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EBV-Infected B Cells in Infectious Mononucleosis: Viral Strategies for Spreading in the B Cell Compartment and Establishing Latency

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Summary

Infection of humans with Epstein-Barr virus (EBV) may cause infectious mononucleosis (IM). Analysis of single EBV-infected cells from tonsils of IM patients for rearranged immunoglobulin genes revealed two strategies of EBV for rapid and massive spread in the B cell compartment: the direct infection of many naive as well as memory and/or germinal center B cells and the expansion of the latter cells to large clones. In IM, the generation of virus-harboring memory B cells from naive B cells passing through a germinal center reaction likely plays no role. Members of clones can show distinct morphologies and likely also EBV gene expression patterns, and this ability implies a mechanism by which EBV-harboring cells can evade immune surveillance and establish a pool of persisting EBV-infected B cells.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous, tumorigenic herpes virus infecting more than 90% of the human population (Rickinson and Kieff, 1996). During childhood, primary infection by EBV is usually asymptomatic and leads to a lifelong persistence of the virus. Primary infection in adolescents and young adults can cause infectious mononucleosis (IM), a self-limiting, lymphoproliferative disease (Henle et al., 1968). In the course of the disease, one in 10^4 circulating B cells is infected by EBV (Tosato et al., 1984; Rocchi et al., 1977). In response to spreading of EBV, there is a massive proliferation of T cells, including EBV-specific cytotoxic T cells (Callan et al., 1996, 1998), which eliminate most EBV-infected cells. However, some resting EBV-infected B cells lacking expression of most or all EBV-encoded immunogenic proteins can evade immune surveillance (Miyashita et al., 1997; Babcock et al., 2000 [this issue of *Immunity*]). In this way a balance between rare EBV-harboring B cells (one in 10^5–10^6 peripheral B cells [Wagner et al., 1992; Miyashita et al., 1995]) and EBV-specific T cells is established during persistence of the virus.

EBV-carrying cells in tonsils of IM patients vary morphologically and include small- to medium-sized lymphocytes (the major population of EBV-positive cells), lymphoblasts, and rare, very large, and occasionally multinucleated cells that resemble morphologically and by the expression of CD30 Hodgkin and Reed-Sternberg (HRS) cells of Hodgkin's disease (HD) (Anagnostopoulos et al., 1995; Reynolds et al., 1995). The EBV-infected cells vary not only in their morphology but also show different expression patterns of EBV-encoded genes. These expression patterns are consistent with the three classical programs of EBV gene expression (Rickinson and Kieff, 1996). Three classes of cells can be distinguished: (1) cells, mostly of small size, expressing EBER transcripts but neither LMP1 and LMP2A nor EBNA2 (latency I); (2) lymphoblasts and HRS-like cells expressing EBER transcripts, LMP1, and LMP2A but not EBNA2 (latency II); and (3) small- to medium-sized cells and some HRS-like cells positive for EBER transcripts, LMP1, LMP2A, and EBNA2 (latency III) (Niedobitek et al., 1997a, 1997b; unpublished data). While, at least late after infection, most if not all EBV-bearing cells represent B cells, T cells and epithelial cells may initially also harbor EBV (Sixbey et al., 1984; Tokunaga et al., 1993; Anagnostopoulos et al., 1995).

In addition to the mentioned classical terminology of latent EBV gene expression, another nomenclature of EBV latency programs has been developed based on the behavior of the virus in normal B cells during persistent infection in vivo (Thorley-Lawson et al., 1996; Babcock et al., 2000). According to this hypothesis, EBV infects in tonsils naive IgD^+ B cells, which are driven into proliferation and express all EBV-encoded latent genes (growth program, classical latency III). The virus gains access to the compartment of memory B cells, as these EBV-bearing naive B cells proceed in differentiation like noninfected B cells; in T cell–dependent antibody responses, antigen binding cells are activated by antigen and T helper cells and are driven into the germinal center (GC) reaction, where mutations are introduced into their rearranged V region genes in the course of cellular proliferation (Rajewsky, 1996). Mutant cells expressing antigen receptors with high affinity for the immunizing antigen subsequently differentiate into plasma cells or are selected into the pool of long-lived, recirculating memory cells. During this differentiation process, EBV-bearing cells change the expression pattern of EBV-encoded genes. GC centroblasts and centrocytes as well as reactivated memory B cells express EBNA1, LMP1, and LMP2A (classical latency II). Resting recirculating memory B cells show another type of latency, which ensures escape from immune surveillance. This program is characterized by expression of EBER transcripts and perhaps LMP2A and EBNA1 (latency program) (Qu and

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Rowe, 1992; Tierney et al., 1994; Chen et al., 1995; Miyashita et al., 1997; Babcock et al., 2000).

EBV is associated with malignancies such as Burkitt's lymphoma, posttransplantation lymphoproliferative disorders, nasopharyngeal carcinoma, and rare T cell lymphomas (Rickinson and Kieff, 1996). A role of EBV has also been suggested in the pathogenesis of HD because HRS cells, the malignant cells of HD, carry clonal copies of EBV in about 50% of the cases (Rickinson and Kieff, 1996). A potential association between HD and IM is implied from the observation that patients with a history of IM have a 3- to 4-fold increased risk of developing HD (Rosdahl et al., 1974; Munoz et al., 1978). In a fraction of classical HD cases, the HRS cells carry "crippling" mutations that render originally productive V region genes nonfunctional (Kanzler et al., 1996; Küppers and Rajewsky, 1998). Since loss of B cell receptor expression in GC B cells normally results in cell death by apoptosis, HRS cells, at least in these cases, thus appear to be derived from "crippled" GC B cells that were rescued from apoptosis through some transforming event (Kanzler et al., 1996; Küppers and Rajewsky, 1998). In EBV-positive cases of HD, this initial transforming event might be infection by EBV and subsequent expression of LMP2A, the viral oncogene LMP1, or both (Kanzler et al., 1996; Caldwell et al., 1998; Kulwichit et al., 1998).

It is still unknown how EBV establishes the pool of EBV-carrying cells during acute infection and persistence. Moreover, a possible relationship between HRS-like cells of IM and the malignant cells of HD in terms of their cellular derivation remains elusive. To approach these questions, we micromanipulated single, morphologically and phenotypically distinct EBV-infected cells from frozen tissue sections of two cases of IM and analyzed them for immunoglobulin V gene rearrangements. This allowed us to characterize the composition of the EBV-harboring B cell population in terms of clonality and stage of differentiation and thus to trace the pathway of viral spread in the B cell compartment.

Results

Histological Distribution of EBV-Infected Cells

Tonsillar sections of six cases of IM were stained for EBER transcripts or CD30 expression in order to define the localization of EBV-infected cells and HRS-like cells in the tissue. Besides their mainly interfollicular location often adjacent to crypt epithelium (the entry site of EBV into the tonsil) and areas of necrosis, it became evident that EBV-positive cells in five of the six cases are restricted to some interfollicular regions (Figure 1A; data not shown). In only one case were most EBV-harboring cells evenly distributed throughout the tissue section (Figure 1B).

In addition, tonsillar sections of five of the six patients with IM were stained for expression of LMP1 in combination with EBNA2 protein or for EBER transcripts (in three cases in combination with CD30) (Figure 1; data not shown). By means of these double stainings and based on the assumption that all EBV-infected cells are positive for EBER transcripts, EBV-harboring cells can be divided into the following phenotypically different subtypes (see also Introduction): (1) fewer than 10% of cells express EBER transcripts but not LMP1 and EBNA2 (latency program or classical latency I; mainly small- to medium-sized CD30$^-$ cells); (2) 20%–30% of cells express EBER transcripts and LMP1 but not

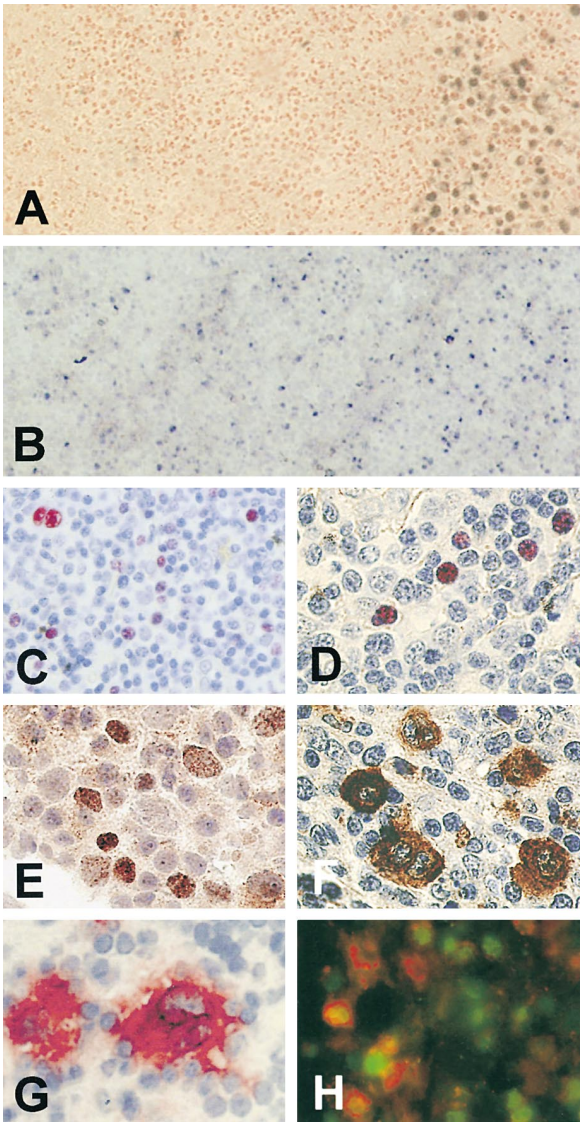

Figure 1. Phenotypic Characterization of EBV-Infected Cells

Stainings were performed on tonsillar sections.

(A) Overview of a paraffin section of case 1 stained for EBER transcripts (red staining). A part of one region with EBV-infected cells is shown.

(B) Overview of a frozen section of case 2 stained for EBER transcripts (blue-purple staining). The dissemination of EBV-bearing cells is shown.

(C) Overview of a paraffin section of case 1 stained for EBER transcripts (red staining). The phenotypic variation of EBV-infected cells is shown.

(D) Paraffin section of case 1 stained for EBER transcripts (red staining); small EBV-infected cells are shown.

(E) Frozen section of case 2 stained for EBNA2 (brown staining)

(F) Paraffin section of case 1 stained for LMP1 (brown staining)

(G) HRS-like cells from a frozen section of case 1 stained for CD30 (red staining).

(H) Frozen section of case 2 stained for LMP1 (red fluorescent staining) and EBNA2 (green fluorescent staining).

EBNA2 (classical latency II; mononucleated [occasionally CD30$^+$] lymphoblasts and large, often multinucleated CD30$^+$ HRS-like cells, sometimes with irregularly shaped nuclei); and (3) 10%–20% of cells express EBER transcripts, LMP1, and EBNA2 (classical latency III; small- to medium-sized CD30$^-$ cells and, rarely, CD30$^+$ HRS-like cells). In addition, 50%–60% of EBER$^+$ cells (or perhaps somewhat fewer if cells with single dots of LMP1 signal are regarded as LMP1 positive) are small- to medium-sized cells expressing EBNA2 but not LMP1. This might represent a transitory stage shortly after infection since in newly infected B cells expression of EBNA2 precedes expression of LMP1 (Alfieri et al., 1991; Niedobitek et al., 1997a) (Figure 1). Between 1% and 5% of EBV-bearing cells acquire an HRS-cell like morphology.

PCR Analysis of Isolated EBV-Infected Cells

EBV-infected cells of two of the six cases of IM were analyzed at the molecular level. These two cases differ on the one hand by the time of tonsillectomy in the course of the disease (case 1, 3 days after onset of symptoms; case 2, more than 14 days after onset of symptoms) and on the other hand by the distribution of EBV-harboring cells. As with most tonsils analyzed, the first case showed local restriction of EBV-infected cells (see above); in this biopsy four areas with EBV-carrying cells were identified (Figures 1A and 2A). In the second case, EBV-bearing cells were found throughout the tissue (Figures 1B and 2B).

Besides gaining insight into the overall composition of EBV-harboring cell populations in terms of clonality and differentiation stage, we were also interested in the nature of the cells positive for LMP1 or EBNA2. These subsets of EBV-infected cells were included in the analysis since we wanted to find out whether distinct subsets of EBV-bearing cells (with regard to clonal composition and stage of development) can be defined by these markers. For example, it has been speculated that EBNA2 expression is restricted to naive EBV$^+$ B cells (Babcock et al., 2000). In addition, HRS-like cells were analyzed in order to study their possible relationship to HRS cells in HD. Therefore, for the first case, EBER$^+$ cells (representing the overall population of EBV-infected cells), LMP1$^+$ cells (representing cells in latency II or III), and EBNA2$^+$ cells (representing cells in latency III and/or newly infected cells) were isolated (Figure 1; for morphological and immunophenotypic criteria of cell isolation see Table 1). In addition, HRS-like cells were micromanipulated (Figure 1G; Table 1). For the second case, small- to medium-sized EBV-infected cells were micromanipulated from sections stained for both CD30 and EBER transcripts. This staining allowed us to isolate cells of the main population of EBV-bearing cells (CD30$^-$, EBER$^+$ cells) and to exclude the rare CD30$^+$ HRS-like cells (Table 1). The latter cells were micromanipulated from CD30-stained sections (Figure 1G; Table 1). In addition, EBNA2$^+$ cells were isolated (Figure 1E; Table 1).

These isolated single cells were analyzed by PCR for V_H and V_λ gene rearrangements (Table 2) (V_κ genes were not analyzed, for reasons outlined in the Experimental Procedures). In order to confirm EBV infection of the cells, some cells were analyzed in parallel for the presence of a fragment of the EBV-encoded *EBNA1* gene. In both cases, the EBNA1-specific PCR product could be amplified from the vast majority of cells positive for V gene rearrangements, and this fact demonstrated that the cells are indeed infected by the virus (Table 2). Single micromanipulated CD3$^+$ T cells, EBER$^-$ cells, and CD20$^+$ B cells as well as aliquots of the buffer covering the sections during micromanipulation served as controls. In both cases PCR products for rearranged V genes or the fragment of the *EBNA1* gene were amplified only very rarely from negative controls. They probably result from contaminated fragments of B cells or EBV-infected cells (Table 2).

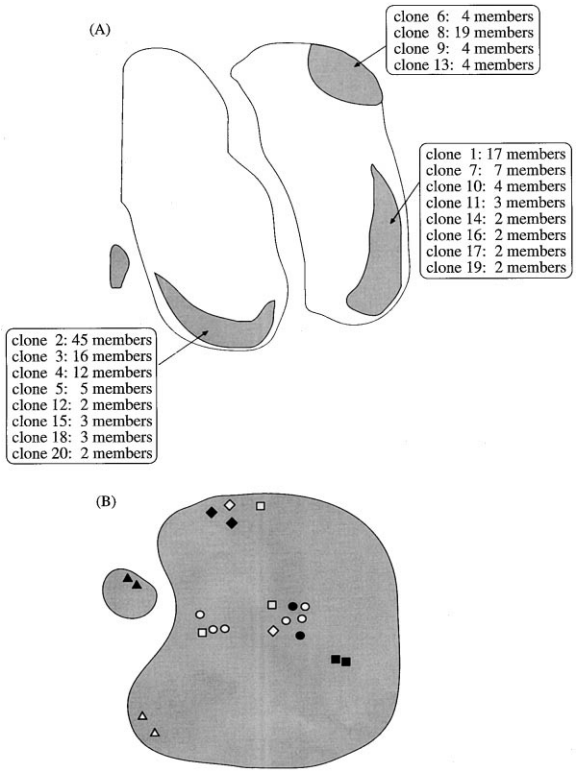

Figure 2. Diagrammatic Representation of Tonsillar Sections of the Two Analyzed Cases of IM Indicates the Distribution of Clonally Related Cells

Regions where EBV-infected cells are mainly located are shown in gray. The location of cells with unique V gene rearrangements is not shown.

(A) Case 1: in this case EBV-harboring cells are predominantly found in the four regions indicated. The clone number, the number of members, and the location of the clones are indicated in boxes. Clonally related cells were found evenly distributed inside the respective regions.

(B) Case 2: EBV-infected cells are evenly distributed throughout the section. Individual members of a given clone are marked with the same symbol.

Naive as Well as Memory and/or GC B Cells Are Infected by EBV

From the first case, 224 V gene rearrangements were amplified from 203 of 698 cells analyzed, and from the second case, 79 V gene rearrangements were amplified

Table 1. Cell Types Analyzed

Case	Isolated Subset	Cell Type	Morphological Criteria for the Isolation of Cells	Immunophenotypic Criteria[a] for the Isolation of Cells
1	EBER$^+$	overall population of EBV-infected cells	—	EBER$^+$ or CD30$^-$ EBER$^+$[b]
	EBNA2$^+$	cells in latency III or newly infected cells	—	EBNA2$^+$
	LMP1$^+$	cells in latency II or III	—	LMP1$^+$
	HRS-like	HRS-like cells	large, often multinucleated irregularly shaped nucleus	CD30$^+$ or CD30$^+$ EBER$^+$
2	EBER$^+$	overall population of EBV-infected cells excluding HRS-like cells	small- to medium-sized	CD30$^-$ EBER$^+$
	EBNA2$^+$	cells in latency III or newly infected cells	—	EBNA2$^+$
	HRS-like	HRS-like cells	large, often multinucleated, irregularly shaped nucleus	CD30$^+$

[a] Including in situ hybridization for EBER transcripts.
[b] From the CD30 and EBER double staining only small CD30$^-$ EBER$^+$ cells were isolated (approximately 30% of the total number of EBER$^+$ cells).

from 67 of 367 cells analyzed (Tables 2 and 3). The relatively low amplification efficiency for some of these populations (e.g., 20 V gene rearrangements were amplified from 120 EBNA2$^+$ cells from case 1; see Table 2) may be due to technical matters such as DNA instability during handling of sections, or it may indicate that some of the EBV-infected cells are not B but T cells (Tokunaga et al., 1993; Anagnostopoulos et al., 1995). However,

Table 2. Summary of the Single-Cell Analysis from Two Cases of IM

Case	Cell Type	Number of Cells/ Samples Analyzed[a]	Number of Gene Rearrangements		Number of EBNA1-Positive Samples per V_H and/or V_λ-Positive Cells Analyzed[b]
			V_H	V_λ	
1	All	698	224		90/95
	EBER$^+$	176[d]	60[e]	6	52/56
	EBNA2$^+$	120	20	n.d.	n.d.
	LMP1$^+$	142	36	6	3/3
	HRS-like	260[d]	72[e]	24	35/36
2	All	367	79		49/54
	EBER$^+$	100	20	8	26/26
	EBNA2$^+$	140	13	n.d.	n.d.
	HRS-like	117	31	7	23/28
	Controls				
1	B cells[c]	39	11	5	n.d.
	EBER$^-$[c]	47	n.d.	n.d.	0/47
	T cells	35	1	0	0/20
	Buffer	210[d]	3[f]	1	0/110
2	B cells	50	11	4[f]	14/50
	EBER$^-$	33	n.d.	n.d.	1/33
	T cells	121[d]	6	3[f]	6/71[g]
	Buffer	121[d]	1	0	1/79

[a] The following PCR analyses were carried out: (1) 96 EBER$^+$ cells, 37 LMP1$^+$ cells, 22 HRS-like cells, 47 EBER$^-$ cells, one T cell and 41 buffer controls of the first case and 80 EBER$^+$ cells, 70 HRS-like cells, 45 B cells, 25 EBER$^-$ cells, 47 T cells, and 54 buffer controls of the second case were analyzed after preamplification with a combination of V_H/V_λ and EBNA1 primers; (2) 50 EBER$^+$ cells, 50 HRS-like cells, and 35 buffer controls of the first case were analyzed after preamplification with a combination of V_H and EBNA1 primers; (3) 91 HRS-like cells, 19 T cells, and 34 buffer controls of the first case and 20 EBER$^+$ cells, five B cells, eight EBER$^-$ cells, six T cells and six buffer controls of the second case were analyzed with a combination of V_H/V_λ and EBNA1 primers; (4) 47 HRS-like cells, 18 T cells, and 19 buffer controls of the second case were analyzed with a combination of V_HL/V_λ and EBNA1 primers; (5) 30 EBER$^+$ cells, 105 LMP1$^+$ cells, 97 HRS-like cells, 39 B cells, 15 T cells, and 61 buffer controls of the first case were analyzed with a combination of V_H and V_λ primers; (6) 120 EBNA2$^+$ cells and 39 buffer controls of the first case were analyzed with V_H primers, (7) 140 EBNA2$^+$ cells, 50 T cells, and 42 buffer controls of the second case were analyzed after preamplification with V_H primers.
[b] For controls, all analyzed samples are considered.
[c] One to three CD20$^+$ B cells or EBER$^-$ cells were transferred into one tube; altogether 39 B cells and 47 EBER$^-$ cells were analyzed.
[d] 126 EBER$^+$ cells, 273 HRS-like cells, 136 buffer controls of case 1 and 71 T cells and 79 buffer controls of case 2 were analyzed for V_λ gene rearrangements (in addition to V_H rearrangements).
[e] Two double sequences are not considered.
[f] One sequence is identical to a clonal V_H3 rearrangement obtained from 22 cells; all other rearrangements from control cells were not clonally related to sequences from HRS-like cells or other EBV-infected cells. One $V_\lambda1$ rearrangement amplified from a T cell is identical to a rearrangement obtained from a CD20$^+$ B cell.
[g] The amplification of fragments of the *EBNA1* gene from 6 of 71 T cells analyzed is compatible with infection of T cells.
HRS-like cells and other EBV-infected cells were analyzed together with T cells, B cells, EBER$^-$ cells, and aliquots of buffer covering the sections during micromanipulation.

Table 3. Sequence Analysis of Ig Gene Rearrangements Amplified from Single EBV-Infected Cells of Two Cases of IM

Case	Cell Type	Number of Cells Positive	Percent of Cells with Mutated V Gene Rearrangements	Percent of Cells Belonging to Clones[a]	Number of Clones with Mutated V Gene Rearrangements	Average Mutation Frequency of Gene Rearrangements[b]	
						V_H	V_λ
1	All cells	203	96	78	20/20	6.3	3.9
	$EBER^+$	64	97	86		7.9	6.0[e]
	$EBNA2^+$	19	100	74		4.8	
	$LMP1^+$	33	91	61		5.5	5.4[e]
	HRS-like	87	95	79		5.7	3.2
2	All cells	67[c]	85	32	8/8[d]	6.1	2.6
	$EBER^+$	26	81	50		5.2	2.2
	$EBNA2^+$	13	92	0[e]		5.7	
	HRS-like	28[c]	85	27		6.8	3.1

[a] The following clones are composed of phenotypically distinct members: clone 2 of case 1: 23 small- to medium-sized EBV-infected cells (12 $CD30^-$ $EBER^+$ cells, ten $EBNA2^+$ cells, one $LMP1^+$ cell) and 22 HRS-like cells (eight $CD30^+$, eight $LMP1^+$ and six $CD30^+$ $EBER^+$ HRS-like cells); clone 3 of case 1: five small- to medium-sized cells (three $EBNA2^+$ cells and two $LMP1^+$ cells) and 11 HRS-like cells (six $CD30^+$, three $LMP1^+$ and two $CD30^+$ $EBER^+$ HRS-like cells); clone 4 of case 1: 1 small $CD30^-$ $EBER^+$ cell and 11 HRS-like cells (seven $CD30^+$ and four $CD30^+$ $EBER^+$ HRS-like cells); clone 5 of case 1: three small- to medium-sized cells (one $CD30^-$ $EBER^+$ cell and two $LMP1^+$ cells) and two HRS-like cells (one $CD30^+$ and one $CD30^+$ $EBER^+$ HRS-like cells); clone 7 of case 1: six small- to medium-sized cells (five $EBER^+$ cells and one $LMP1^+$ cell) and one $CD30^+$ HRS-like cell; clone 20 of case 1: one small $EBNA2^+$ cell and one $CD30^+$ $EBER^+$ HRS-like cell; clone 1 of case 2: four small- to medium-sized $CD30^-$ $EBER^+$ cells and one HRS-like cell; clone 3 of case 2: one small- to medium-sized $CD30^-$ $EBER^+$ cell and two HRS-like cells.

[b] Considering only mutated V gene rearrangements.

[c] From two samples both a mutated and an unmutated in-frame V_H region gene were amplified. This indicated the presence of (fragments of) two B cells in these tubes. These samples were not further taken into consideration.

[d] One "mutated" clone is defined by four members with unmutated V gene rearrangements and two members with two and three mutations each. In addition, for one of the clones classified as "mutated" this assignment is uncertain since the two members of this clone harbor only a single shared nucleotide difference to the most homologous known germline gene, which may represent a polymorphism. Moreover, since this clone is defined by a V_λ gene rearranged without N-sequences to $J_\lambda 6$, it cannot be excluded that these two rearrangements are derived from two clonally independent cells.

[e] These values are considered not to be significantly different from the corresponding values obtained from other cellular subsets due to the low number of sequences.

since no $EBNA2^+CD3^+$ T cells could be identified in any of four cases of IM that were analyzed for cells simultaneously expressing CD3 and EBNA2 (data not shown) and since the amplification efficiency in the present study is largely within the range typical for the amplification of Ig gene rearrangements from single, micromanipulated B cells (Küppers et al., 1993), most EBV-harboring cells and especially EBNA2-expressing cells are likely B cells.

In case 1, sequence analysis of V_H and V_λ gene rearrangements revealed that 96% of the cells carry somatically mutated V region genes, while 4% are characterized by unmutated V gene rearrangements. The average mutation frequency of mutated V_H and V_λ region genes was 6.3% and 3.9%, respectively (Table 3). These values are typical for GC or memory B cells (Goossens et al., 1998; Klein et al., 1998b). The fraction of cells with mutated V genes and the average mutation frequencies were similar for the subtypes of EBV-infected cells (Table 3). Hence, two distinct populations of EBV-infected cells were identified: (1) a small number of cells carrying unmutated V region genes and likely representing naive B cells and/or recent GC immigrants that have not yet acquired somatic mutations and (2) a main population of cells having mutated V gene rearrangements and representing memory and/or GC B cells. Although at the time of their isolation the EBV-bearing cells were not located inside GCs, the possibility that (some of) these cells had been infected inside GCs has to be taken into consideration because tonsillar GCs often vanish during the course of IM.

From case 2, 85% of cells carry somatically mutated V gene rearrangements with average mutation frequencies of 6.1% and 2.6% for mutated V_H and V_λ gene rearrangements, respectively (Table 3). No significant differences with regard to the fraction of mutated cells and the mutation frequencies were observed between the three distinct populations of cells analyzed. Thus, in this case of IM as well, B cells of different stages of development are infected by EBV.

The ratio of replacement to silent mutations (R/S value) in framework regions (FRs) of productive V gene rearrangements can be taken as a sign for selection of the respective B cells for antigen receptor expression. The intrinsic R/S value for random mutagenesis without selection (as in out-of-frame rearrangements) is usually about 3 (Klein et al., 1998a). After selection within GCs, cells are characterized by lower R/S values because R mutations are usually counterselected within FRs. The average R/S value in FRs of the productive V gene rearrangements analyzed here (1.7 in both cases analyzed; clonally related cells counted only once) is in between the R/S values typical for selected memory B cells (1.0–1.6) and the average R/S value of GC centroblasts (1.8), which are not yet fully selected for expression of a high-affinity antigen receptor (Klein et al., 1998a).

Clonal Expansion of EBV-Infected Cells

In the first case, 78% of the cells from which V gene rearrangements were amplified could be assigned to 20 clones with 2–45 members (Table 3; Figure 2A). These clones are characterized by the following features: (1)

members of a given clone are locally restricted to one of the four regions where EBV-infected cells were predominantly found; (2) with the exception of few nucleotide exchanges (two V gene rearrangements with one difference each among approximately 40,000 bp V gene sequence amplified from 172 V genes of clonally related cells), which are likely due to Taq DNA polymerase errors or rare spontaneous somatic mutation, no intraclonal sequence diversity was observed among clonally related rearrangements; and (3) V gene rearrangements of all clones are somatically mutated (average mutation frequency of V_H and V_λ gene rearrangements: 7.2% and 4.6%, respectively). This mutation frequency is comparable to that of the general population of EBV-bearing cells. Consequently, the preferential clonal expansion of memory and/or GC B cells results in an overrepresentation of these cells among EBV-carrying cells (if clonally related cells are counted only once, 86% of the cells belong to the memory and/or GC B cell compartment, as compared to 96% if all cells are counted individually). Taken together, EBV-infected memory and/or GC B cells expand in a locally restricted manner without undergoing (further) somatic hypermutation.

In the second case of IM, eight clones with 2–6 members were identified (Figure 2B). Although EBV-bearing cells were disseminated throughout the tissue in this case, the distribution of clonally related cells was also locally restricted (Figure 2B). In comparison to the first case, a lower fraction of cells (32%) could be assigned to clones (Table 3). Clone 1 consists of four members with unmutated V gene rearrangements and two members with two and three mutations, respectively. Hence, this clone might represent an expanding GC founder B cell that started mutating the V gene rearrangements upon proliferation (Lebecque et al., 1997). Since the other seven clones also have mutated V gene rearrangements, there seems to be a preferential clonal expansion of memory and/or GC B cells in the second case, as well. However, occasional proliferation of naive B cells after infection by EBV in vivo cannot be excluded (see legend to Table 3). In case 2, cells belonging to clones with mutated V gene rearrangements are characterized by an average mutation frequency of 6.3% for their V_H genes, and this frequency is similar to that of the overall population of EBV-infected memory B cells (Table 3; the average mutation load of three mutated V_λ genes is 1.5%). No intraclonal diversity (with the exception of the five nucleotide exchanges in clone 1) was observed among members of clones. If both cases are taken together, most if not all large clones seem to be derived from EBV-bearing memory and/or GC B cells, which proliferate largely without ongoing somatic hypermutation. In addition, members of clones are located in proximity to each other.

Phenotypic Variations within Clones of EBV-Infected B Cells

The identification of clones of EBV-harboring cells in the present study allowed us to address the question of whether clonally related EBV-positive cells can show distinct morphology. Eight clones isolated from the two patients are composed of phenotypically distinct members. For example, clone 2 of the first case is defined by 23 small EBV^+ cells (micromanipulated from sections stained for EBER transcripts, LMP1, or EBNA2) and 22 HRS-like cells (see legend to Table 3 for composition of the clones). In the first case, the number of clones with phenotypically distinct members is most likely underestimated since HRS-like cells and small $CD30^-$, $EBER^+$ cells were mainly micromanipulated from different regions.

Comparison between HRS-like Cells of IM and HRS Cells of Classical HD

In cases of B cell–derived classical HD, HRS cells are a clonal population of mature B cells characterized by mutated V gene rearrangements (average mutation frequency of V_H gene rearrangements: 11.2%), an average R/S value in FRs of productive V gene rearrangements of 1.7, and obviously crippling mutations such as nonsense mutations in potentially functional V gene rearrangements in approximately 30% of the cases (Klein et al., 1998a; Küppers and Rajewsky, 1998). In comparison, the R/S value in FRs of productive rearrangements amplified from HRS-like cells in IM (1.6) resembles that of HRS cells in HD, while the average mutation frequency of mutated V_H gene rearrangements is lower (6.9%; clonally related cells were considered only once here and below). In contrast to HRS cells of HD, HRS-like cells of IM do not represent a single clone, and 14% of these cells carry unmutated V region genes. Furthermore, no crippling mutations were observed among mutated, potentially functional V gene rearrangements amplified from more than 50 HRS-like cells of IM.

Discussion

EBV Infection of Naive as Well as Memory and/or GC B Cells

Although it has been known since 1968 that EBV causes IM (Henle et al., 1968), we still know remarkably little about the cellular target(s) of the virus in vivo. The derivation of EBV-infected cells in IM has been analyzed by immunohistochemistry and in situ hybridization. Most of these studies showed that EBV-harboring cells and especially HRS-like cells are mainly B cells (Isaacson et al., 1992; Reynolds et al., 1995; Niedobitek et al., 1997a). In the two cases of IM investigated here, many EBV-infected cells and HRS-like cells were also identified as B cells.

Sequence analysis of V region genes amplified from EBV-infected B cells shows that cells with unmutated as well as mutated V genes are infected. The latter constitute 86% and 89% of the cells in the two cases investigated (clonally related cells count only once). Cells with unmutated V gene rearrangements likely represent naive, antigen-unexperienced cells or derive from the small population of GC founder cells, which were not yet targeted by somatic hypermutation (Lebecque et al., 1997).

On the basis of the present study, one can speculate about the derivation of EBV-infected cells with mutated V gene rearrangements. According to the scenario proposed by Babcock et al. (1998) for virus persistence resulting from asymptomatic infection, the pool of EBV-infected cells with mutated V gene rearrangements might be formed by infection of naive cells, which are driven

into a GC reaction after infection and EBV-induced proliferation. These cells might then acquire somatic mutations within their V region genes and differentiate to memory B cells (Babcock et al., 1998). If this scenario also holds true for IM, then one would expect to find members of clones that show extensive intraclonal V gene diversity due to somatic hypermutation during proliferation inside the GC. However, among 173 cells belonging to 27 clones, only a single clone shows some intraclonal diversity. Hence (with rare exceptions), EBV-infected B cells proliferate without undergoing somatic hypermutation. Consequently, it is unlikely that in IM the pool of EBV-carrying B cells with mutated V region genes is mainly generated from EBV-infected naive B cells, which subsequently underwent somatic hypermutation inside the GC. However, besides direct infection of memory B cells, EBV may infect GC B cells that have already acquired somatic mutations. It may also cause a shut down of the hypermutation activity in these infected cells. That EBV infection might indeed interfere with the GC reaction is indicated by the finding that LMP1 expression blocked GC formation in a transgenic mouse (Uchida et al., 1999). The possible direct infection of GC B cells is supported by the findings that the average R/S value of EBV-infected cells in IM is in between the average R/S value of selected memory B cells and GC cells and that in IM rare EBV-bearing cells are sometimes observed inside GCs (Anagnostopoulos et al., 1995; Niedobitek et al., 1997a; Araujo et al., 1999; unpublished data). As pointed out above, this possibility is not contradicted by the observation that the EBV-carrying cells analyzed were not located inside GCs since the follicular structures of tonsils are usually disrupted during IM (Niedobitek et al., 1992; Anagnostopoulos et al., 1995). In summary, we think that in IM, unlike in virus persistence resulting from asymptomatic infection (and perhaps distinct from the events that take place during the first days after infection and that result in IM), the pool of EBV-harboring cells with somatically mutated V region genes results from direct infection of memory cells and likely also of GC B cells. Differences in the behavior or load of the virus or in the strength of the EBV-specific T cell response could be the reason for the different pathways of EBV dissemination in IM compared to virus persistence, which becomes also evident by the finding that EBNA2-positive cells of IM are mostly GC and/or memory B cells, whereas only naive IgD^+ B cells express EBNA2 during latency (Babcock et al., 2000).

The Pool of EBV-Infected Cells is to a Great Extent Formed by Proliferation of EBV-Harboring Memory and/or GC B Cells

In both cases of IM analyzed here, clones of EBV-infected B cells were detected. Thus, the pool of EBV-carrying cells is formed not only by infection of a large number of B cells, but also to a great extent by presumably virus-driven proliferation of these cells. That expansion of EBV-carrying cells can be induced by EBV is shown by in vitro studies demonstrating that B cells transformed by the virus give rise to lymphoblastoid cell lines (Diehl et al., 1969; Casali and Notkins, 1989), and it is further indicated by the observation that LMP1 and EBNA2 can induce B cell proliferation (Zimber-Strobl et al., 1996; Kilger et al., 1998).

Whereas all of the 20 clones identified in case 1 and all of the eight clones of case 2 (with one potential exception) are derived from memory or GC B cells, a fraction of EBV-infected cells not assigned to clones likely represents naive B cells. Hence, for unknown reasons, there seems to be a preferential expansion or survival of EBV-carrying memory and/or GC B cells in IM. The latter may be induced by EBV to differentiate into memory B cell–like cells. The overrepresentation of EBV-infected cells of a memory phenotype (which are presumably long-lived) might be an important aspect of the viral strategy to establish a lifelong persistence (Babcock et al., 1998).

In both cases, members of clones are mainly found in proximity to each other. This may indicate that during acute infection proliferating EBV-infected cells have little tendency to migrate, although it is quite possible that members of these clones still migrate out of the tonsil. At least for LMP1-positive cells, the clustering of clonally related cells might be due to LMP1-induced upregulation of adhesion molecules (Wang et al., 1990). The fraction of cells assigned to clones differs between the two cases analyzed. This could be due to the fact that, in case 2, tonsillectomy was performed later in the course of the disease. Thus, since the T cell response was longer lasting in this case compared to case 1, it is possible that the majority of EBV-infected cells has been eliminiated by cytotoxic T cells, so that the pool of EBV-bearing cells is likely mainly formed by newly infected cells. In line with this idea, most EBV-infected cells (approximately 90% of EBER-positive cells) in case 2 express EBNA2 but not LMP1, which is typical for cells early after infection (Alfieri et al., 1991). Alternatively, the two cases may be infected by two distinct EBV variants that differ in their ability to induce B cell proliferation (Rickinson et al., 1987).

Variation in Morphology (and Gene Expression Pattern) among Clonally Related EBV-Infected Cells

We show that morphologically distinct, EBV-harboring cells of IM, namely small- to medium-sized EBV-infected cells and HRS-like cells, do not represent distinct subsets in terms of their cellular derivation, since they can be members of the same clone. Moreover, members of clones most likely also differ in terms of EBV gene expression patterns. This speculation is based on the observation that HRS-like cells mostly show a latency II expression pattern, whereas small- to medium-sized EBV-infected cells are mainly in latency I or III (see above). Switches in EBV gene expression programs among members of clones have also been shown for EBV^+ lymphoblastoid and Burkitt's cell lines in vitro (Kieff, 1996; Szeles et al., 1999). In addition, the phenotypically different subtypes of EBV-infected cells analyzed here are composed of comparable fractions of naive and GC and/or memory B cells (Table 3). Thus, a particular morphology and/or latency program of EBV-encoded genes is not restricted to a special B cell differentiation stage.

Distinct Origin of HRS-Like Cells of IM and HRS Cells of HD

HRS cells in classical HD generally carry mutated V gene rearrangements and may survive without antigen

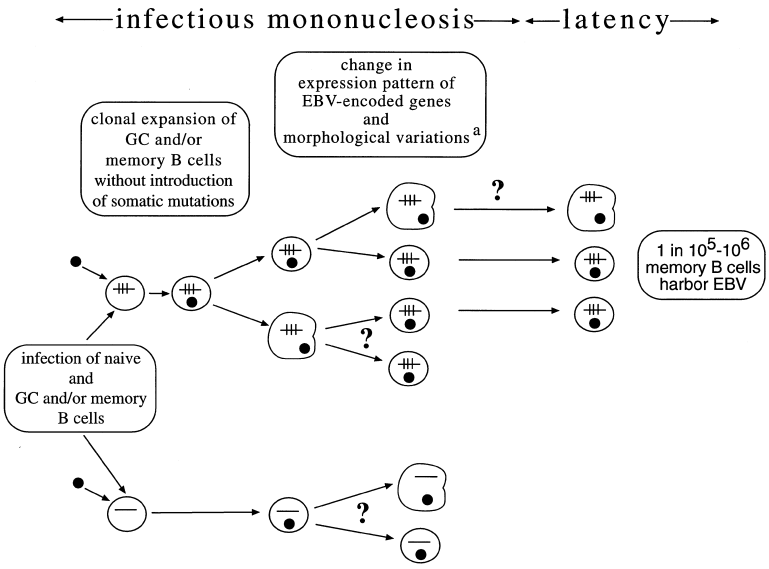

Figure 3. Scenario for the Generation of the Pool of EBV-Infected Cells during IM and the Establishment of a Lifelong Infection

Closed circles represent the EBV genome, and open circles represent cells. The horizontal lines indicate a V gene rearrangement; vertical lines within the circles indicate somatic mutations. Although naive as well as memory and/or GC B cells are infected, expansion to large clones seems to be restricted to the (post) GC B cell compartment. It is unclear whether naive B cells can expand to clones (implied by a question mark). The relative distribution of naive and (post) GC B cells with HRS-like morphology (indicated as cells with irregular cell shape) shown in this scheme does not mirror the situation found in IM. Question marks indicate that it is unclear whether HRS-like cells can persist after IM or change their phenotype to small EBV-infected cells. The possibility that HRS-like cells might persist is implied by a patient who presented with a B cell chronic lymphocytic leukemia (B-CLL) and a clonal population of intermingled HRS-like cells 2 years after diagnosis of IM (Kanzler et al., 2000). Since the HRS-like cells were clonally unrelated to the B-CLL, the HRS clone might originate from an HRS-like cell of IM. Moreover, it is uncertain whether HRS-like cells can proliferate. Some EBV-harboring memory B cells may survive after IM and build the initial pool of EBV-infected memory B cells of latent infection (Babcock et al., 1998). The lowercase "a" indicates that members of clones can show distinct morphology, namely small- to medium-sized EBV-infected cells and HRS-like cells.

receptor expression, as indicated by the findings of downregulation of immunoglobulin transcription and "crippling" mutations such as nonsense mutations or deletions resulting in loss of the correct reading frame in approximately 30% of cases (Kanzler et al., 1996; Marafioti et al., 2000). In IM no obviously crippling mutations were found in about 50 mutated in-frame V gene rearrangements of HRS-like cells. Although it is possible that HRS-like cells of IM may give rise to an HRS cell of HD, perhaps after reentering a GC and acquiring unfavorable mutations, the present study shows that HRS-like morphology is not directly correlated with the derivation from a "crippled" GC B cell. Cells with HRS-like morphology must not have even been targeted by somatic hypermutation since cells with unmutated V genes were also found among HRS-like cells. Thus, it is still unclear what determines HRS cell morphology.

The EBV Strategy during IM and in the Establishment of a Lifelong Infection: A Scenario

During persistence, EBV resides in the memory B cell compartment in the peripheral blood (Babcock et al., 1998; Ehlin-Henriksson et al., 1999). In tonsils, however, IgD^+ B cells are also infected (Babcock et al., 1998). Although it is not clear whether the EBV-infected IgD^+ cells are indeed naive cells given that a fraction of IgD^+ B cells represents memory B cells (Klein et al., 1998b), the following scenario has been proposed: EBV infects naive IgD^+ B cells in the tonsils and induces the newly infected cells to proliferate like lymphoblastoid cells in vitro. After downregulation of EBNA2, some of these naive EBV-bearing cells enter a GC reaction and later differentiate into memory B cells so that EBV gains access to the memory B cell pool (Babcock et al., 2000).

Based on the present study, for the acute primary EBV infection an alternative scenario emerges for the strategy EBV has evolved to quickly and efficiently create a large pool of EBV-positive B cells (Figure 3): EBV forms a large pool of virus-harboring cells by infection of large numbers of naive as well as memory and GC B cells (and perhaps other cells, such as T cells). The population of EBV-carrying cells is further increased by virus-driven B cell proliferation, which is largely restricted to GC and/or memory B cells. Alternatively, it is possible that EBV-bearing naive as well as GC and/or memory B cells proliferate and that naive EBV-harboring B cells are preferentially eliminated by cytotoxic T cells, which would result in the overrepresentation of GC and/or memory B cells among expanding EBV-positive cells. In the course of this clonal expansion, EBV-carrying cells can change their morphology and the expression pattern of EBV-encoded genes. B cells that become resting and downregulate most EBV-encoded, immunogenic proteins can evade immune surveillance. Due to the overrepesentation of memory and/or GC B cells among EBV-bearing B cells (the latter cells may be induced by EBV to differentiate into resting memory B cell–like cells) and to the longevity of memory B cells, it is likely that some EBV-infected memory B cells survive after IM and establish the initial pool of EBV-harboring cells of persistence. The same scenario might also hold true for asymptomatic primary infection, which may differ from IM simply by the number of cells involved.

Experimental Procedures

Tissue and Clinical Data

Tonsillectomies were perfomed in all six cases analyzed because the patients developed IM complications. Patient 1 was a 15-year-old female with a 3 day history of IM. EBV serology confirmed the diagnosis of IM (anti-VCA IgM and IgG). Patient 2 was a 15-year-old female who presented with a more than 14 day history of tonsilitis. The diagnosis of IM was verified by EBV serology (anti-VCA IgM and IgG). The four additional cases of IM were analyzed by immunohistochemistry and in situ hybridization. Two patients, one

18-year-old female and one 16-year-old male, had been symptomatic about 3 days before surgery, and two males, 33 and 17-years old, had been symptomatic 14 and 17 days before tonsillectomy, respectively. The diagnosis of IM was also confirmed by serology in these four cases.

Immunostaining, In Situ Hybridization, and Micromanipulation

Immunostainings were performed on 7–10 μm thick frozen or paraffin sections as described before (Küppers et al., 1997) by using antibodies against CD30, LMP1, CD20 (BerH2, CS1–4, and L26: DAKO, Hamburg, Germany), EBNA2 (Biotest, Dreieich, Germany), CD3 (OKT3: Ortho Diagnostic Systems, Raritan, NJ; or polyclonal rabbit anti-human CD3 antibody: DAKO) and T cell receptor β chain (βF1: T cell Science, Cambridge, MA). Secondary antibodies were biotinylated and visualized by avidin – coupled either to alkaline phosphatase or to horseradish peroxidase (DAKO). Fast red (DAKO) was used as a substrate for alkaline phosphatase, and DAB (Sigma-Aldrich, Deisenhofen, Germany) served as a substrate for horseradish peroxidase.

For simultaneous staining of either LMP1 and EBNA2 or CD3 and EBNA2 the fluorescent staining technique was applied by using FITC-conjugated goat anti-rat IgG antibodies (Santa Cruz Biotechnology, CA) for the detection of EBNA2$^+$ cells and Alexa Fluor-coupled goat anti-mouse IgG antibodies (Molecular Probes, OR) or Cy3-conjugated goat anti-rabbit IgG antibodies (Dianova, Hamburg, Germany) for the detection of LMP1$^+$ cells or CD3$^+$ cells, respectively. The LMP1-specific stainings were performed by using two different anti-LMP1 antibodies (CS1–4, DAKO and S12, kindly supplied by D. A. Thorley-Lawson) and gave comparable results.

To detect EBV-infected cells, in situ hybridization was performed with in vitro transcribed digoxygenin-labeled probes specific for EBV-encoded small nuclear RNAs (EBER 1 and 2) (Niedobitek et al., 1991). In situ hybridization was performed on paraffin sections as previously described (Kanzler et al., 2000). When in situ hybridization was carried out on frozen sections, the following modifications were introduced: before fixation in 4% paraformaldehyde and dehydration, sections were heated to 91°C–95°C for 3 min, fixed in 4% paraformaldehyde and treated with 0.5 μg/ml of Pronase (Boehringer Mannheim, Mannheim, Germany). BCIP/NBT, BCIP/NBT/INT, or fast red (DAKO) served as staining substrates.

To distinguish HRS-like cells from other EBV-infected cells, a combination of CD30 immunostaining and EBER in situ hybridization was carried out in some experiments. Frozen sections were fixed for 3 min at 91°C–95°C followed by 12–15 hr incubation with 4% paraformaldehyde before CD30 immunostaining was performed as described (Küppers et al., 1997). To avoid unspecific staining and degradation of EBER transcripts, 100 μg/section of yeast tRNA (Boehringer Mannheim) and 16 U/section of RNAsin (Promega, Madison, WI) were added to solutions. DAB was used as a substrate for horseradish peroxidase. The CD30-stained sections were fixed for 4 hr in 4% paraformaldehyde, and the EBER in situ hybridization was continued as described above.

With the exception of EBNA2-expressing cells that were isolated by a combination of microdissection with the PALM laser (P.A.L.M. Mikrolaser Technology, Bernried, Germany) and micromanipulation, all single cells were micromanipulated from stained sections as described previously (Küppers et al., 1993), transferred into 20 μl of 1× Expand High Fidelity PCR buffer (Boehringer Mannheim) with 1 ng/μl of 5S rRNA (Boehringer Mannheim), and stored at −20°C. As a control for the PCR, single CD3$^+$ T cells, EBER$^-$ cells, and CD20$^+$ B cells that had been isolated either from the same sections used to micromanipulate EBV-infected cells or from adjacent sections were used, as well as aliquots of buffer covering the section during micromanipulation.

Primer Extension Preamplification

The whole genomic DNA of some micromanipulated single cells and controls was amplified following the PEP protocol with a random 15-mer oligonucleotide (Zhang et al., 1992; Kanzler et al., 2000) in order to analyze these cells with different combinations of primers and to preserve DNA for future analysis.

Single-Cell PCR and Sequence Analysis

V_H and V_λ gene rearrangements were amplified by a seminested PCR approach using family-specific V gene primers together with two sets of the respective J gene primers as described, with the following modifications: (1) concentration of each primer in the first round is 13.6 or 15 nM, (2) in the second round the annealing temperature is 61°C for all V_H gene families (Kanzler et al., 1996; Braeuninger et al., 1997; Küppers et al., 1997; Bräuninger et al., 1999; Kanzler et al., 2000). Rearranged V_κ genes were not amplified since in λ-expressing cells (nonfunctional) V_κ gene rearrangements are usually inactivated and consequently unmutated (Klein et al., 1998a). Therefore, the amplification of V_κ genes is less suitable for the analysis of a population of cells in regard to the presence of somatic mutations in rearranged V genes and thus a GC derivation. For some cells a fragment of the EBV-encoded *EBNA1* gene was amplified in parallel. Thereto, two EBNA-specific primers (EBNA1F: 5′-GGT CGC CGG TGT GTT CGT ATA TGG-3′, EBNA1R1: 5′-GCG GCA GCC CCT TCC ACC ATA G-3′) were added to the primer mix of the first round. The second round for the EBNA1 fragment was carried out in a separate reaction with primers EBNA1F and EBNA1R2 (EBNA1R2: 5′-AGG GAG GCA AAT CTA CTC CAT CGT C-3′). The PCR program for the *EBNA1* fragment differed from the second round of the V_H PCR only by the number of cycles (35 cycles).

The analysis of some CD30$^+$ HRS-like cells of the second case was performed using family-specific V_H leader (L) (Braeuninger et al., 1997), V_λ, V_κ, and EBNA1 primers in the first round. In the second round, V_κ gene rearrangements were not analyzed. The PCR was carried out as described for a combination of V_HL, V_κ, 3′ J_H, and 3′ J_κ primers (Kanzler et al., 2000) with the following changes: (1) addition of V_λ, 3′ J_κ, EBNA1F, and EBNA1R1 primers, (2) primer concentration of 50 nM, and (3) 2 mM $MgCl_2$ for V_HL4.

If a PEP reaction was performed (see above), the following modifications were introduced to the protocol of the first round: (1) the gene-specific PCRs were carried out with 4 μl aliquots of these PEP reactions, (2) the reaction started with 10 min denaturation at 95°C, (3) separate first rounds for V_H/EBNA1 and V_λ were carried out, and (4) 1× Promega PCR buffer and 2.5 U Promega Taq DNA polymerase in storage buffer A (Promega) were used.

PCR products were gel purified and sequenced on an ABI 377 sequencer (PE Applied Biosystems, Weiterstadt, Germany). Sequence analysis was performed using DNASIS software (Pharmacia, Freiburg, Germany) and the V-BASE database (http://www.mrc-cpe.cam.ac.uk/imt-doc/restricted/DNAPLOT.html).

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EMBL Accession Numbers

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