Distribution and Phenotype of Epstein-Barr Virus-Infected Cells in Inflammatory Bowel Disease

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Little is known about Epstein-Barr virus (EBV) infection of colon mucosa, particularly in inflammatory bowel diseases. Crohn's disease and ulcerative colitis are thought to differ in T-helper lymphocyte composition and cytokine secretion patterns. Some of the implicated cytokines are growth factors for EBV-infected cells. We examined colon mucosa for differences in the distribution and phenotype of EBV-infected cells. Colon tissues with Crohn's disease (n = 31) or ulcerative colitis (n = 25) and controls (n = 60) were characterized by in situ hybridization and immunohistology for six EBV gene products as indicators of latent and replicative EBV infection. The cells were additionally phenotyped by combined detection of the EBV transcripts and B- or T-cell antigens. B lymphocytes predominated as the site of latent EBV infection in the colon and were most numerous in ulcerative colitis. In active ulcerative colitis, EBV-positive lymphocytes accumulated under and within the epithelium and displayed evidence for replicative infection. The patterns of mucosal EBV gene expression indicate local impairment of virus-specific T-cell responses in active ulcerative colitis. Detection of EBV may help to discriminate between active ulcerative colitis and other inflammatory bowel diseases. Colon mucosa is a potential site of EBV replication and may be relevant for EBV transmission. (Am J Pathol 2000, 157:51–57)
likewise influence the local response to the EBV as mirrored by EBV gene expression.

We studied mucosal biopsy and resection specimens with the diagnoses of CD, UC, collagenous colitis, chronic nonpurulent appendicitis, and diverticulitis as well as colon tissue distant to primary colorectal adenocarcinomas for the presence of EBV-harboring cells to test whether active mucosal lesions of CD and UC differ in their content of EBV-infected cells. These cells were further characterized for the presence of gene products indicative of replicative infection and phenotyped by double- and triple-labeling techniques. Latently EBV-infected cells were detected by in situ hybridization using probes specific for the nonpolyadenylated small nuclear EBV-encoded RNA transcripts, EBER1 and EBER2, which are transcribed at high copy numbers in every known condition of EBV latency facilitating their detection even in paraffin-embedded tissues. Cells switching to productive infection were identified by detection of the BZLF1 protein or of BHLF1 transcripts, the presence of which precedes expression of all structural viral genes.1

**Materials and Methods**

**Tissues**

Formalin-fixed, paraffin-embedded specimens from colon tissues of a total of 116 cases (Table 1) and tonsillar tissue from four patients with the clinical diagnosis of infectious mononucleosis were drawn from the files of the University Institutes of Pathology in Berlin, Hamburg, and Frankfurt am Main. Tissue blocks were from colon resection, hemicolecotomy, or colectomy specimens with the exception of 21 appendectomy specimens and serial biopsies available from two cases of CD, 13 cases of UC, and eight cases of collagenous colitis. The diagnoses of CD or UC were established on the basis of clinical, radiological, and morphological criteria. A combination therapy of prednisolone and sulfasalazine had been administered to most CD and UC patients at the time of surgery or biopsy. All cases of CD displayed moderate to severe inflammatory activity with focally accentuated leukocytic infiltrates of high density, occurrence of aphthous and fissural ulcers, fistulae, segmental transmural fibrosis, and occasional formation of granulomas. UC specimens displayed moderate to severe inflammatory activity primarily restricted to the lamina propria and submucosal layers with ulcers, wide-spread depletion of goblet cells, epithelial regeneration with mild nuclear atypia, and formation of pseudopolyps. The diagnosis of infectious mononucleosis was confirmed by serology and characteristic clinical presentation.

**Immunohistology**

Four-μm sections of paraffin-embedded tissue blocks were stained by the immunoalkaline phosphatase method using new fuchsin as chromogen. The monoclonal reagents were CS1-4, a cocktail of four antibodies specific for LMP1, antibody PE2 against EBNA2, antibody BZ.1 specific for BZLF1 (ZEBRA) protein, and the antibody L26 (CD20). CD3 antigen was detected with a polyclonal rabbit antibody. All primary antibodies as well as rabbit-anti-mouse immunoglobulin and immunoalkaline phosphatase-complex were purchased from DAKO (Glostrup, Denmark). CS1-4, PE2, BZ.1, and anti-CD3 antibodies required high pressure cooking for antigen retrieval (3 minutes in 10 mmol/L citrate buffer) to obtain staining in paraffin sections. Tonsillar tissue with infectious mononucleosis served as positive controls for detection of EBV gene products.3

**Probes**

Fluorescein isothiocyanate-labeled oligonucleotides specific for BHLF1 transcripts were obtained from DAKO. EBER1- and EBER2-specific pBluecript-based vectors were used for the generation of single-stranded RNA probes.18,19 EBER1 and EBER2 probes were used in combination to increase sensitivity. For the preparation of human immunoglobulin light chain (IgLC) RNA probes the 550-bp StsI fragment containing the IgLC type κ (IgLCκ) gene constant segment,20 and the 600-bp BglII/BamHI fragment containing the IgLC type λ (IgLCλ) gene C2 constant segment,21 respectively, were subcloned

**Table 1.** Frequency of EBV-Positive Lamina Propria Cells in IBD and Controls

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Specimen*</th>
<th>EBV+ cases/no. of cases</th>
<th>EBV+ cells/0.5 cm² (no. of cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>R/E</td>
<td>9/12 (75%)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6/13 (46%)</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>15/25 (60%)</td>
<td>10</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>R</td>
<td>25/29 (86%)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0/2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>25/31 (81%)</td>
<td>6</td>
</tr>
<tr>
<td>Collagenous colitis</td>
<td>B</td>
<td>0/8</td>
<td>8</td>
</tr>
<tr>
<td>Chronic appendicitis</td>
<td>E</td>
<td>3/21 (14%)</td>
<td>18</td>
</tr>
<tr>
<td>Chronic diverticulitis</td>
<td>R</td>
<td>5/12 (42%)</td>
<td>7</td>
</tr>
<tr>
<td>Adenocarcinoma, adjacent mucosa</td>
<td>R</td>
<td>9/19 (47%)</td>
<td>10</td>
</tr>
<tr>
<td>tumor stroma</td>
<td>R</td>
<td>8/19 (42%)</td>
<td>11</td>
</tr>
</tbody>
</table>

*Specimen type: R, resection; E, excision; B, biopsy specimen.
into pGEM1 (Promega Biotec, Madison, WI). Phages with the IgLC\(_x\) and IgLCA genomic fragments, were the kind gift of Dr. P. Leder, Cambridge, MA. After linearization with the appropriate restriction enzymes (Gibco BRL, Karlsruhe, Germany), \(\text{[}^{35}\text{S}\text{]}\)-labeled anti-sense or sense (control) run-off transcripts were generated using either SP6, T3, or T7 RNA polymerases and \(\text{[}^{35}\text{S}\text{]}\)-uridine-5'-\(\alpha\)-thio)-triphosphate (1250 Ci/mmol, New England Nuclear, Dreieich, Germany) or, alternatively, digoxigenin-11-uridine-5'-triphosphate (Boehringer Mannheim, Mannheim, Germany) at a concentration of 0.5 mmol/L.\(^{19}\) The average specific activity of radioactively labeled probes was \(1.3 \times 10^9\) cpm/\(\mu\)g.

### In Situ Hybridization

The hybridization with either \(\text{[}^{35}\text{S}\text{]}\)-labeled, digoxigenin-labeled, or combinations of \(\text{[}^{35}\text{S}\text{]}\)-labeled and digoxigenin-labeled probes on paraffin sections and autoradiography were carried out as described.\(^{18,19}\) Digoxigenin-labeled probes were detected by immunohistology with a digoxigenin-specific alkaline phosphatase-conjugated antibody F\(_{ab}\) fragment (Boehringer Mannheim). In situ hybridization with BHLF1-specific oligonucleotides was performed as recommended by the manufacturer (DAKO). For combined detection of IgLC and EBER, immunohistological detection of digoxigenin-labeled EBER probes was followed by autoradiography for up to 28 days of exposure.

### Sequential Immunohistochemistry and in Situ Hybridization

Antibodies were used in freshly prepared RPMI 1640 medium (Gibco BRL), pH 7.5, containing 10 mg/ml bovine serum albumin, 1.0 mg/ml yeast RNA, and 5000 U/ml heparin ammonium salt (Sigma, Munich, Germany) to inhibit RNase activity. To estimate the RNA loss during immunostaining procedures, adjacent tissue sections were subjected to in situ hybridization not preceded by immunostaining. The autoradiographic exposure times for slides subjected to the double-labeling procedure were adjusted accordingly. In situ hybridization using sense probes for EBER or IgLC genes alone showed only weak nonspecific background. Prolonged exposure times up to 3 months ensured that negative results were not because of premature termination of the exposure. Cells presenting with grain counts four times above background signal as defined by hybridization with sense-strand control probes were considered specifically labeled.\(^{18,19}\)

For triple labeling, immunohistology was performed with a peroxidase-labeled antibody L26 (CD20, EPOS; DAKO) before hybridization with a combination of digoxigenin-labeled EBER probes and \(\text{[}^{35}\text{S}\text{]}\)-labeled IgLC\(_x\) and IgLCA probes. Slides were treated with 0.3% \(\text{H}_2\text{O}_2\) for 3 minutes, rinsed, incubated with the antibody for 45 minutes, and washed. Immobilized antibody was detected with diaminobenzidine (DAB) chromogen before hybridization.

To avoid loss of hybridization signal when applying the polyclonal CD3-specific antibody, the procedure was reversed. Digoxigenin-labeled EBER probes were hybridized and detected with peroxidase-labeled antibody F\(_{ab}\) fragments (Boehringer Mannheim) after blocking with 0.3% \(\text{H}_2\text{O}_2\). Detection with DAB chromogen was followed by a 3-minute high pressure cooking procedure and routine anti-CD3 immunohistology using the immunoalkaline phosphatase technique.

### Statistics

Statistical evaluation was performed using the Craddock-Flood chi-square test.\(^{22}\)

### Results

#### Distribution and Prevalence of EBV-Infected Cells in Colon Mucosa

EBER-positive cells were detected in colon tissue specimens of 57 out of 116 (49%) patients. All of the EBER-positive cells displayed the morphology of lymphocytes. Labeling of epithelial cells was not noted in any instance. EBER-positive cells were absent from all of the serial biopsies of eight patients with collagenous colitis. The number of EBV-infected cells did not exceed five cells per 0.5 cm\(^2\) in chronic appendicitis, diverticulitis, and uninvolved mucosa from adenocarcinoma rectectases, but was slightly increased relative to uninvolved mucosa in the stroma of 3 out of 19 adenocarcinomas (Table 1). In CD, EBER-positive cells were detected in 25 of 31 (81%) cases, but the average number of cells per section area was only slightly elevated compared to mucosa from appendicitis and diverticulitis cases. EBER-positive cells were randomly distributed within the lamina propria of diverticulitis, appendicitis, and CD cases. In CD, a proportion of EBER-positive cells was found around lymphoid follicles in the lamina propria (Figure 1) and deep within the bowel wall. Fifteen of 25 (60%) of the UC specimens displayed EBER-positive cells, and several of the positive cases displayed a significantly increased load of EBER-positive cells as compared to CD (\(P < 0.002\)) and controls (\(P < 0.001\)) (Figure 2). In contrast to CD and controls, EBER-positive cells were clustered in sub- and intraepithelial location in UC cases in areas with moderate to high inflammatory activity (Figure 2). Comparison of figures obtained with radioactive and nonradioactive detection of EBER transcripts revealed no differences in sensitivity between these methods. In general, intramucosal EBER-positive cells showed the morphology of small lymphocytes. In three UC cases with high inflammatory activity, occasional mitotic figures and slightly enlarged nuclei were noted in a small proportion of EBER-positive lymphocytes.

### Phenotype and EBV Gene Expression

LMP1- and EBNA2-expressing cells were not detectable in the colon specimens whereas these antigens were...
found in sections of infectious mononucleosis tonsils (data not shown). In two of four UC cases with severe inflammatory activity and an increased density of EBER-positive cells of >21 and >50 per 0.5 cm² section area, respectively, labeling for BZLF1 protein and BHLF1 transcripts revealed nuclear staining in a small proportion of the sub- and intraepithelial lymphocytes, indicating entry into the lytic cycle of EBV infection (Figure 2). In contrast, all of three CD cases and both of two carcinoma cases with elevated burden of EBV-infected lymphocytes displayed no signs of replicative infection when tested in serial sections for BZLF1 and BHLF1 expression (data not shown). CD3 antigen was detected in large proportions of intramucosal lymphocytes. EBER- and CD3-specific signals were not co-localized, however. In contrast, 25 to 30% of EBER-positive cells stained for the B-cell antigen CD20. By combined in situ hybridization using radioactively-labeled IgLC probes and digoxigenated EBER probes; >90% of EBER-positive cells were identified as B cells with a slight excess of IgLC-κ over IgLC-λ-positive cells.

Discussion

Studying the distribution and phenotype of EBV-infected cells in colon tissues, we found differences between CD, UC, and controls that may provide further insight into the biology of inflammatory bowel disease (IBD) and EBV infection. In conditions morphologically diagnosed as chronic, nonpurulent appendicitis and diverticulitis of the sigmoid, in biopsies with collagenous colitis as well as in colon mucosa adjacent to invasive adenocarcinoma, only few EBER-positive small lymphocytes were found in scattered distribution in approximately one third of the cases. A similar distribution was observed in CD specimens with, however, slightly elevated occurrence of EBV-positive cells as 81% of the CD cases displayed occasional EBER-positive cells. This seems to indicate an uncharacteristic expansion of the pool of circulating latently virus-infected cells accompanying the hyperplasia of mucosal lymphatic tissue in CD and may also reflect individual variation in absolute numbers of EBV-positive lymphocytes. The situation is reminiscent of the distribution of EBV-infected cells in hyperplastic lymph nodes and tonsils, proportions of which regularly display an increased frequency of EBER-positive cells in reactive lesions even under conditions unrelated to EBV infection such as toxoplasmosis. The findings are well in line with a polymerase chain reaction study of viral genomes in CD and UC which found an increased prevalence of EBV in UC over CD in five of six and six of 10 cases, respectively, as well as with a more recent study of 16 IBD cases...
displaying increased numbers of EBER1 positivity in non-
epithelial cells in each 60% of UC and CD cases.\textsuperscript{24}

In active UC, we observed a distribution of EBV-infected
cells and a pattern of EBV gene expression different from
CD and controls. Although some of the biopsies
did not display any EBER-positive cells, cases with

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{EBV gene expression and phenotype of EBV-infected cells in cases of ulcerative colitis. A small proportion of subepithelial and intraepithelial infected lymphocytes express the early lytic cycle gene products BZLF1 protein (A) and BHFL1 transcript (B) detected by immunohistology with the antibody BZ.1 or an oligonucleotide probe, respectively. The colon mucosa displays flattened epithelium with reduced numbers of goblet cells and a leukocyte infiltrate containing polymorphonuclear cells in the crypt lumen. A proportion of EBER-positive cells (red signal) display CD20 expression detectable by immunohistology (brown cytoplasmic and membrane signal) indicating the B-lymphoid nature of these infected cells (C and D). Note the subepithelial distribution of many EBER-positive lymphocytes and the presence of one infected cell (arrow) in the crypt lumen (C) as well as a mitotic figure in an EBER-positive cell (arrow; D). When detection of EBER and IgLC transcripts is combined, the majority of EBV-infected cells can be identified as B-lymphocytes with polytypic IgLC expression pattern. ~50% of EBER-positive cells are labeled by the IgLC-\(\alpha\) probe (E), although a small proportion of EBER-positive cells does not display IgLC expression even after extended autoradiographic exposure time (F). Two EBER- and IgLC-positive cells are marked by arrows (F). Autoradiographic exposure time, 14 days (E) and 24 days (F). Original magnifications, \(\times120\) (A and B), \(\times60\) (D), \(\times75\) (C, E, F).}
\end{figure}
UC specimens tested for BZLF1 and BHLF1 expression provided evidence not only for latent but also for replicative EBV infection of lymphocytes. In contrast, all of the CD and carcinoma cases with increased prevalence of EBV-infected lymphocytes showed no signs of EBV replication. The relative frequency of such EBV-producing cells was similar to tonsils with infectious mononucleosis.\textsuperscript{3,4,17} As previously observed in infectious mononucleosis, BZLF1- and BHLF1-positive cells in UC were small and occurred predominantly in sub- or intraepithelial locations.\textsuperscript{3,4,17} In one instance, EBV-infected cells were found within the crypt lumen. At variance with infectious mononucleosis tonsils and suggesting a restricted expression of latent genes (ie, latency type I) in UC, expression of the EBV latent gene products, EBNA2 and LMP1, was not detectable in our study specimens, even though the presence of occasional EBV-harboring T cells could not be identified. The phenotypic characteristics of mucosal EBV-infected cells also mirror closely the situation observed in lymph nodes and tonsils.\textsuperscript{3,18}

The apparently increased tolerance toward EBV antigens in these UC cases with high numbers of EBV-positive cells does not seem to represent an iatrogenic artifact, because the immunosuppressive therapy was not significantly different from the other IBD cases. The high load with EBV-infected cells indicates a microenvironment particularly favoring the polyclonal expansion of EBV-infected cells and permitting their entry into the lytic cycle of viral infection. Immediate early and early lytic-cycle antigens are known to be targets of EBV-induced cytotoxic T-cell responses.\textsuperscript{26} The pattern of EBV infection may therefore be related to an altered reactivity to EBV, and perhaps to infection with other viruses as well.

Recent research provided evidence for altered immune responsiveness in the intestinal lamina propria of IBD patients. Specifically, an immune reaction dominated by T\textsubscript{H}1 cytokine-producing T cells was identified in CD, whereas a T\textsubscript{H}2 pattern and elevated serum levels of soluble CD30 protein were found in UC.\textsuperscript{18,25} EBER- and CD3-positive cells, ie, EBV-infected T cells, could not be identified though the presence of occasional EBV-harboring T cells cannot be excluded. The phenotypic characteristics of mucosal EBV-infected cells also mirror closely the situation observed in lymph nodes and tonsils.\textsuperscript{3,18}

In conclusion, latently and productively EBV-infected cells are detectable in many, but not all cases of IBD, and are more frequently detectable in active UC than in CD or controls. EBV is unlikely to have a role as an etiological agent in IBD. However, intramucosal expansion of EBV-infected cells may be an indicator for a local impairment of antiviral immunity. After the onset of the disease triggered by as yet unknown factors,\textsuperscript{37} EBV infection may influence the make-up of the inflammatory infiltrate in UC and may contribute to self-perpetuation of the disease as well as to the development of autoimmune disease. The characterization of EBV-reactive T cells may therefore shed light on the pathogenesis of the immune dysfunctions associated with UC, and detection of subepithelial and intraepithelial clusters of EBV-infected cells may help to discriminate between active UC and other IBD. Moreover, colon mucosa is a potential site of EBV replication and may be relevant for EBV transmission.
Acknowledgments

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References

41. Epoca 2000, Vol. 157, No. 1