Epstein-Barr virus reactivation in renal transplant recipients

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Summary

Renal transplant recipients at Tygerberg Hospital were investigated to determine the importance of Epstein-Barr virus (EBV) as a pathogen in these patients. All 106 patients investigated were shown to have EBV antibodies before transplantation and most had serological evidence of reactivation of the infection after transplantation. A mild clinical illness was present in a few patients concomitant with EBV reactivation, which may suggest that this virus has a role in the morbidity of some renal transplant recipients. Lymphoblastoid cell lines were established from 11 renal transplant recipients; 5 of these cell lines were shown to be virus producers and 1 is thought to have unique properties.

Material and methods

Patients

The records of 147 patients who underwent renal transplantation at Tygerberg Hospital during the period May 1976 - August 1985 were reviewed. Particular note was taken whether symptoms of a viral-aetiologic nature, which could be associated with a virus, particularly EBV, had developed after transplantation. Sera from most of these patients had been stored in the virology laboratory and were available for retrospective EBV antibody testing. Twenty of these patients were investigated monthly for EBV infection during the period September 1984 - August 1985.

Serological investigations

Sera were tested for IgG and IgM antibodies to EBV capsid antigen (VCA), early antigen (EA) and nuclear antigen (EBNA) by indirect immunofluorescence using standard methods.

Lymphocyte cultures

Blood for the culture of lymphocytes was collected in tubes containing lithium heparin (Teklab). The lymphocytes were separated by centrifugation on Histopaque 1077 gradients (Sigma). One per cent washed sheep red blood cells were added to the lymphocytes, which caused the T lymphocytes to form rosettes. The B lymphocytes were separated by centrifugation on Histopaque 1077 gradients and cultured at a concentration of 10^6 cells per millilitre in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 2% L-glutamine (Merck), penicillin 100 IU/ml and streptomycin 60 μg/ml in plastic disposable plates (Costar 3524). The cultures were incubated at 37°C in an atmosphere containing 5% CO2. About half the medium was replaced every 2-3 days.

Electron microscopy

 Cultured lymphocytes were washed once with phosphate-buffered saline and then fixed in 1% glutaraldehyde in Sorenson's buffer (pH 7.3) for 24-48 hours. Lymphocytes for scanning electron microscopy were washed three times in Sorenson's buffer and 3 x 10^6 cells were then collected on grids, which had previously been treated with Poly-L-lysine. The grids were placed in a humidified atmosphere overnight, dehydrated in a graded series of alcohol and critical-point-dried using CO2. Cells for transmission electron microscopy were post-fixed with 1% osmium tetroxide in Sorenson's buffer. After dehydration in a graded series of alcohol the preparations were embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

EBV antigen tests

Acetone-fixed cells were stained for EBNA by anticomplementary immunofluorescence as described by Reedman and Klein. VCA staining was performed by indirect immunofluorescence using EA-negative, VCA-positive serum. The reaction was followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG.

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Cytogenetic studies
Metaphase spreads were prepared and stained as described by Jarvis et al. and karyotypes constructed.

Virus detection
Cells cultured for 14 days at 37°C in 200 ml medium were sedimented at 1000 rpm for 20 minutes. The supernatant was then centrifuged at 20,000 rpm and the pellet resuspended in tris-HCl-buffered saline plus 1% bovine serum albumin. This material was placed on top of a discontinuous gradient of 10% and 50% potassium tartrate in 1M CaCl/0,1M tris HCl/10 mM ethylenediamine tetra-acetic acid buffer (pH 7,4), and centrifuged at 25,000 rpm for 2 hours at 5°C. The opalescent band which formed was layered on a 20 - 30% potassium tartrate linear gradient and centrifuged for 17 hours at 25,000 rpm. Samples of the opalescent band were then placed on a Formvar carbon-coated grid, stained with 2% phosphotungstic acid and examined in an electron microscope.

Transformation of umbilical cord blood lymphocytes
The ability of virus isolates to transform umbilical cord blood lymphocytes was tested. A 200 ml volume of cell suspension which had been cultured for 14 days at 37°C was sedimented. The supernatant was then filtered through a 0,45 μm filter and 0,2 ml added to 5 x 10⁸ umbilical cord blood lymphocytes in 2 ml culture medium. Cultures were examined daily and when transformation was observed the cells were examined for EBNA, VCA and virus production.

Results
Clinical findings
Examination of the records of 147 renal transplant patients revealed that 2 had developed clinical symptoms of hepatitis, associated with raised CMV antibody titres in 1 case. Ten patients developed an infectious mononucleosis-like illness with a sore throat and fever; of these 3 were tested for EBV-specific antibody and found to have high titres. Of 4 other patients who had raised EBV antibody levels 1 had vertigo, 1 had decreased renal function with anaemia and weight loss, and 2 had abnormal haematological findings (Table I). Four patients developed malignant lesions but none of these was associated with EBV infection.

Serological findings
Stored sera taken from 106 patients immediately before renal transplantation were positive for VCA IgG antibodies (Table II), most having titres over 320. Of 29 of these tested none was positive for VCA IgM. Thirty-five patients with low VCA IgG antibody titres were retested 3 months after transplantation and 12 showed a rise in these antibodies. Of the 20 patients investigated monthly all were positive for EBNA and VCA-IgG antibodies before transplantation. The VCA-IgG antibody titres of these patients rose after transplantation and remained at about 1280. One patient developed VCA-IgM antibodies. The immunofluorescence test for EA antibody became positive in 15 of these 20 patients during the period of investigation, indicating EBV reactivation.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Symptoms</th>
<th>Anti-VCA IgG</th>
<th>Anti-VCA IgM</th>
<th>Anti-EA</th>
<th>Lymphoblastoid cell line established</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fever, sore throat</td>
<td>1280</td>
<td>&lt;10</td>
<td>&gt;10</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Fever, sore throat</td>
<td>1280</td>
<td>&lt;10</td>
<td>&gt;10</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>Fever, sore throat</td>
<td>1280</td>
<td>&lt;10</td>
<td>&gt;10</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>Vertigo</td>
<td>1280</td>
<td>&lt;10</td>
<td>&gt;10</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Raised lymphocyte count, toxic granulation +</td>
<td>1280</td>
<td>&lt;10</td>
<td>&gt;10</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>Decreased renal function, anaemia, weight loss</td>
<td>1280</td>
<td>&lt;10</td>
<td>&gt;10</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>Atypical lymphocytes, toxic granulation +</td>
<td>1280</td>
<td>&lt;10</td>
<td>&gt;10</td>
<td>No</td>
</tr>
</tbody>
</table>

**TABLE II. RENAL TRANSPLANT RECIPIENTS WITH EBV-SPECIFIC ANTIBODIES**

<table>
<thead>
<tr>
<th>Retrospective tests</th>
<th>Anti-VCA IgG</th>
<th>Anti-VCA IgM</th>
<th>Anti-EBNA</th>
<th>Anti-EA</th>
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<tr>
<td>Pre-transplantation (positive antibody titre)</td>
<td>106/106</td>
<td>0/29</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Post-transplantation rise in titre</td>
<td>12/35</td>
<td>0/35</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sequential tests</th>
<th>Anti-VCA IgG</th>
<th>Anti-VCA IgM</th>
<th>Anti-EBNA</th>
<th>Anti-EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-transplantation (positive antibody titre)</td>
<td>20/20</td>
<td>0/20</td>
<td>20/20</td>
<td>4/20</td>
</tr>
<tr>
<td>Post-transplantation rise in titre</td>
<td>20/20</td>
<td>1/20</td>
<td>ND</td>
<td>15/20</td>
</tr>
</tbody>
</table>

ND = not done.
Lymphoblastoid cell lines

Lymphoblastoid cell lines were established from peripheral blood of 11 patients. In wet preparations of these cell lines the characteristic microvilli and pseudopodia were seen by light microscopy (Fig. 1). On scanning electron microscopy the typical morphological features of lymphoblastoid cells were seen (Fig. 2). Five cell lines were shown by electron microscopic examination of cell supernatants and ultrathin sections of cells to be virus pro-

Fig. 1. Phase-contrast photomicrograph of a wet preparation of a lymphoblastoid cell line showing microvilli and pseudopodia (bar represents 40 \( \mu \)m).

ducers (Fig. 3). Between 5% and 10% of the cells of each of these cell lines were VCA-positive by immunofluorescence. The cytoplasm of virus producer cell lines had a foamy appearance in Giemsa-stained preparations, whereas the non-producers did not (Fig. 4).

The ability of these 5 virus isolates to transform cord blood lymphocytes was tested and 1 was positive. These transformed cord blood lymphocytes were EBNA- and VCA-positive and produced EBV. None of the cell lines investigated had any detectable cytogenetic abnormalities. The VCA antibody levels of all patients from whom lymphoblastoid cell lines were formed were high (titres between 640 and 2,560) during the entire period of investigation, and all these patients also became EA antibody-positive. Mild clinical symptoms were present in 4 of these patients (Table I).
Fig. 4. Photomicrographs of Giemsa-stained preparations of lymphoblastoid cell lines. Top: producer cell line showing the foamy appearance of the cytoplasm. Bottom: non-producer cell lines without this foamy appearance.

Discussion

EBV infection is associated with a number of clinical syndromes including malignant disease. Recently it has also been shown to be associated with persistent malaise.\textsuperscript{15,16} After primary infection the virus becomes latent in the body and may become reactivated following immune suppression, resulting in B-cell lymphoproliferative disease.\textsuperscript{7} In this study of 147 renal transplant patients, all 106 tested retrospectively for EBV antibodies were found to be positive for VCA-IgG antibodies before renal transplantation, indicating previous EBV infection. The majority of these patients (78%) had high serological titres to the virus (> 320). Three months after transplantation 23% of those with low antibody levels showed a rise in titre to high levels (> 1280) indicating reactivation of the virus. All patients investigated monthly for a further 1 year were found to show serological evidence of reactivation of latent EBV infection. Some of these patients developed clinical symptoms of a viral infection which could be attributed to EBV. Whether the development of symptoms of vertigo, anaemia, decreased renal function and weight loss can be ascribed to the reactivation of EBV can only be elucidated by further study. In none of the patients investigated could EBV reactivation be related to the type of immunosuppressive therapy used, including cyclosporin. Previous studies have shown that a small number of renal transplant recipients develop solid tumours histologically classified as polymorphic diffuse B-cell hyperplasia or polymorphic B-cell lymphoma. Four patients in this study developed malignant lesions but none was associated with an EBV infection.

In this study there was some evidence to suggest an association between reactivation of EBV and the development of clinical symptoms in a few renal transplant recipients. The retrospective analysis of clinical records is often difficult to interpret in relation to laboratory results. However, these findings have created a greater awareness of the possible clinical significance of EBV reactivation, and if the importance of this virus in renal transplantation is to be determined, detailed prospective clinical, haematological and virological studies should be carried out.

Eleven lymphoblastoid cell lines were established from these patients. They were shown to have morphological features typical of lymphoblastoid cells on scanning and transmission electron microscopy. None had any cytogenetic abnormalities. Five of these cell lines were VCA-positive and were shown by electron microscopy to produce EBV. In most lymphoblastoid cell lines established from patients the viral genome is latent and rarely undergoes a replicative cycle; however, in a small number of lymphoblastoid cell lines the virus replicative cycle is spontaneously activated in about 10% of cells. These are known as producer lines.\textsuperscript{17} The proportion of VCA-positive cells (5-10%) of the 5 producer lymphoblastoid cell lines established in this study is comparable to that of the marmoset cell line B 95-8,\textsuperscript{18} which is used universally as an EBV producer for the study of the virus. Virus from one lymphoblastoid cell line transforms human cord blood lymphocytes. EBV from previously established producer cells, e.g. B 95-8, transforms cord blood cells, but these cells are then only EBNA-positive and do not produce virus. The cord blood cells which were transformed by virus from one of our producer lymphoblastoid cell lines was VCA-positive and shown by electron microscopy to produce EBV. This cell line therefore appears to be unique and is under further investigation.

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REFERENCES

Plasma C-peptide and insulin responses to intravenous glucagon stimulation in idiopathic haemochromatosis


Summary

Beta-cell reserve was investigated in 15 patients with proven idiopathic haemochromatosis (IHC) (7 normoglycaemic haemochromatotic patients, 4 non-insulin-requiring diabetics and 4 insulin-requiring diabetics) by measuring the response of plasma C-peptide, insulin and glucose to a 2 mg intravenous bolus of glucagon, and compared with that in 5 lean normal subjects. The corresponding C-peptide/insulin molar ratios after glucagon were significantly reduced in the normoglycaemic IHC group. These results suggest the presence of at least two abnormalities of insulin metabolism in IHC — a progressive reduction in beta-cell function and a diminished rate of removal of insulin by the liver.

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and then by the non-insulin-requiring diabetics, although there were no significant differences. In contrast, C-peptide/insulin molar ratios after glucagon were significantly reduced in the normoglycaemic IHC group. These results suggest the presence of at least two abnormalities of insulin metabolism in IHC — a progressive reduction in beta-cell function and a diminished rate of removal of insulin by the liver.

Diabetes has been reported in up to 80% of patients with idiopathic haemochromatosis (IHC) and the relative contributions of cirrhosis, pancreatic islet cell damage, predisposition to inherited diabetes mellitus and true peripheral insulin resistance to its pathogenesis have been investigated. Although carbohydrate intolerance has most commonly been ascribed to the progressive destruction of beta cells by iron deposits, recent evidence suggests that the initial abnormality of glucose metabolism in conditions of iron overload may be hyperinsulinemia as a result of insulin resistance. In the present study, beta-cell reserve was assessed by measuring C-peptide secretion after glucagon stimulation in groups of IHC patients with varying degrees of glucose intolerance.

Subjects and methods

We investigated 12 men and 3 women with IHC. Their mean age was 55 years (range 42 - 70 years) and their mean ideal body