Early Stage of Epstein-Barr Virus Lytic Infection Leading to the "Starry Sky" Pattern Formation in Endemic Burkitt Lymphoma

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• ***Context.*—Burkitt lymphoma (BL) is histologically characterized by a "starry sky" appearance, representing scattered macrophages that have phagocytosed cell debris among proliferating lymphoma cells. As is well known, almost all the neoplastic cells of endemic BL are infected with Epstein-Barr virus (EBV). Previous studies have indicated that most of the EBV in B cells is latent, and few virus particles enter the lytic cycle.**

***Objective.*—To examine the histologic relationship between EBV infection stages and the formation of the starry sky pattern in African endemic BL tissues.**

***Design.*—Tissue samples from 44 patients with African endemic BL were examined with immunohistochemistry and in situ hybridization. We used EBV-encoded small RNA (EBER) as a marker of latent infection, and *Bam*HI H left frame 1 (BHLF1) and *Bam*HI Z EBV replication activator (ZEBRA) as lytic cycle markers.**

***Results.*—In all cases, signals for EBER were found in most neoplastic lymphocytes, and in 73% of cases, signals for BHLF1 and/or ZEBRA were recognized in the lymphoma cells within and around the lacunae in starry sky figures. The mean number of lacunae per unit area in cases positive for lytic cycle markers was significantly higher than that in negative cases ($P < .001$).**

***Conclusions.*—Our findings suggest that EBV-infected lymphoma cells in the lytic cycle, which eventually lapse into cell death, are phagocytosed prior to their rupture by macrophages that have migrated into the parenchyma. We emphasize that transition of EBV-infected lymphoma cells to the lytic cycle is one of the histomorphogenetic factors influencing the formation of starry sky pattern in endemic BL.**

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Burkitt lymphoma (BL) is one of the non-Hodgkin malignant lymphomas that often affects extranodal regions.[1,2] Burkitt lymphoma is a high-grade B-cell lymphoma, and the tumor consists of medium-sized cells with basophilic cytoplasm and numerous mitotic figures. It is well known that BL is histologically characterized by a "starry sky" appearance, which is due to the scattering of macrophages that appear within lacunae surrounded by proliferating lymphoma cells. Burkitt lymphoma is clinically divided into endemic BL, sporadic BL, and immunodeficiency-associated BL.[2,3] Endemic BLs consist of Epstein-Barr virus (EBV)–infected B lymphocytes.[4,5] All BLs, whether associated with EBV or not, show chromosomal translocation between the c-*myc* gene and the immunoglobulin gene loci, resulting in constitutive expression of c-Myc.[6–8] The c-*myc* is an oncogene that encodes a transcription factor and drives cell proliferation in multiple human cancers. Thus, this translocation is pivotal in BL development. It is hypothesized that EBV infection has an important role in the translocation process.[6–8] Moreover, holoendemic malaria is considered to facilitate EBV infection by reducing EBV-specific T-cell immunity. Therefore, malaria is thought to be an indirect condition leading to EBV infection–mediated constitutive c-*myc* deregulation in endemic regions.[9–11]

In most EBV-infected B lymphocytes the virus remains latent, whereas some virus enters the lytic cycle to produce EBV virions.[12] Although these 2 infection stages have been demonstrated in established cell lines derived from BLs,[13–15] these lines have not been studied to determine the relationship between the infection stages in the BL tissues and the development of their starry sky pattern.

In this study, we examined the histologic relationship between change in the infection phase and development of the starry sky pattern in African endemic BL tissues. To distinguish latent and lytic phases, we used EBV-encoded small RNAs (EBERs) as a marker of latent infection, and *Bam*HI H left frame 1 (BHLF1) and *Bam*HI Z EBV replication activator (ZEBRA) as lytic cycle markers. Ep-

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Table 1. Clinical Data for the Patients With Burkitt Lymphoma

Tumor Site	Sex			Total
	Male	Female	Not Recorded	
Jaw	8	5	0	**13**
Ovary	0	7	0	**7**
Abdomen	4	1	0	**5**
Lymph node	4	1	0	**5**
Orbit	2	1	0	**3**
Neck	1	0	0	**1**
Upper arm	0	1	0	**1**
Colon	0	1	0	**1**
Not recorded	2	4	2	**8**
Total	**21**	**21**	**2**	**44**

stein-Barr virus–encoded small RNAs are the most abundant EBV RNAs located in the cell nucleus and cytoplasm of latently infected cells.[12] Lytic cycle–associated genes are divided into immediate early, early, and late genes.[12,16] The antigens encoded by these genes are transcription factors, enzymes for gene replication, and capsid and envelope proteins. Among the lytic cycle–associated genes, *BHLF1* is one of the early genes.[17,18] ZEBRA is a 33-kd transcription-transactivating nuclear protein encoded by *Bam*HI Z left frame 1 (*BZLF1*), which is one of the immediate early genes of EBV. ZEBRA up-regulates its own promoter, as well as promoters for other immediate-early genes, and converts EBV infection from the latent to lytic state.[19–21] Thus, both lytic cycle markers are considered to be expressed in the initial stage of the lytic cycle, before the viral particles have been dispersed.

MATERIALS AND METHODS

Tissues

We studied 44 cases of African endemic BL that were retrieved from the files of one of the authors (K.T.). Various hospitals in the western part of Kenya (Western, Nyanza, and Rift Valley provinces; 1980–1995) submitted biopsy and surgical specimens for pathologic diagnosis to the Department of Histopathology, Rift Valley Provincial General Hospital, Nakuru, Kenya. The age range of the patients was 1.5 to 19 years (mean age, 6.4 years), and the male-female ratio was 1:1. Table 1 summarizes the clinical data for the 44 BL patients. This was a retrospective study, and the specimens used were archived specimens. This study was authorized by the government of Kenya (research permit OP.13/001/8C224/36; K.T.)

The specimens were fixed in 10% formalin saline solution, embedded in paraffin, sectioned, stained with hematoxylin-eosin and periodic acid–Schiff, and silver impregnated. Paraffin sections from specimen blocks were also prepared for in situ hybridization and immunohistochemistry.

In Situ Hybridization

To discriminate between the tumor cells in latent and lytic stage infections, we used 2 peptide nucleic acid probes for in situ hybridization: EBER probe (Dako Japan, Kyoto) and BHLF1 probe (Dako Japan). The in situ hybridization was carried out using the peptide nucleic acid ISH detection kit (Dako Japan). Deparaffinized sections were pretreated with proteinase K and hybridized with fluorescein isothiocyanate–labeled probes in a humid chamber for 90 minutes at 55°C. After washing with stringent wash solution at 55°C, the sections were incubated with alkaline phosphatase–conjugated anti–fluorescein isothiocyanate antibody. Positive signals were visualized using 5-bromo-4-chloro-3-indolyl-phosphate-*p*-toluidine salt/nitroblue tetrazolium (BCIP/NBT) substrate. The nuclei were counterstained with hematoxylin, and the sections were mounted with aqueous medium.

Immunohistochemistry

Another lytic cycle marker, ZEBRA, was stained with specific antibody (Dako Japan; monoclonal, clone BZ.1, dilution 1:20). The immunohistochemical assay was performed using the avidin-biotin-complex (ABC) method after autoclave pretreatment of sections immersed in citrate buffer. The chromogen 3,3′-diaminobenzidine (Sigma-Aldrich Japan, Tokyo) was used to reveal peroxidase. After the sections were counterstained with hematoxylin, they were dehydrated and mounted in a synthesized medium.

Evaluation of Starry Sky Pattern Relating to Lytic Infection

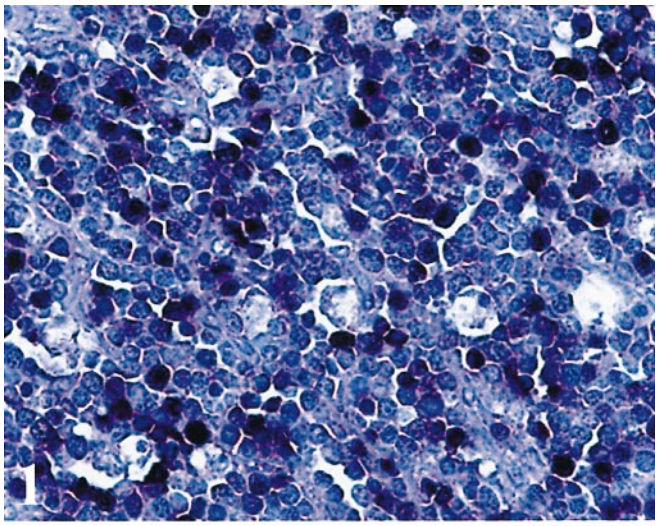
The relationship between starry sky appearance and neoplastic lymphocytes in the lytic cycle was assessed by comparing the number of lacunae per unit area (area density) in sections with and without lytic infection markers. Five nonoverlapping square fields were viewed at ×400 magnification, and area density was measured with the aid of a tetragonal lattice ocular micrometer. The length of a side of the view was 175 μm at this magnification. The mean numbers of lacunae per field in sections with and without lytic markers were compared using a Student *t* test.

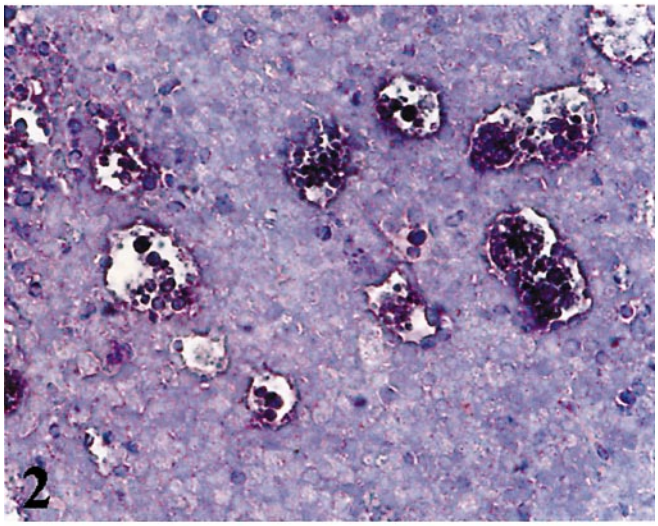
RESULTS

A strong nuclear and cytoplasmic reaction for EBER probe was present in all sections, representing all 44 cases of endemic BL. The EBER positivity of latently infected neoplastic cells was diffuse in the tumor and had no positional relationship to the starry sky figures (Figure 1). Only a few latently infected cells were detected within the lacunae of the starry sky pattern. Twenty-one (48%) of 44 cases showed reactivity for BHLF1. Interestingly, the positive cells and associated debris were mainly located within lacunae, indicating that most lytically infected cells were phagocytosed by the infiltrated macrophages. The additional finding of reactive cells around the lacunae suggested that macrophages were moving toward the lytically infected cells (Figure 2). Although a few cells positive for ZEBRA were scattered throughout the parenchyma, a large majority of the nuclear-positive lymphoma cells had accumulated in the lacunae, similar to the distribution of BHLF1-positive cells. Twenty-one (48%) of 44 specimens were ZEBRA positive (Figure 3).

Almost all lacunae included macrophages and degenerated cells with shrunken or condensed nuclei. Thus, it is possible that necrotic debris in the lacunae nonspecifically reacted with BHLF1 and ZEBRA. However, we believe that our findings were not the result of nonspecific reaction to BHLF1 and ZEBRA, because noticeable, broad necrotic areas of BL tissues demonstrated no reactivity with both lytic markers.

The mean lacunar area densities for specimens staining $BHLF1^+/ZEBRA^+$, $BHLF1^+/ZEBRA^-$, $BHLF1^-/ZEBRA^+$, and $BHLF1^-/ZEBRA^-$ were 20.6, 21.5, 21.4, and 13.3, respectively (Table 2). The mean area density of $BHLF1^-/ZEBRA^-$ specimens was significantly lower than that of the others ($P = .02$ for $BHLF1^-/ZEBRA^-$ vs $BHLF1^+/ZEBRA^+$ or $BHLF1^+/ZEBRA^-$; $P < .001$ for $BHLF1^-/ZEBRA^-$ vs $BHLF1^-/ZEBRA^+$). The mean lacunar area density in $BHLF1^+$ and/or $ZEBRA^+$ cases was 21.2, which was also significantly different from that of the $BHLF1^-/ZEBRA^-$ staining group ($P < .001$).





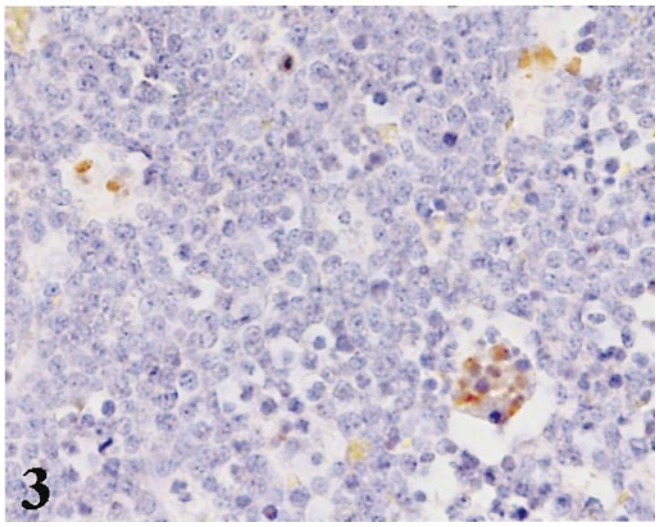


Figure 1. *In situ hybridization for Epstein-Barr virus–encoded small RNA (EBER). Cells expressing nuclear positivity are diffusely present and are distributed independent of macrophages in the lacunae (original magnification ×40).*

Figure 2. *In situ hybridization for* Bam*HI H left frame 1 (BHLF1). Positive signals are found in the cells located within and just around the lacunae (original magnification ×40).*

Figure 3. *Immunohistochemistry for* Bam*HI Z Epstein-Barr virus replication activator (ZEBRA). Reactive cells are phagocytosed by macrophages in the lacunae. This distribution is like that for* Bam*HI H left frame 1 (BHLF1) determined by in situ hybridization (original magnification ×40).*

Table 2. Expression of Latent and Lytic Infection Markers and Their Correlation With Area Densities of Lacunae in Specimens With "Starry Sky" Appearance*

No. of Patients	EBER Expression	BHLF1 Expression	ZEBRA Expression	Mean Area Density of Lacunae†
10	+	+	+	20.6
11	+	+	−	21.5
11	+	−	+	21.4
12	+	−	−	13.3

* EBER indicates Epstein-Barr virus–encoded small RNA; BHLF1, *Bam*HI H left frame 1; and ZEBRA, *Bam*HI Z Epstein-Barr virus replication activator.

† The numbers of lacunae per unit area in BHLF1$^-$/ZEBRA$^-$ cases were significantly lower than in other groups ($P = .02$ for BHLF1$^+$/ZEBRA$^+$ and BHLF1$^+$/ZEBRA$^-$; $P < .001$ for BHLF1$^-$/ZEBRA$^+$).

COMMENT

In 1964, Tony Epstein and Yvonne Barr discovered an unknown herpesvirus infecting cultured cells from African endemic BL,[22,23] and the virus was subsequently named EBV. Thereafter, it became apparent that EBV was associated with other disorders as well, for example, nasopharyngeal carcinoma, Hodgkin disease, and infectious mononucleosis.[3,24] In endemic BL, chromosomal translocation by EBV is considered to play an important role in deregulating c-*myc*.[6,7] Starry sky appearance is a well-known histologic feature of BL; however, to the best of our knowledge, no previous reports have described how the starry sky pattern is formed in endemic BL.

In the present study, we showed that lymphoma cells latently infected with EBV were abundant in all cases of African endemic BL, and their distribution was not associated with that of the lacunae. By contrast, EBV-infected lymphoma cells in the lytic cycle accumulated within or around the lacunae, suggesting that infiltrated macrophages phagocytose lytic EBV-infected lymphoma cells by an active and selective mechanism prior to their rupture. It is known that virus-infected cells undergoing apoptosis are efficiently recognized and ingested by macrophages.[25] However, EBV lytic antigens protect lymphoma cells in the lytic stage from apoptosis,[26] suggesting that EBV-infected BL cells in the lytic cycle may be phagocytosed by a unique mechanism.

The starry sky pattern develops as a result of infiltration of macrophages into the EBV-infected BL parenchyma. The infiltration may be due to macrophage-attracting chemokines (such as macrophage inflammatory protein 1 [MIP-1], monocyte chemotactic peptide 1 [MCP-1], monokine induced by interferon-gamma [Mig], and interferon-alpha–inducible protein 10 [IP-10]) secreted by EBV-infected tumor cells.[27,28] Anchoring of infiltrated macrophages is also a possible histogenetic factor of the starry sky pattern. That macrophage migration inhibitory factor might induce macrophage anchoring is suggested by the fact that expression of migration inhibitory factor is upregulated in glioblastoma multiforme by hypoxia and hy-

poglycemia, a scenario that is also possible in lymphoma tissue.[29] Starry sky patterns also are observed in some kinds of lymphoma that are not associated with EBV, for example, sporadic BL, immunodeficiency-associated BL, and precursor T- and B-cell lymphoblastic leukemia/lymphoma. Focal hypoxia and/or hyponutrition due to rapid tumor proliferation may stop macrophage migration and form starry sky patterns in these lymphomas.

We also observed starry sky patterns in lytic cycle–negative cases, and the mean lacunar area density in lytic cycle marker–positive cases was higher than that in negative cases. These results suggested that lytically infected cells additionally participate in the formation of the starry sky pattern. As mentioned, the cells under the aggravated microenvironment in rapidly proliferating BL induce the formation of the starry sky pattern by means of cytokines. In addition, transition of EBV to the lytic cycle may accelerate the formation of the starry sky pattern. Macrophages in the starry sky pattern may be engaged in clearing lymphoma cells lytically infected with EBV.

Niedobitek et al[30] investigated immunoexpression patterns of not only EBV-latent proteins, but also ZEBRA in endemic BL. They mentioned that isolated scattered tumor cells of endemic BL were positive for ZEBRA. In addition to their findings, we observed that the distribution of ZEBRA-positive cells was related to the lacunae of the starry sky pattern. Moreover, in a previous study of fine-needle aspirate smears of African BL, good response to chemotherapy was demonstrated among cases expressing BHLF1 and/or ZEBRA.[16] However, the authors did not mention the contribution of lytic infection markers in developing the starry sky pattern, because artifacts of smear preparation were thought to alter or obscure the starry sky pattern in this study. Our study, which used paraffin-embedded specimens, demonstrated that the lytic infection marker–positive cases had high densities of lacunae. The simple measurement of number of lacunae per unit area, using routinely stained slides of sectioned endemic BL tissue, might be useful in patient management.

In conclusion, transition of EBV-infected lymphoma cells to the lytic phase is an additional histomorphogenetic factor of starry sky pattern that might prevent EBV from spreading outside infected lymphoma cells.

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