspension cultures on glass slides and HeLa cells grown on coverslips were air dried and fixed with aceticone:methanol (1:1). A human serum with no detectable antibodies to EBV-related antigens was used as a source of complement. An EBNA antibody containing human serum was used in the test in a 1:10 dilution, and it was the same serum used in CF and CEIA tests. Dilutions and washes were made in phosphate-buffered saline (CaCl₂, 100 mg/liter; MgSO₄, 120 mg/liter; KCl, 200 mg/liter; KH₂PO₄, 200 mg/liter; NaCl, 8000 mg/liter; Na₂HPO₄, 1150 mg/liter). After fixation, the slides were washed 3 times in Veronal-buffered saline, treated with 1:10 dilution of fluorescein isothiocyanate (FITC) conjugated with goat anti-human IgG (Hyland Laboratories, Inc., Los Angeles, Calif.) at 37°C for 30 min in a humidified chamber; they were then washed with phosphate-buffered saline, treated with 1:10 dilution of fluorescein isothiocyanate anti-β-galactosidase (Hyland Laboratories, Inc., Los Angeles, Calif.) at 37°C for 30 min, washed again, mounted, and examined by fluorescence microscopy. A control of the complement-binding capacity of cells was done by the ACIF procedure in the absence of serum containing EBV antibody and in the presence of a negative serum. As a negative control, an EBV-negative serum was used.

CEIA Reagents

Enzyme. β-Galactosidase purified from E. coli 3300 (1 mg·g⁻¹) was used. The method of Celada et al. (3, 17) was used.

Antiserum and Complement. We used a β-galactosidase antiserum obtained from immunized donkeys. Undiluted normal donkey serum was used as a source of complement. Both the normal and the immune sera were obtained from Farmitalia-Carlo Erba Analytical Division, Montedison, Milan, Italy. Preliminary titration experiments indicated the optimal concentration of complement, suitable for competition, to be used in the test.

Preparation of Probe Complex. The optimal concentrations of β-galactosidase and anti-β-galactosidase were determined as described in detail previously (10). The probe complex was prepared by mixing 1 volume of β-galactosidase and 4 volumes of antiserum.

Conjugatin-covered Plastic Beads. The preparation of this solid phase was done as extensively described previously (10).

Substrate. o-Nitrophenyl-β-galactopyranoside (2.3 mm; Merck AG, Darmstadt, Germany) was used as the substrate for β-galactosidase; the product (o-nitrophenol) was determined spectrophotometrically at 420 nm.

CEIA Procedures

Determination of Optimal Antigen-Antibody Dilution. Preliminary determinations were performed to determine the optimal antigen-antibody dilution, following the criteria previously reported (10). The best dilution of antigen, which gave the greatest discrimination in absorbance at 420 nm between the positive and the negative control sera, was chosen. The same criteria were followed to establish the optimal serum dilution.

Test Procedure. To 0.1 ml of a 1:2 dilution of serum, 0.1 ml of appropriate dilution of antigen was added. Dilutions were made with Veronal-buffered saline. Then 0.2 ml of anti-β-galactosidase antiserum and 0.05 ml of β-galactosidase plus 0.1 ml of undiluted complement were added. After the addition of a conglutinin-covered plastic bead to each tube, the mixture was incubated at 37°C for 1 hr and then at room temperature for 3 hr. Beads were washed 3 times in Veronal-buffered saline, and the probe complex bound was measured by adding 1 ml of o-nitrophenyl-β-galactopyranoside (2.3 mm). After 30 min of incubation at 37°C, the reaction was stopped by adding 1 ml of 0.4 M Na₂CO₃, and the results were read photometrically at 420 nm. Of fundamental importance to the interpretation of the test results was the inclusion in each run of 2 controls, 0.9% NaCl solution plus antigen and 0.9% NaCl solution plus serum.

RESULTS

Preliminary ACIF staining for EBNA was done on the cell cultures tested. All 5 lymphoblastoid lines previously known to carry EBV had positive EBNA staining. In contrast, negative results were obtained with the 3 EBV-free cultures. No nonspecific complement binding occurred, and none of the EBV-negative sera gave nuclear staining.

Table 1 summarizes the results obtained when the various cell lines were tested.

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Origin</th>
<th>Test method</th>
<th>CF</th>
<th>VCA</th>
<th>EA</th>
<th>ACIF</th>
<th>CEIA</th>
</tr>
</thead>
</table>
| Raji     | BL*    | A + - - +   |    |     |    |     | 0.072 ± 0.009*
|          |        | B - - - -   |    |     |    |     | 0.408 ± 0.04 |
|          |        | C - - - -   |    |     |    |     | 0.422 ± 0.02 
| A.P.     | N      | A + - - +   |    |     |    |     | 0.194 ± 0.03 |
|          |        | B - - - -   |    |     |    |     | 0.410 ± 0.02 |
|          |        | C - - - -   |    |     |    |     | 0.438 ± 0.04 
| R.G.     | N      | A + - - +   |    |     |    |     | 0.204 ± 0.03 |
|          |        | B - - - -   |    |     |    |     | 0.418 ± 0.04 |
|          |        | C - - - -   |    |     |    |     | 0.448 ± 0.05 
| T.A.     | N      | A + - - +   |    |     |    |     | 0.168 ± 0.02 |
|          |        | B - - - -   |    |     |    |     | 0.440 ± 0.05 |
|          |        | C - - - -   |    |     |    |     | 0.462 ± 0.05 
| IM3      | IM     | A + - - +   |    |     |    |     | 0.088 ± 0.02 |
|          |        | B - - - -   |    |     |    |     | 0.398 ± 0.02 |
|          |        | C - - - -   |    |     |    |     | 0.400 ± 0.02 |
| Molt-4   | L      | A - - - -   |    |     |    |     | 0.432 ± 0.04 |
|          |        | B - - - -   |    |     |    |     | 0.422 ± 0.02 |
|          |        | C - - - -   |    |     |    |     | 0.488 ± 0.05 
| HeLa     | C      | A - - - -   |    |     |    |     | 0.444 ± 0.04 |
|          |        | B - - - -   |    |     |    |     | 0.440 ± 0.04 |
|          |        | C - - - -   |    |     |    |     | 0.452 ± 0.05 
| Lymphocytes | N   | A - - - -   |    |     |    |     | 0.422 ± 0.02  
|          |        | B - - - -   |    |     |    |     | 0.416 ± 0.03 |
|          |        | C - - - -   |    |     |    |     | 0.448 ± 0.05 |

* BL: Burkitt's lymphoma; A, tested with positive serum; B, tested with negative serum; C, tested with 0.9% NaCl solution; N, derived from normal donors; IM, derived from infectious mononucleosis; L, derived from leukemia; C, carcinoma.  

b Mean ± S.D.
Comparative absorbances of triplicate determinations made on selected positive and negative sera tested in the presence of various EBV antigens

<table>
<thead>
<tr>
<th>Serum</th>
<th>CF titer</th>
<th>Raji A.P.</th>
<th>R.G.</th>
<th>T.A.</th>
<th>IM3</th>
<th>Molt-4</th>
<th>HeLa</th>
<th>Lymphocytes</th>
<th>Serum control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>0.136</td>
<td>0.206</td>
<td>0.202</td>
<td>0.200</td>
<td>0.140</td>
<td>0.416</td>
<td>0.430</td>
<td>0.422</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>0.266</td>
<td>0.320</td>
<td>0.300</td>
<td>0.308</td>
<td>0.242</td>
<td>0.422</td>
<td>0.444</td>
<td>0.416</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>0.180</td>
<td>0.242</td>
<td>0.216</td>
<td>0.223</td>
<td>0.178</td>
<td>0.416</td>
<td>0.433</td>
<td>0.424</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>0.122</td>
<td>0.216</td>
<td>0.210</td>
<td>0.199</td>
<td>0.137</td>
<td>0.400</td>
<td>0.405</td>
<td>0.401</td>
</tr>
<tr>
<td>5</td>
<td>128</td>
<td>0.100</td>
<td>0.198</td>
<td>0.187</td>
<td>0.200</td>
<td>0.102</td>
<td>0.407</td>
<td>0.401</td>
<td>0.400</td>
</tr>
<tr>
<td>6</td>
<td>256</td>
<td>0.100</td>
<td>0.198</td>
<td>0.176</td>
<td>0.180</td>
<td>0.106</td>
<td>0.407</td>
<td>0.406</td>
<td>0.412</td>
</tr>
<tr>
<td>7</td>
<td>&lt;8</td>
<td>0.412</td>
<td>0.398</td>
<td>0.400</td>
<td>0.416</td>
<td>0.408</td>
<td>0.406</td>
<td>0.406</td>
<td>0.412</td>
</tr>
<tr>
<td>8</td>
<td>&lt;8</td>
<td>0.402</td>
<td>0.406</td>
<td>0.408</td>
<td>0.421</td>
<td>0.418</td>
<td>0.400</td>
<td>0.430</td>
<td>0.416</td>
</tr>
<tr>
<td>Antigen control*</td>
<td>0.440</td>
<td>0.453</td>
<td>0.436</td>
<td>0.416</td>
<td>0.422</td>
<td>0.436</td>
<td>0.428</td>
<td>0.434</td>
<td>0.427</td>
</tr>
</tbody>
</table>

* Tested with 0.9% NaCl solution.

Lines were tested for EBV antigens by the methods described previously, with the positive and the negative sera taken as EBV reference sera. The results are expressed as positive or negative in CF, immunofluorescence, and ACIF, and as mean absorbances ± S.D. of triplicate testing in the CEIA procedure. The absorbance values obtained after 0.9% NaCl solution plus serum incubation (not shown in Table 1) were always >0.400.

Preliminary block titration experiments indicated the optimal dilution of antigens and antisera to use in the CEIA reported. EBV antigens were used undiluted, and sera were used at a 1:2 dilution. Taking into account the results of these block titration experiments and the fact that the various EBV antigens were prepared by the same procedure from a controlled, equal amount of the different cells (10⁹/ml), we concluded that the absorbance values in the CEIA are inversely related to EBV antigen concentration. CEIA results were always in agreement with CF and ACIF results.

Table 2 reports CEIA results obtained with 6 EBV-positive and 2 EBV-negative sera with different CF titers when tested in the presence of various cell antigens. CEIA results were in agreement with CF results. The data reported indicated that all the EBV-positive sera tested reacted only with antigens present in the EBV-transformed lymphoblastoid cell lines. In contrast, no reaction was observed with transformed epithelial cells or with normal and transformed lymphoid cells lacking EBV genetic information. These data suggest that the immune reaction takes place with at least one component common in all the EBV-transformed lines and appear to preclude aspecificities due, for example, to isoantigen-isoantibody interactions. Moreover, CEIA results confirmed the subjective quantitative evaluation of EBNA obtained by fluorescence microscopy. Raji and IM3 lines possessed a stronger ACIF staining as revealed by an intense fluorescent staining and a lower absorbance value in the CEIA.

**DISCUSSION**

Although every cell line investigated herein has not been studied by DNA hybridization, it is generally accepted that CF and ACIF are sensitive probes of EBV association. As a matter of fact, nonproducer lymphoblastoid cell lines contain EBV genetic information that is revealed by the production of virus-determined CF antigen. The data reported in this paper indicated that CEIA detected an EBV-associated antigen in cells carrying EBV, and the overall results suggested that a likely candidate for this antigen would be the previously known CF antigen. In fact, a comparison of CEIA results with those obtained by CF and ACIF techniques revealed strict similarities; such results are not surprising, because the 3 techniques constitute different methodologies for detecting a single class of antibodies, namely, those fixing complement. CEIA detection of EBV-associated CF antigen is a specific and sensitive procedure which allows the demonstration of the EBV carrier state in nonproducer lymphoblastoid cell lines. In light of these findings, CEIA application to EBV research has proved to be effective both in serological surveys and for detecting the presence of virus marker in culture.

We have reported previously (10) that CEIA has a sensitivity practically identical to that of CF but presents several advantages over conventional CF, the most important being the achievement of quantitative results. Moreover, while in the ACIF test variations in the amount of antigen produced are subjectively detected by variation in fluorescence intensity, CEIA allowed more reliable estimation of the antigen produced, providing numerical absorbance results; under optimization and the future availability of international standards, this assay should allow the quantitative estimation of antigen. The procedure may also be applied to the detection of EBV-specific CF antigen in cell extracts of biopsies. All these characteristics make CEIA a promising tool in EBV research.

**REFERENCES**

EBNA Detection by CEIA


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