Morphology, Immunophenotype, and Distribution of Latently and/or Productively Epstein-Barr Virus-Infected Cells in Acute Infectious Mononucleosis: Implications for the Interindividual Infection Route of Epstein-Barr Virus

By Ioannis Anagnostopoulos, Michael Hummel, Cornelia Kreschel, and Harald Stein

The present study was undertaken to unequivocally demonstrate the morphology, immunophenotype, and localization of Epstein-Barr virus (EBV)-infected cells as well as the type of infection (latent versus productive) in tonsils of acute infectious mononucleosis. Paraffin sections from nine cases with clinical, serologic, and morphologic evidence of EBV infection were analyzed for the detection of small transcripts, designated EBER1 & 2, and BHLF1 by in situ hybridization (ISH) using nonisotopically labeled probes. ISH was combined with immunohistology, employing a broad panel of antibodies against B-, T-, epithelial-, macrophage-, and follicular dendritic cell (FDC)-antigens. All EBER-positive cells could be identified as lymphocytes, as they did not exhibit any morphologic or immunologic characteristics of epithelial cells, macrophages, or FDCs. A preferential accumulation of EBER-positive cells was noted around crypts, within surface squamous epithelium, and in the surroundings of necrosis. The majority of these lymphocytes could be shown to be B cells, which morphologically included Reed-Sternberg (RS)-like cells, immunoblasts, medium-sized lymphoid cells, as well as cells with plasmacytoid differentiation. In all cases, a varying number of EBER-positive T cells could be identified. ISH for BHLF1-RNA detection showed that almost all cases contained single positive small lymphoid cells, indicating a transition from latent to productive infection cycle. Such cells could also be detected within the crypt epithelium reaching up to its surface. Additional screening of 123 oropharyngeal mucosa samples from patients without evidence of acute EBV-infection, using the polymerase chain reaction for EBV-DNA detection combined with EBER- and BHLF1-ISH showed single latently infected lymphocytes in only one case. Our data imply that infected lymphocytes and not epithelial cells are, in fact, the reservoir for EBV infection, and that these are the cells that participate in the interindividual virus transfer.

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Address reprint requests to Harald Stein, MD, Institute of Pathology, Klinikum Benjamin Franklin, Free University Berlin, Hindenburgdamm 30, 12200 Berlin, Germany.

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EBV is a ubiquitous DNA herpes virus that infects more than 90% of the population worldwide. As in the case of other viruses, EBV can cause a productive infection marked by a high copy number of viral DNA with synthesis of infectious virions or a latent infection in which EBV is present in the nucleus of the infected cell in the form of episomal DNA and no detectable virions are produced.

The first human disease entity found to be caused by EBV was acute infectious mononucleosis (IM). Many experimental studies provided indirect evidence that the target cells in IM could be B cells. In more recent years, in situ labeling studies have been performed to visualize the infected cells directly and to analyze their nature and distribution. Most studies of this kind employed radioactive DNA probes specific for internal DNA repeats of the EBV genome. The first human disease entity found to be caused by EBV was acute infectious mononucleosis (IM). Many experimental studies provided indirect evidence that the target cells in IM could be B cells. In more recent years, in situ labeling studies have been performed to visualize the infected cells directly and to analyze their nature and distribution. Most studies of this kind employed radioactive DNA probes specific for internal DNA repeats of the EBV genome. The first of these studies showed an EBV infection in only some lymphoid cells distributed in the interfollicular areas of tonsils, whereas a third study published more recently, reported that not only lymphoid cells, but also macrophages, endothelial cells, follicular dendritic cells (FDC), and even pharyngeal epithelium carried the virus. In a subsequent study performed by our group, RNA probes specific for small EBV-encoded nuclear transcripts (designated EBER1 & 2) were used in order to identify all latently EBV-infected cells in acute and chronic EBV infection. The advantage of in situ hybridization (ISH) with EBER-specific probes lies in its remarkably increased sensitivity due to the fact that the EBER transcripts are present in each single, latently infected cell at a very high copy number (up to 10⁶). The EBER-ISH-study confirmed the lymphoid morphology and the interfollicular distribution of the infected cells and showed, unlike the ISH using DNA probes, a larger number of infected cells to be present. Through double-labeling for EBER and CD20, the majority of infected cells could be shown to be B cells.

All the studies previously mentioned incorporated the application of ISH with radioactive probes, which is coupled with the problem of the morphology of labeled cells being obscured due to an accumulation of grains. Because of this, the conflicting reports about the number of different cell types labeled with EBV-specific probes, as well as the surprising finding that the neoplastic cells of various T-cell non-Hodgkin's lymphomas (T-NHL) more frequently carry the virus than those of B-cell non-Hodgkin's lymphomas (B-NHL), and because studies for productive EBV infection have not been performed, a reinvestigation of IM would appear necessary to clarify the types, morphology, and distribution of EBV-infected cells, as well as the type of infection (latent and/or productive) in acute EBV infection. In the present study, these questions were addressed by taking advantage of the high sensitivity and excellent morphologic preservation provided by ISH with nonradioactively labeled EBER and BHLF1 probes for the demonstration of latent (EBER1 & 2) and productive (BHLF1) infection. The BHLF1 probe not only allows the detection of productively infected cells, but also shows those cells that are in the...
process of switching from a latent to productive infection. To clarify the type of infected cells with precision, EBER-ISH was combined with immunohistology using various cell type specific/characteristic monoclonal antibodies.

MATERIALS AND METHODS

Biologic Specimens

Tonsillar specimens from nine patients with the clinical diagnosis of IM were taken from the files of the Institute of Pathology, Benjamin Franklin Medical Centre of the Free University of Berlin, Germany. All patients had a clinical history of acute IM, which was confirmed in six cases by the heterophil antibody test. There were six males and three females, ranging in age from 4 to 36 years. All patients had undergone tonsillectomy for severe obstructive tonsillitis and had made an uneventful recovery from their illness. The tonsils were fixed in buffered formalin and embedded in paraffin by routine histologic procedures. All investigations were performed on sections of whole tonsils comprising superficial mucosa, lymphoid tissue, adjacent connective tissue, and salivary glands. A large number of specimens containing oropharyngeal mucosa from patients without clinical or laboratory findings of an acute EBV-infection were analyzed as controls. They included 25 samples of salivary glands, 20 of nasal mucosa, 25 of gingiva, 10 of tongue, 35 of tonsils, and eight of buccal mucosa.

The morphologic changes in all IM cases were quite similar to one another. In addition to a follicular hyperplasia, an expansion of the interfollicular zone was noted, leading to a distortion rather than an effacement of the tonsillar lymphoid tissue architecture. These expanded interfollicular zones were characterized by a polymorphous lymphoid cell hyperplasia with variable numbers of small- to medium-sized transformed lymphocytes, immunoblasts, occasional Reed-Sternberg (RS)-like cells, and plasma cells at various stages of differentiation. Small necrotic foci were found in all cases and were usually adjacent to and entering into crypts, while large areas of fresh necrosis were present in three cases.

In Situ Hybridization

In situ hybridization for the detection of EBV-encoded small nuclear RNAs (EBER1 and EBER2) was performed as previously described in detail. Briefly, 3 to 5-μm thick sections taken from paraffin-embedded tissue specimens were used for hybridization after dewaxing, rehydration, and proteinase K digestion. The hybridization was performed overnight at 42°C in a humid chamber using a solution containing 50% formamide and 3 to 5 ng of digoxigenin (DIG)-labeled in vitro transcribed EBER1 and EBER2 sense (negative control) or antisense RNA-probes. After hybridization, the slides were washed and treated with RNase A to dispose of unspecific bound RNA probes, resulting in an extraordinarily low background signal. The detection of specifically bound, DIG-labeled antisense RNA probes was achieved by DIG-specific monoclonal antibodies, coupled with alkaline phosphatase and subsequent development with Naphthol-As-Biphosphate and New Fuchsin. EBV-infected cells became visible by their red nuclei.

For the detection of BHLF1-RNA, a mixture of three oligonucleotides complementary to two abundant immediate-early/early mRNAs encoding proteins belonging to the subgroup Ea-D of EBV-encoded antigens, were used for ISH. The procedure was performed according to the manufacturer's protocol (DAKO, Glostrup, Denmark), the only exception being that we used Naphthol-As-Biphosphate/New Fuchsin instead of nitro-bue tetrazolium/bron-chloro-indoxyl-phosphate (NBT/BCIP) for the detection of BHLF1-expressing cells. To confirm the specificity of this assay system and to exclude the binding of the BHLF1 mRNA specific oligonucleotides to viral DNA, we performed a number of controlled experiments using totally EBV-infected Raji cell line cells and the productively infected cell line B95-8. As expected, the application of the BHLF1-ISH was totally negative on Raji cells and positive on 5% to 10% of the B95-8 cells, whereas the EBER probes produced practically the reverse results. DNA probes, being homologous to a part of the IRI of EBV, did not produce detectable signals in the B95-8 cell line cells when applied under conditions used for BHLF1-ISH. As an additional positive control for the BHLF1-ISH assay, we included specimens of oral hairy leukoplakia occurring in human immunodeficiency virus (HIV)-positive patients.

Double Labeling

To determine the nature of the infected cells, we performed a double-labeling procedure using immunohistology (IH) with a panel of monoclonal antibodies (Table 1) and EBER-ISH. The immunohistoalogic demonstration of the different antigens involved the avidin-biotin-complex method (DAKO), which was applied before EBER-ISH. All steps were performed under RNase-free conditions. After IH development, the slides were washed in phosphate-buffered saline and submitted to the EBER-ISH procedure as described above, differing only in that a development using NBT/BCIP (BioRad, Munich, Germany) was employed. As a result of this procedure, EBV-infected cells of different phenotype became visible by their deep-blue nuclei and brown cytoplasmic membrane staining. In addition, adjacent sections were immunostained with antibodies to IgL κ, IgL λ, and J-chains using the APAAP (alkaline phosphatase anti-alkaline phosphatase) method as described.

Polymerase Chain Reaction (PCR)

The two-step nested primer PCR was performed under conditions and with the same set of primers as described previously. Briefly, 10-μm thick paraffin sections obtained under conditions preventing DNA-cross-contamination were dewaxed and digested with proteinase K. Aliquots of the digested material were used for PCR after boiling for 15 minutes. Amplification products were examined with a 2% ethidium bromide agarose gel for the presence of appropriate bands, blotted on nylon membranes (Hybond N, Amersham, Germany), and hybridized with 32P terminal-transferase labeled internal nucleotide. The positive controls used were the Namalwa cell line harboring 1 to 2 copies of EBV and material from five IM samples, while the EBV-negative cell line HUT102 and DNA-negative samples served as negative controls. The amplification of a section of the β-globin gene served to determine the amplifiability of the extracted DNA. The sensitivity of our PCR approach was determined by an experiment using serial dilutions of the Namalwa-cell line DNA, in which only a single copy of EBV-genome could be detected.

RESULTS

EBER–In Situ Hybridization

The ISH with nonsitotopically labeled EBER1 & 2 probes led to a strong nuclear signal without any background and with excellent morphologic preservation of the labeled and unlabeled cells in all nine cases of IM studied. This allowed a subtle morphologic evaluation of the labeled samples. All EBER-positive cells were found to correspond to lymphoid cells, while all other cellular components including squamous epithelia, glanular epithelial cells, endothelial cells, fibrocytes, granulocytes, macrophages, FDC etc were constantly EBER-negative. The morphology of the EBER-positive cells ranged from immunoblast-like and RS-like to small- and medium-sized lymphoid cells and cells with features of plasma cellular differentiation. The EBER label was also present in many cells undergoing mitotic division.
The EBER-positive cells were usually not homogeneously distributed in the tonsillar tissue. EBER-positive lymphoid cells were present in the interfollicular regions, usually sparing the germinal centres (Fig 1A). Occasional EBER-positive small lymphocytes were localized in the follicle mantle in all instances, while rare (less than three) EBER-positive cells were seen in few germinal centers of only four cases. The vast majority of the labeled lymphoid cells had accumulated around necrotic areas and tonsillar crypts, infiltrating the crypt epithelium (Figs 2A and 3A). EBER-positive cells

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<tr>
<th>Antibody Designation</th>
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<tr>
<td>L26</td>
<td>CD20 Pan B cell (except plasmacytoid differentiated cells and plasma cells)</td>
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<td>CD45RB B-cell subset, including certain plasmablasts and proplasma cells</td>
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<td>CD21 FDC in paraffin sections</td>
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<td>Anti-J-chains</td>
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Fig 1. (A through C) Distribution and immunophenotype of EBV-infected (i.e., EBER-positive) cells in the subepithelial area of a human tonsil affected by IM. (A) Double labeling for EBER (blue-black nuclei) and CD20 (brown membrane staining) shows that large numbers of EBV-infected B cells are localized in the perilinterfollicular zone and are absent from germinal centers (GC). Original magnification × 400. (B and C) The simultaneous labeling for EBER (blue-black nuclei) and the TCR β chain (brown membrane staining) shows that T cells can become infected by EBV in IM (original magnification × 800). An EBV-infected, medium-sized T cell and a large T-cell blast are shown.

Fig 3. Demonstration of latently and productively infected lymphoid cells in the crypt epithelium of a tonsil affected by IM. (A) Numerous latently infected (EBER-positive) lymphoid cells have invaded the crypt EP and have spilled over into the crypt lumina (indicated by CR), which is released into the oropharynx. Original magnification × 400. (B and C) One productively infected (BHLF1-positive) lymphoid cell in the middle layer (B) and one at the surface (C) of the crypt EP. Original magnification × 850.
Distribution and immunophenotype of EBV-infected cells in the epithelium of a tonsil affected by IM. (A) Demonstration of numerous EBV-infected (EBER-positive) cells (red-brown nuclei) in all layers of the tonsillar epithelium (EP). Original magnification × 600. (B) Double labeling for EBER (blue-black nuclei) and cytokeratin (brown cytoplasm). The EBER-positive cells appear to be cytokeratin-negative. Original magnification × 600. (C) Double labeling for EBER and CD20. Most EBER-labeled cells present in the tonsillar EP do not express the CD20 molecule. Original magnification × 600. (D) Double labeling for EBER and the B-cell marker Ki-B3 (CD45RB). Most, if not all, EBER-positive cells present within the tonsillar EP proved to express the Ki-B3 antigen, indicating their B-cell nature. Original magnification × 600.
could also be identified on the surface of tonsillar epithelium and within the necrotic material filling tonsillar crypts (Fig 3A). The morphology of the EBER-positive cells within the epithelium was because of their small, round nucleus and their small cytoplasmic rim most consistent with that of lymphoid cells. However, on morphologic grounds, it was not possible to exclude EBER-expression in basal cells of the squamous epithelium.

Single EBER-positive small lymphoid cells could be detected in the stroma of gingival mucosa in one of the 123 control group cases that proved through PCR to contain DNA sequences specific for EBV.

**Double-Labeling Studies**

**B-cell markers.** Many of the EBER-positive cells were also CD20-positive; they showed the morphology of medium-sized lymphoid cells, immunoblasts, and occasionally Hodgkin and RS (HRS)-like cells. These cells were often arranged in sheets in the perifollicular areas (Fig 1A) and also in the surroundings of necrotic foci. The rarely observed EBER-positive cells in germinal centers were also CD20-positive. However, in all cases, a large number of EBER-positive cells were CD20-negative. These cells were most notably found in large numbers both within and adjacent to the tonsillar crypt epithelial cells (Fig 2C) and also around necrotic foci. Also, the occasional small EBER-positive lymphocytes in the follicular mantle zone were CD20-negative. The vast majority of the EBER-positive and CD20-negative cells could be identified as B cells, plasmablasts, and proplasma cells and some as plasma cells because of their morphologic features and reactivity with the monoclonal antibody Ki-B3 directed against the CD45RB epitope (Fig 2D). The plasma cellular differentiation of the CD20-negative and Ki-B3-positive cells could be confirmed by the demonstration of polytypic cytoplasmic IgL and J-chains in many of the Ki-B3-positive cells by immunostaining adjacent sections. In addition, the infected immunoblasts and some of the HRS-like cells showed a labeling with the Ki-B3 antibody.

**T-cell markers.** In all nine cases, EBER-positive lymphocytes expressing T-cell characteristic/specific markers could be identified. The number of EBV-infected T cells was considerably lower than that of the infected B cells and varied considerably between the cases: In four cases, only some EBER-positive small T lymphocytes were detectable; three cases contained some EBER-positive T cells that were usually small to medium-sized (Fig 1B), occasionally blastosoid in morphology; in two further cases, a large number of EBV-infected T cells were present, often exhibiting the morphology of Immunoblasts (Fig 1C) and RS cell-like cells. The EBV-infected T cells were predominantly located in the interfollicular areas.

**Macrophage/FDC markers.** Double-labeling experiments for EBER, the macrophage marker CD68, and the FDC marker CD21 produced good results in four cases. In none of these cases was the EBER signal colocalized with the staining for macrophage and/or FDC.

**Cytokeratin markers.** This analysis did not show any convincing colocalization of EBER and cytokeratin signals (Fig 2B) in any of the nine cases studied, indicating the nonepithelial nature of EBV-infected cells present in the crypt and surface squamous epithelium.

**In Situ Hybridization for BHLF1-RNA**

BHLF1-expressing cells could be detected in eight of the nine infectious mononucleosis cases studied. The BHLF1 cells usually had the morphology of small lymphocytes with a round nucleus and condensed chromatin; only occasionally could a larger nucleus with a prominent nucleolus be identified. The number of these BHLF1-positive lymphocytes was relatively low, ie, one to five per section. These cells were mostly detected in the vicinity of necrotic areas and never within germinal centres. Only two cases contained more than five positive lymphocytes per section. In these cases, BHLF1-expressing lymphocytes were also found within tonsillar epithelia (Fig 3B), sometimes reaching the epithelial surface (Fig 3C). None of the specimens of the oropharyngeal mucosa of the control group of cases contained any BHLF1-RNA-expressing cells.

**PCR**

EBV-specific sequences were detected in only one of the 123 oropharyngeal samples after application of PCR with the corresponding primers. The EBV-carrying cells in the EBV-DNA-positive sample could be identified as lymphoid cells by EBER-ISH.

**DISCUSSION**

In the present study, we analyzed biopsy samples involved by acute IM with a highly sensitive ISH using nonradioactive RNA probes and oligonucleotides for the cellular localization and the anatomical distribution of the latent and productive EBV infection, respectively. Because the nonradioactive signal is crisp and constantly restricted to the areas where the probe binds, the cytologic features of the infected cells could be much better evaluated when compared with previous radioactive ISH studies, in which the morphology of the labeled cells is usually obscured by the silver grains. By combining the ISH with immunohistology, using a panel of cell-type specific/characteristic monoclonal antibodies, the infected cell types could be reliably immunophenotyped. The results obtained are relevant for the following reasons.

First, the present study confirms previous DNA-ISH investigations by Weiss et al and Niedobitek et al showing that, in IM, EBV infection is restricted to cells with the morphology of lymphoid cells of various sizes. The restriction of the infection to lymphoid cells was unequivocally confirmed by double labeling showing that there was no EBV-infected cell with an immunophenotype other than that of lymphoid cells. This finding challenges the results of the report by Prange et al in which an EBV infection not only in lymphoid cells, but also in macrophages, endothelial cells, FDC, and pharyngeal epithelia is described.

The second relevant finding of the study is that, in IM, among the lymphoid cells, not only B cells, but also T cells, are infected. This is shown by the detection of cells simultaneously expressing EBER and CD45RO and/or the β-chain of T-cell receptor (TCR). These EBV-infected T cells had the morphology of small- to medium-sized lymphoid cells...
in the majority of cases and were present in all of the IM cases studied, usually at a low number. Similar findings have also been recently reported by a Japanese group that investigated the phenotype of EBER-positive cells in lymph nodes of IM patients. In view of our recent observation that the neoplastic cells in T-NHL are more frequently infected than in B-NHLs, the presence of EBV-infected T cells in IM is not surprising. It is tempting to assume that the EBV-infected T cells in IM might be the normal equivalents for the EBV-infected tumor cells in peripheral T-cell lymphomas.

The third finding of interest is that the majority of infected lymphoid cells that were negative for the Pan B-cell marker CD20, the T-cell markers CD45RO and FO1 could be labeled with the B-cell antibody, Ki-B3. Many of the Ki-B3-positive infected B cells showed morphologic and immunophenotypic (ie expression of polytypic cytoplasmic immunoglobulin light chains and J-chains) features of plasmacellular differentiation. This corroborates with in vitro studies on the differentiation of EBV-infected cells, which appear to be a model of acute EBV infection in vivo. However, in a previous study, we were unable to show any plasmacellular differentiation of EBV-infected lymphocytes. This was most probably due to a blurring of the morphologic characteristics of the EBER-positive cells through the grains caused by the radioactively labeled probes used for ISH. The EBV-infected cells showing a plasmacellular differentiation might represent a certain type of plasma cells because Ki-B3 is consistently negative with most plasma cells and their precursors. However, Ki-B3 decorates plasmablasts, proplasma cells, and even plasma cells in certain cases of hyperplasia of extranodal EBV blasts with plasma cell differentiation, and the plasma blasts, proplasma cells, and plasma cells in angioimmunoblastic T-cell lymphoma (unpublished observations). This observation favors the view that the plasma cells and precursors expressing the Ki-B3 epitope represent a pregerminall center, extragranularly developed, short-lived plasma cell subset, and the plasma cells and precursors devoid of the Ki-B3 epitope correspond to the germinal-center born, long-lived plasma cell population. Taken together, these observations comply with the notion that EBV usually infects early B cells (eg, cord blood B cells) and that these early EBV-infected B cells cannot usually enter the germinal center, as evidenced by sprouting of germinal centers from EBV infection in all our IM cases and the IM cases studied by other authors.

The fourth important finding of this study is that latently and productively infected lymphoid cells do not only occur in mass in interfollicular regions and in the direct surroundings of necrosis, but also within the squamous epithelium of the crypts and even within the crypt lumina themselves. The EBV-infected cells present in the epithelium had the morphology of small- to medium-sized lymphoid cells and none of them displayed cytologic features of mature epithelial cells. To exclude EBER expression in macrophages, FDC, and in the basal cells of the epithelium, the nuclei of which can resemble those of lymphoid cells, double-labeling studies for EBER and markers for epithelial cells (cyto-keratin), macrophages (CD68), FDC (CD21), as well as for B- and T-cell lineage were performed. None of the EBV-infected cells located within the epithelium expressed cyto-keratin and/or macrophage and/or FDC antigens, but nearly all of them were positive for one, if not both, of the Pan B-cell antigens. The absence of EBV infection from epithelium is in accordance with previous studies by Weiss et al and our group, in which the EBV infection was visualized by demonstrating EBV-DNA with radioactive ISH.

To further substantiate the absence of EBV from oropharyngeal epithelial cells, we investigated a larger number of oromucosa samples of different sites, including those of tonsils, gingiva, tongue, and salivary glands from patients without an acute EBV infection. In all instances except one, PCR and EBER-ISH did not produce any EBV-specific signals. However, the exception was shown through EBER-ISH to contain the EBV genome in some lymphoid cells, whereas the epithelial cells were devoid of any EBER signal. These data call into question current models of the mode of the EBV infection and EBV persistence, which envisaged, based on studies made in the 1970s, the oropharyngeal and/or the salivary gland epithelium as a primary site and reservoir for EBV infection, from which B cells were thought to be secondarily infected. As a consequence of this concept, it is still widely believed (and stated in textbooks) that the EBV reservoir in the oropharyngeal epithelial cells is responsible for the transmittance of EBV from one individual to another. Our findings showing latent and more importantly productive infection in lymphoid cells on the surface of tonsillar epithelium and within the crypt themselves, but not in any epithelial cells, rather suggest that the interindividual EBV infection might be predominantly or even selectively mediated by the transfer of EBV-infected lymphoid cells, eg, by kissing, from person to person, and that EBV-infected epithelial cells are not required for the oral infection route. To further substantiate or disprove this possibility, appropriate experiments are currently being undertaken, which involve the analysis of the type of infected cells found in oral lavages of patients with IM.

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