Epstein–Barr Nuclear Antigen (EBNA) Carrying Lymphocytes in Human Palatine Tonsils

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SUMMARY

The presence of Epstein-Barr virus (EBV) antigens in human palatine tonsil-derived lymphocytes (TDL) was investigated using the indirect fluorescent antibody (FA) technique. The TDL were screened for the presence of EBV early antigen (EA), virus capsid antigen (VCA), and EBV nuclear antigen (EBNA).

In 76% of the patients diagnosed as recurrent exudative tonsillitis, and in 33% diagnosed as recurrent tonsillitis and/or serous otitis media, EBNA was demonstrated in the purified TDLs. No EA- or VCA-producing cells were found in either the glass adsorbed or TDL cell preparations from all of the patients. These data suggest that in our patient sample, the tonsils may serve as a reservoir for EBV carrying lymphocytes and a basis for recurrent disease.

INTRODUCTION

There is general agreement regarding the causal relationship between Epstein–Barr virus and infectious mononucleosis (Henle, Henle & Diehl, 1968; Neiderman et al. 1968); however, the possible role of EBV in other infectious diseases remains unsettled. Significant titre changes to EBV in acute and convalescent sera were not demonstrated in studies of patients with a variety of acute virus infections (Neiderman et al. 1970; Beltran et al. 1971). In attempts to establish a relationship between EBV and infectious diseases Evans, Neiderman & McCollum (1968) and Henle & Henle (1970) tested acute tonsillitis patients but excluded patients with exudative tonsillitis. These studies added little support for the role of EBV in acute tonsillitis. That there may be an association between EBV and exudative tonsillitis was reported by Tischendorf et al. (1970) and recently Veltri, Sprinkle & McClung (1975) reported that significant titre changes to EBV occurred in patients with exudative tonsillitis. Additional evidence in support of this association was the finding that EBV induced exudative tonsillitis in gibbons (Werner et al. 1972). These findings and the availability of a convenient method to demonstrate the EBV nuclear antigen (Reedman & Klein, 1973) prompted this investigation into the possibility that EBV may be localized in the tonsil lymphocytes of patients with a history of recurrent tonsillitis.

METHODS

Subjects. The patient sample consisted of 18 males and 17 females ranging in age from 2 to 44 years, the mean age being 11 years. The patients were selected for tonsillectomy based upon three or more bonified attacks of clinical tonsillitis in a year with or without
exudate on the palatine tonsils. Seventeen patients, mean age of 13.88 years, had clinically diagnosed, recurrent exudative tonsillitis. Eighteen patients having recurrent tonsillitis and/or serous otitis media had a mean age of 5.83 years. No patient was clinically ill at the time of tonsillectomy.

Preparation of tonsil derived lymphocytes (TDL). Tonsil derived lymphocytes were extracted from the palatine tonsils of above subjects in accordance with the method of Sloyer, Veltri & Sprinkle (1973). The sediment containing lymphoid cells was resuspended in 10 ml of Hanks balanced salt solution (BSS) and layered on to gradients consisting of 24 parts of 9% Ficoll and 10 parts of 34% Hypaque. These gradients were centrifuged at 400 g for 40 min. The buffy layer was resuspended in 10 ml of BSS and placed in a 150 cm² glass prescription bottle for 1 to 2 h at 37 °C to remove adherent polymorphonuclear cells and macrophages. The cell suspensions from these prescription bottles were layered on a second Ficoll-Hypaque gradient and centrifuged as described above. The buffy layer was harvested again and the TDLs were washed three times in BSS. The washed TDLs were adjusted to 4 x 10⁶ cells/ml.

Subsequent to centrifugation of the second gradient, the cells which had adhered to the prescription bottle were removed with BSS, washed three times, and adjusted to 4 x 10⁶ cells/ml. Cell suspensions were placed in a 0.05 ml vol. on a 1 x 3 inch pre-cleaned slide. One-half of the slides prepared from the TDLs and glass adherent cells were fixed in acetone-methanol (1:1); the remaining half of the slides were fixed in acetone at -20 °C. Slides were then stored at -80 °C for later testing.

Matched slides from the cells in the lymphocyte buffy layer of both gradients and the glass adherent cells were also fixed and stained by the Wright's stain (Todd-Sanford, 1974) for cellular differential analysis.

Assay of TDLs for T and B lymphocytes. The purified TDLs and peripheral circulating lymphocytes (PCL) from seven patients were assayed for their ability to form rosettes with sheep red blood cells (Jondal, Holm & Wigzell, 1972), a property associated with T lymphocytes. The TDLs and PCLs were also assayed for the presence of surface-bound immunoglobulins (Jondal & Klein, 1973) which are associated with B lymphocytes.

Detection of EBV antigen producing cells. The presence of EA- and/or VCA-producing cells was determined by the indirect fluorescent antibody test as modified by Veltri et al. (1975). The TDLs and glass adherent cells were treated with a 1:10 dilution of (1) EA⁺ VCA⁺, (2) EA⁻ VCA⁺ and (3) EA⁻ VCA⁻ EBNA⁻ control sera which were kindly provided by Dr Berge Hampar, Viral Carcinogenesis Branch, National Cancer Institute, Bethesda, Maryland. These same control sera were used in all tests.

Detection of EBNA. The acetone-methanol fixed slides were examined by using the anti-complement immunofluorescence test (ACIF) of Reedman & Klein (1973). Cells exhibiting a diffuse finely granular fluorescence confined to the nucleus were considered positive (Fig. 1). The same EA⁻ VCA⁻ EBNA⁻ human complement source and serum were used throughout the test. Slides containing P₃HR-1 cells or Raji cells were included in each test as positive controls. Also positive and negative serum controls were included with every test.

Detection of serum antibodies to EBV antigens: a serum sample was obtained at tonsillectomy. These serum samples were assayed for the presence of antibodies to EBV–EA and VCA by using the modified indirect fluorescent antibody test described by Veltri et al. (1975) with proper negative and positive serum controls. Substantiation of the EA serological response obtained in our patient sample was achieved by re-titrating all EA positive sera by using Raji cells treated with 5-iododeoxyuridine (IuDR). The Raji cells
EBNA carrying lymphocytes in human tonsils

Fig. 1. Human tonsil derived lymphocytes exhibiting diffuse granular nuclear fluorescence indicative of EBNA.

were grown in the dark in the presence of 100 µg/ml of IuDR for 72 h at 37°C, harvested and slides prepared. Such cells synthesize only EA in the presence of inhibitors of DNA synthesis (Gergely, Klein & Ernberg, 1971; Hampar, Derge & Showalter, 1974; Long, Derge & Hampar, 1974).

RESULTS

Cytological analysis of human tonsil derived cells

The viability of the tonsil cells was determined by trypan blue dye exclusion before the cells were layered on to the first Ficoll–Hypaque gradient and after each subsequent step. The average viability of the TDLs in the recurrent tonsillitis and/or serous otitis media patients and the recurrent exudate tonsillitis patients was 95.0% and 95.7%, respectively. There was no significant difference in viability of lymphocytes isolated from the different patient groups. Using Wright’s stain, slides of glass adherent cells were found to consist primarily of polymorphonuclear (PMN) cells and plasma cells with not more than 10% lymphocytes. The cells in the buffy layer obtained from the second Ficoll–Hypaque gradient described above contained 94.0% ± 2.5% lymphocytes.

The lymphocytes in the second buffy layer were further enumerated into T and B lymphocyte populations in seven of the patients. Table 1 shows percentages of T and B lymphocytes in TDL preparations paired with those obtained from the peripheral circulating lymphocytes (PCL).

In comparison to the PCL, the B lymphocytes, derived from tonsils, were significantly elevated at α = 0.05 as determined by the Wilcoxon matched paired signed-ranks tests. The TDL and the PCL had similar T lymphocyte populations.

Production of EBV antigens by tonsil-derived cells

The acetone fixed slides of cells from the glass adsorption procedure and the TDLs were assayed by the indirect FA test. Approx. 1000 cells/slide were counted. No EA- or VCA-producing cells were detected. Although both cell types exhibited dim background
Table 1. *T and B lymphocytes distribution in paired tonsil-derived lymphocytes and peripheral circulating lymphocytes*

<table>
<thead>
<tr>
<th>Patient</th>
<th>% rosettes</th>
<th>% B-cells</th>
<th>% rosettes</th>
<th>% B-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>69</td>
<td>22</td>
<td>NT*</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>51</td>
<td>21</td>
<td>32</td>
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<tr>
<td>7</td>
<td>32</td>
<td>65</td>
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<td>47</td>
</tr>
<tr>
<td>Mean</td>
<td>27</td>
<td>58</td>
<td>32</td>
<td>42</td>
</tr>
</tbody>
</table>

* Not tested.

Table 2. *Detection of EBNA in TDL and correlation with antibodies to EA and VCA in patient’s sera*

<table>
<thead>
<tr>
<th>Patient’s diagnosis</th>
<th>No. of patients</th>
<th>EBNA-positive† no. (%)</th>
<th>No. of patients</th>
<th>EA-positive no. (%)</th>
<th>VCA-positive no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET‡</td>
<td>17</td>
<td>13 (76)</td>
<td>17</td>
<td>8 (47)</td>
<td>15 (88)</td>
</tr>
<tr>
<td>RT-SOM§</td>
<td>18</td>
<td>6 (33)</td>
<td>15</td>
<td>0 (0)</td>
<td>8 (53)</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>19 (54)</td>
<td>32</td>
<td>8 (25)</td>
<td>23 (72)</td>
</tr>
</tbody>
</table>

* All sera were screened at 1:10 for EA antibodies and 1:20 for VCA antibodies.
† Percentage patients with EBNA-positive TDLs.
‡ RET = recurrent exudative tonsillitis.
§ RT-SOM = recurrent tonsillitis and/or serous otitis media.
|| No sera obtained from three patients.

fluorescence, none exhibited the characteristic brilliant cytoplasmic fluorescence associated with EA- and VCA-producing cells.

In the 35 TDL preparations tested 54% contained EBNA-positive TDLs ranging from 5 to 46% per 500 lymphocytes counted (Table 2). A total of 6 of 18 (33%) of patients with recurrent tonsillitis and/or serous otitis media had EBNA-positive TDLs. The age range of this group was 3 to 12 years with a mean of 5.83 years. In the recurrent exudative tonsillitis patient group, 13 of 17 (76%) had EBNA-positive TDLs and their age range was 2 to 44 years with a mean of 13.88 years. Also the recurrent exudative tonsillitis group of patients had antibodies to EA and/or VCA, whereas the recurrent tonsillitis and/or serous otitis patients had antibodies to only VCA. Sixteen patients in both groups had no EBNA in their TDLs and nine of these possessed no antibodies to EA or VCA. The remainder had antibodies, but to VCA only.

**DISCUSSION**

The findings reported in this study indicate that EBNA is present in tonsil-derived lymphocytes (TDL) from 76% of the patients with recurrent exudative tonsillitis and from 33% of patients with recurrent tonsillitis and/or serous otitis media. Of the 35 patients studied 19 (54%) had tonsils that contained from 5 to 46% EBNA positive TDLs. It is of importance that EBV lymphoblastoid cell lines maintained in vitro contain about 90%
EBNA-positive lymphocytes (Reedman & Klein, 1973) of B lymphocyte origin (Jondal et al. 1972). The significant variability and reduced numbers of EBNA-positive TDLs may be due to the complex cell populations including fibroblasts, PMNs, monocytes, macrophages, plasma cells, T and B lymphocytes which would cause a dilution effect. Also, the majority of these cells lack the necessary EBV receptor sites found on B lymphocytes (Jondal et al. 1972). Furthermore, continued B lymphocyte lymphopoiesis in human tonsils (Good et al. 1967; Koburg, 1967) and the fact that human tonsils have no afferent but only efferent lymphatic drainage (Koburg, 1967) may relate to these variable results. Additional research dealing with in vivo cellular transformation as evidenced by the EBNA-positive TDLs is an obvious necessity.

The serological correlation of antibodies to EA and/or VCA in sera of all patients with TDLs carrying EBNA indicate previous exposure to EBV. Only the RET patients exhibited a serological response to EA as well as VCA which may indicate recent exposure to EBV (Miller, 1974; Veltri et al. 1975) or a condition similar to one observed in Burkitt’s lymphoma and nasopharyngeal carcinoma (Pope, Horne & Wetters, 1969; Vonka, Benyesh-Melnick & McCombs, 1970; ZurHausen & Schulte-Holthausen, 1970; Nonoyama & Pagano, 1971; Reedman & Klein, 1973). True latency as with the adenoviruses in human tonsils (Strohl & Schlesinger, 1965a, b) necessitates proving EBV coded antigens other than EBNA such as EA, VCA and complete infectious virus are not being produced. No EA or VCA coded antigens of EBV were demonstrated in these studies but no assays for complete infectious virus were performed. If complete infectious virus is produced, then the tonsils are probably serving as a reservoir for EBV-producing lymphocytes. In either case the clinical relevance of these investigations to problems in infectious and neoplastic diseases cannot be denied.

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