

Expression of the Epstein-Barr virus in lymphoproliferative diseases of the lung

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Abstract. There have been few studies regarding the etiology of lymphoproliferative disorders of the lung. To examine the possible involvement of the Epstein-Barr virus (EBV) in these diseases, EBV mRNAs, proteins and DNA, were detected. Two non-Hodgkin's lymphomas (NHL) originating in the lung, 5 mucosal-associated lymphoid tissue lymphomas (MALT lymphoma) of the lung, 1 lymphoid hyperplasia of the lung and 1 lymphoid interstitial pneumonia (LIP), were subjected to mRNA *in situ* hybridization, indirect immunofluorescence staining and PCR. mRNA *in situ* hybridization using *Bam*HIW, *Bam*HIY1Y2, the Epstein-Barr virus nuclear antigen (EBNA) and the EBV encoded small non-polyadenylated RNA (EBER1) probe revealed signals in all the examined samples, although some samples showed weak signals by using the EBER1 probe. Indirect immunofluorescence staining using the anti-leader protein, anti-EBNA2, the anti-latent member protein-1 and anti-*Bam*HIZ coding

leftward reading frame-1 antibodies showed definitive fluorescence. PCR also revealed EBV DNA in these specimens. EBV infected all the lymphoproliferative diseases of the lung irrespective of the histological or clinical stages. Furthermore, EBV infected not only the atypical lymphocytes but also the macrophages in the tissues of these diseases. These results suggest that the expression of EBV could be involved in the pathogenesis of many lymphoproliferative diseases of the lung.

Introduction

Lymphoproliferative disorders of the lung contain a wide spectrum of diseases, from lymphoid interstitial pneumonia (LIP) to malignant lymphoma such as non-Hodgkin's lymphoma (NHL). Among the patients with LIP or lymphoid hyperplasia of the lung, there are some who suffer from a recurrence of these diseases and many years later, develop NHL. Approximately 5% of the LIP cases transform to lymphoma (1). Although the mechanism that triggers this progression remains unclear, we suspect that recurrent or long-term infection could be correlated with progression.

The Epstein-Barr virus (EBV) is a ubiquitous virus that infects both adults and adolescents throughout the world. It is a well-established causative agent of infectious mononucleosis, and is associated with endemic Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and diffuse large B-cell lymphoma in immunosuppressed hosts (2). We have shown that EBV is related to many other human cancers as well (3-12). In thyroid carcinoma, EBV correlates with the tumor progression of papillary to undifferentiated carcinoma (11). The transforming genes of EBV are suspected to be in the regions of the EBV-determined nuclear antigen-2 (EBNA2) (13,14), and the latent membrane protein-1 (LMP1) (15). EBNA-coding sequences commonly share the *Bam*HIW region which is repeated ~10-fold in an EBV genome, and the *Bam*HIY1Y2 region. EBV encoded small non-polyadenylated RNAs (EBER1) are abundantly present

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Abbreviations: EBV, Epstein-Barr virus; PCR, polymerase chain reaction; EBNA LP, Epstein-Barr virus nuclear antigen-leader protein; EBNA2, Epstein-Barr virus nuclear antigen-2; LMP1, latent membrane protein-1; EBER1, EBV encoded small non-polyadenylated RNAs; BZLF1, *Bam*HIZ coding leftward reading frame-1; NHL, non-Hodgkin's lymphoma; MALT lymphoma, mucosal-associated lymphoid tissue lymphoma; LIP, lymphoid interstitial pneumonia

Key words: *in situ* hybridization, lung lymphoma, mucosal-associated lymphoid tissue lymphoma, lymphoid interstitial pneumonia

in latently EBV-infected cells (16). The *Bam*HIZ coding leftward reading frame-1 (BZLF1) protein initiates the switch from latent to lytic infection (17).

In order to examine the relationship between EBV and lymphoproliferative diseases of the lung, we performed mRNA *in situ* hybridization using 4 different EBV probes and indirect immunofluorescence staining using 4 different monoclonal antibodies against EBV. The *Bam*HIW and EBNA2 probes, and anti-EBNA2 and anti-LMP1 antibodies were selected to detect the transformation by EBV. Furthermore, the *Bam*HIY1Y2 probe and the anti-leader protein (LP) antibody could also be associated with EBV transformation (18).

EBV expression was detected in all the lymphoproliferative diseases of the lung. The EBV DNA was also confirmed by PCR. The results presented herein indicate that the mRNAs and proteins of EBV are expressed in lymphoproliferative diseases of the lung, suggesting that EBV could be a causative agent of these disorders.

Materials and methods

Patients. Formalin-fixed paraffin-embedded tissue samples from 2 patients with NHL, 5 patients with mucosal-associated lymphoid tissue lymphoma (MALT lymphoma), 1 patient with lymphoid hyperplasia and 1 patient with LIP, were selected from the 1994 to 2002 files at Osaka Medical Center for Cancer and Cardiovascular Diseases. All were B-cell type lymphoproliferations of the lung. For the negative controls, 8 samples of normal edge of lung cancers were examined. All were Japanese. None of them had features indicative of an immunocompromised state. Informed consent was obtained from each patient.

Probes. *Bam*HIW probes were transcribed from 2.27 kb EBV *Bam*HIW fragments from which the 'Alu-family'-like sequence had been deleted. The *Bam*HIW fragment of EBV is a highly repetitive sequence that contains the mRNA leader sequence for EBNA2 (19). The fragment was cloned into the pBluescript II SK⁺ vector. The cDNA of the *Bam*HIY1Y2 region was also cloned into pBluescript II SK⁺, and the size of this cDNA was 153 bp. The antisense and sense probes were labeled with digoxigenin-11-UTP by *in vitro* transcription with T3 and T7 polymerases, respectively, using a commercial kit (Boehringer Mannheim, Mannheim, Germany). EBNA2 cDNA (14802-48583 including a spliced sequence) and EBER1 cDNA (6629-6795) were synthesized with RT-PCR by the method reported by Tierney *et al.* (20), and cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The sizes of these cDNAs were 386 and 167 bps, respectively. The antisense and sense RNA probes were labeled with digoxigenin-11-UTP by *in vitro* transcription with SP6 and T7 polymerases using a commercial kit (Boehringer Mannheim). The labeled *Bam*HIW and EBNA2 riboprobes were then fragmented to about 100 bases in length by alkaline hydrolysis. The sense probe served as the negative control.

Messenger RNA *in situ* hybridization. Paraffin sections were prepared from formalin-fixed tissues from biopsied or surgically resected materials. Serial sections, of 3-5 μ m, were cut. After dewaxing and dehydration with graded ethanol, the

slides were treated with 0.2N HCl for 15 min at room temperature (RT) and rinsed with phosphate-buffered saline (PBS) for 5 min at RT. Then they were treated with 50 μ g/ml proteinase K in PBS for 15 min at 37°C, and immersed in 2 mg/ml glycine in PBS for 10 min at RT. They were then refixed with 4% paraformaldehyde in PBS for 15 min at RT and washed twice with PBS for 3 min at RT. Then they were treated with 0.1 M triethanolamine (pH 8.0) for 10 min at RT. After washing with PBS, the sections were dehydrated with ethanol and hybridized for 40 h at 37°C for EBNA2 and EBER1, 39°C for EBNA LP and at 45°C for *Bam*HIW in 4X SSC, 50% formamide, 1X Denhardt's solution, 5% dextran sulfate, 0.5 mg/ml salmon sperm DNA, 0.5 mg/ml yeast tRNA and 10 mM dithiothreitol. After hybridization, the sections were washed twice with 2X SSC for 30 min and then twice with 0.5X SSC for 20 min with gentle shaking at RT. They were then blocked with 1% skim milk (Difco) in 100 mM Tris, 0.15 M NaCl (pH 7.5) for 30 min at RT. Next, they were reacted with 1:100 diluted (for EBNA2) and 1:200 diluted (for *Bam*HIW, *Bam*HIY1Y2 and EBER1) alkaline phosphatase-labeled anti-DIG antibody (Boehringer Mannheim) in blocking buffer for 2 h at RT. After washing, the sections were incubated with Nitroblue tetrazolium and X-phosphate (Boehringer Mannheim) in buffer containing 0.1 M Tris, 0.1 M NaCl, 0.005 M MgCl₂ and 1 mM levamisole (pH 9.6) for 16 h at RT. Visualization was stopped with EDTA, and then the slides were dehydrated with graded ethanol and xylene, and sealed with malinol. These methods are a modified version of those previously reported (3-12,18).

Double staining for macrophage marker and *Bam*HIW mRNA *in situ* hybridization. The slides were examined *Bam*HIW *in situ* hybridization using the same method as described above. Subsequently, they were reacted with the antibody, CD68, purchased from Dako (Glostrup, Denmark), and stained with diaminobenzidine according to the manufacturer's instructions.

Indirect immunofluorescence staining. The formalin-fixed paraffin-embedded tissue samples and control specimens were cut into serial sections of 3-5 μ m. After dewaxing and dehydration, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) for 10 min at 120°C to detect EBNA2, EBNA LP and BZLF1. Alternatively, the samples were digested for 15 min with proteinase K at a concentration of 1 mg/ml in 50 mM Tris-HCl (pH 7.6) at RT to detect LMP1. The sections were then blocked with 10-times-diluted EBV-negative human serum and 20% normal goat serum for 30 min at RT. The monoclonal anti-EBNA2 antibody, PE2, the anti-LMP1 antibody, CS1-4, the anti-BZLF1 antibody (Dako) and the anti-EBNA LP antibody, JF186 (21), were diluted 10x with PBS and reacted for 90 min at 37°C. After washing with PBS, biotinylated goat anti-mouse IgG (Vector, Burlingame, CA, USA) was diluted 75x with PBS and reacted for 60 min at 37°C. After washing, streptavidin-fluorescein isothiocyanate conjugate (Bethesda Research Laboratory, Gaithersburg, MD, USA) diluted 200x was reacted for 45 min at RT. After washing, the slides were sealed with Perma Fluor (Japan Tanner, Kobe, Japan). The negative controls of the tissue samples were reacted with 10-times-diluted normal mouse

Case	Age	Sex	Histology	ISH				IF				PCR
				<i>Bam</i> HIW	Y1Y2	EBNA	EBER1	EBNA2	LP	BZLF1	LMP1	
1	53	M	NHL	++	+	++	++	w+	+-	+	w+	+
2	51	F	NHL	+	+	+	w+	+	+	+	++	+
3	54	M	MALToma	++	w+	w+	w+	+	w+	+	+-	+
4	61	M	MALToma	++	++	++	+	w+	-	w+	-	NT
5	66	F	MALToma	+	+	+	w+	+	+	+	w+	NT
6	50	M	MALToma	++	++	++	w+	w+	+	w+	w+	NT
7	62	F	MALToma	+	+	+	w+	+	+	+	w+	+
8	51	M	Lym.hyperpl.	+	++	++	+	w+	-	-	+	NT
9	37	M	LIP	++	+	+	w+	+	w+	w+	+	NT

NHL, non-Hodgkin's lymphoma, diffuse large B-cell type; MALToma, mucosal-associated lymphoid tissue lymphoma; Lym.hyperpl., lymphoid hyperplasia; LIP, lymphoid interstitial pneumonia; ++, strongly positive; w+, weakly positive; +-, equivocal; NT, not tested.

IgG. These methods are identical to those we have used to study other human cancers (3-12,18).

Nested PCR. DNA was extracted from the frozen tissues. The primers used for the 1st round of PCR were: 5'CCAGA CAGCAGCCAATTCTC3' (nucleotide positions of *Bam*HIW 1087-1106), and 5'CCTAAGAAGGCACCGGTC3' (1503-1520). The primers used for the 2nd round of PCR were: 5'GTAAGAGGGGGTCTTCTAC3' (1196-1214), and 5'CCA GAGGTAAGTGGACTT3' (1399-1416). The resulting 2nd round product was 220 bp. The PCRs were carried out in a thermal cycler Gene Amp 9600-R (Perkin Elmer). The reaction mixture contained 100 ng to 1 μ g template DNA or 10 ng positive control DNA (Namalwa and Raji), 0.1 nmol of each primer, and 200 μ M each of dATP, dGTP, dCTP and dTTP in 1X amplification buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂; 0.01% W/V gelatin) and 2.5 U Taq or LA Taq polymerase (Perkin Elmer/TaKaRa) in a total volume of 100 μ l. The samples were treated for 5 min at 94°C and amplified by 40 cycles of PCR, each consisting of 1 min of denaturing at 94°C, 30 sec of annealing at 55°C, and 1 min of extension at 72°C. After treating for 10 min at 72°C, the DNA was concentrated by ethanol precipitation, electrophoresed on a 1.8% agarose gel in TAE buffer (40 mM Tris-HCl, pH 8.0, 5 mM sodium acetate, 1 mM EDTA) and stained with 0.5 μ g/ml ethidium bromide. This method has already been described (3).

Results

The results are summarized in Table I. In *Bam*HIW mRNA *in situ* hybridization, signals were detected in the cytoplasm in all the sections hybridized with the antisense probe (Fig. 1B) but not with the sense probe (Fig. 1C). This result indicates that EBV infected all the lymphoproliferative disorders of the lung irrespective of the histological or clinical diagnosis. In EBNA LP (*Bam*HIY1Y2) mRNA *in situ* hybridization, signals were detected in the cytoplasm in all the sections hybridized with the antisense probe (Fig. 1D) but not with

the sense probe (Fig. 1E). Signals were weak in case 3. In EBNA2 mRNA *in situ* hybridization, signals were also detected in the cytoplasm in all cases hybridized with the antisense probe (Fig. 1F) but not with the sense probe (Fig. 1G), and signals were weak in case 3. In EBV RNA *in situ* hybridization, signals were detected in the nuclei in all cases hybridized with the antisense probe (Fig. 1H) but not with the sense probe, and the signals were weak in cases 2, 3, 5, 6 and 9. The normal part of well-differentiated adenocarcinoma of the lung showed few signals with the antisense probe of EBV RNA (Fig. 1I).

We further examined the EBV protein expression by indirect immunofluorescence staining, due to higher sensitivity than immunohistochemistry. Immunofluorescence staining using the anti-EBNA2 antibody was prominent in the nuclei of lymphoma cells (Fig. 2A) or atypical lymphocytes, although fluorescence was weak in cases 1, 4, 6 and 8. Immunofluorescence staining using the anti-BZLF1 antibody revealed fluorescence in the nuclei of lymphoma cells (Fig. 2B) or atypical lymphocytes. Cases 4, 6 and 9 showed weak fluorescence. Immunofluorescence staining using the anti-LMP1 antibody revealed fluorescence in the membranes of lymphoma cells (Fig. 2C) or atypical lymphocytes. Cases 1, 5, 6 and 7 showed weak fluorescence and case 4 showed no fluorescence. Immunofluorescence staining using the anti-EBNA LP antibody was prominent in the nuclei of lymphoma cells (Fig. 2D) or atypical lymphocytes, although fluorescence was weak in cases 3 and 9, and cases 4 and 8 showed no fluorescence.

EBV DNA was detected by PCR. DNA was extracted from the resected cancer tissues (T) and the normal cut end (N) of each of the 4 of the 9 cases. The content of DNA was confirmed with primers of the housekeeping gene, cyclin A (Fig. 3A). EBV DNA was amplified in the region of *Bam*HIW in case 7, but not in cases 1N, 2T, and 3T (Fig. 3B). Re-examination of PCR with the enzyme LA Taq amplified EBV DNA in the cases of 2T and 3T. Case 1N could not be amplified in this experiment and as it possibly contained little lymphocytic infiltration (Fig. 3C).

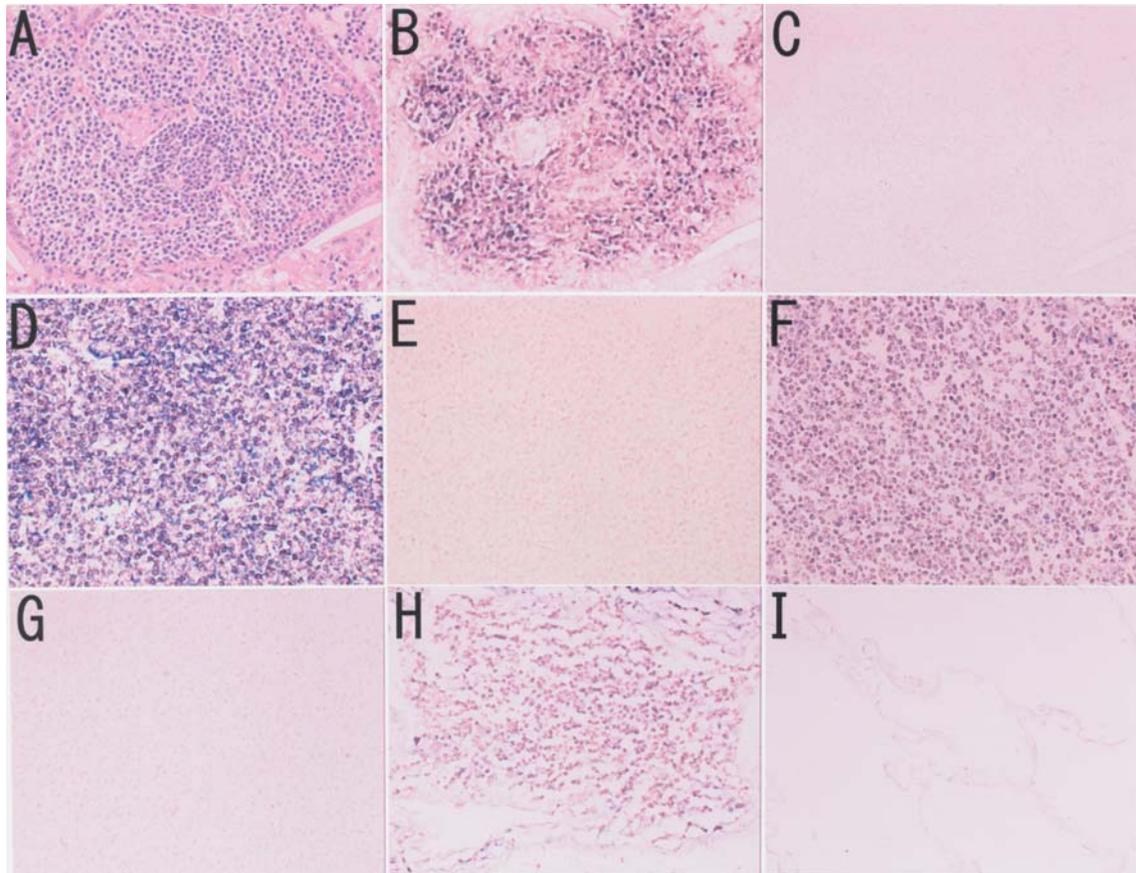


Figure 1. mRNA *in situ* hybridizations of lymphoproliferative diseases of the lung. (A) H&E staining of case 5. (B) mRNA *in situ* hybridization with the BamHIW antisense probe of case 5. (C) mRNA *in situ* hybridization with the BamHIW sense probe of case 5. (D) mRNA *in situ* hybridization with the EBNA LP antisense probe of case 4. (E) mRNA *in situ* hybridization with the EBNA LP sense probe of case 4. (F) mRNA *in situ* hybridization with the EBNA2 antisense probe of case 1. (G) mRNA *in situ* hybridization with the EBNA2 sense probe of case 1. (H) mRNA *in situ* hybridization with the EBER1 antisense probe of case 7. (I) mRNA *in situ* hybridization with the EBER1 antisense probe of the normal part of well differentiated lung adenocarcinoma (negative control). x200. A, B and C, D and E, F and G are the same field of sequential sections, respectively.

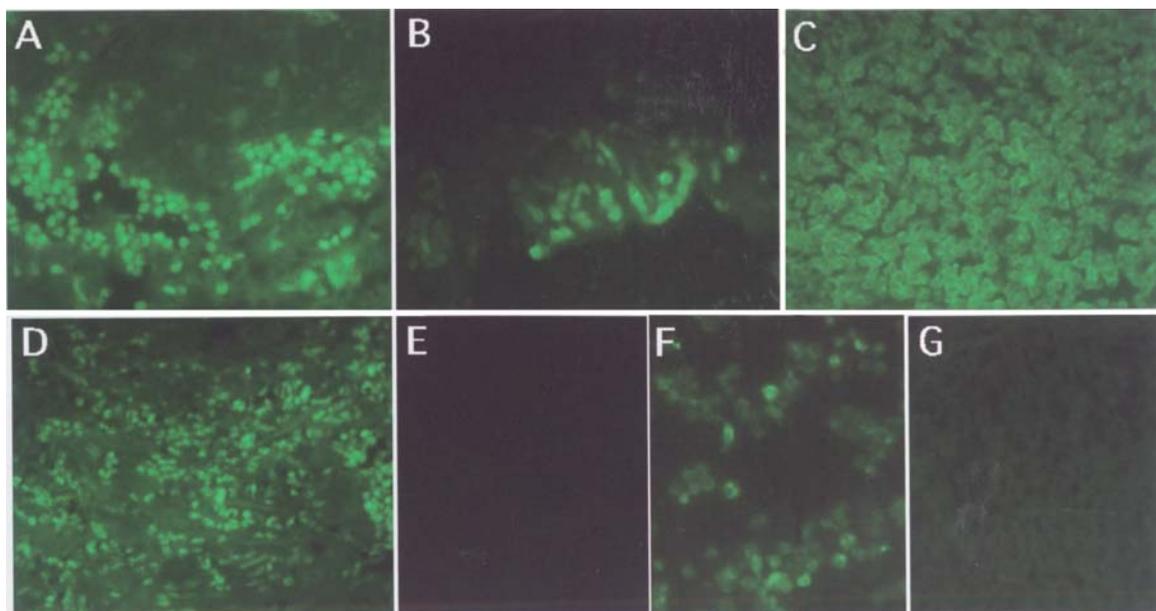


Figure 2. Indirect immunofluorescence staining of lymphoproliferative diseases of the lung. (A) Immunofluorescence with the monoclonal antibody against EBNA2 of case 1. (B) Immunofluorescence with the monoclonal antibody against BZLF1 of case 5. (C) Immunofluorescence with the monoclonal antibody against LMP1 of case 1. (D) Immunofluorescence with the monoclonal antibody against EBNA LP of case 6. (E) Immunofluorescence with the monoclonal antibody against EBNA LP of the EBV-negative B-cell line, BJAB (negative control for EBNA LP). (F) Immunofluorescence with the monoclonal antibody against EBNA LP of the EBV-positive B-cell line, B95-8 (positive control for EBNA LP). (G) Immunofluorescence with the normal mouse IgG of case 6 (negative control). x200.

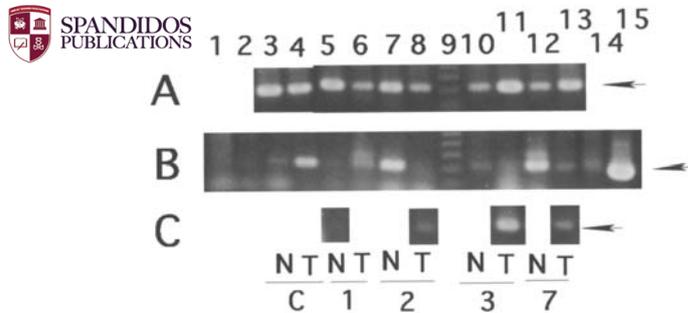


Figure 3. Results of PCR. Lane 1, no DNA; Lane 2, DNA extracted from the tonsils of an EBV-negative child; Lane 3, normal tissue of the reference case (lung cancer); Lane 4, cancerous tissue of the reference case (lung cancer); Lane 5, normal tissue of case 1; Lane 6, tumor tissue of case 1; Lane 7, normal tissue of case 2; Lane 8, tumor tissue of case 2; Lane 9, molecular weight markers; Lane 10, normal tissue of case 3; Lane 11, tumor tissue of case 3; Lane 12, normal tissue of case 7; Lane 13, tumor tissue of case 7; Lane 14, positive control DNA of the EBV-positive cell line, Namalwa; Lane 15, positive control DNA of the EBV-positive cell line, B95-8. (A) Amplification with the primer of cyclin A for the detection of extracted DNA with the enzyme Taq polymerase (TaKaRa, Kyoto, Japan). (B) Amplification with the primer of *BamHIW* with Taq polymerase. (C) Amplification with the primer of *BamHIW* with LA Taq polymerase (TaKaRa).

In these experiments, we observed many alveolar macrophages in or around the lymphoproliferative regions. These macrophages expressed EBV by both the method of *in situ* hybridization and immunofluorescence. To confirm the macrophages, double staining for the macrophage marker, CD68, and *BamHIW in situ* hybridization, were performed. Large macrophages in or around the lymphoma or lymphoproliferative region were double stained (Fig. 4). Furthermore, indirect immunofluorescence staining with anti-BZLF1 revealed fluorescence in much larger cells, suspected to be macrophages. These results suggest that in the tissue of lymphoproliferative disorders, not only lymphoma cells and atypical lymphocytes, but also alveolar macrophages express EBV and that these macrophages express proteins associated with EBV proliferation as well as transformation.

Discussion

In this study, we showed that EBV infected all the lymphoproliferative disorders, and that the EBV-infected cells of these disorders were not only lymphocytes but also macrophages.

In the literature, the respiratory tract has been proved to be major reservoir for EBV (22). There are some reports of EBV detection in LIP (23-26) and in MALT lymphoma (27), whereas negative results have also been reported (28). In the DNA *in situ* hybridization using the *BamHIW* probe, 9 out of the 14 cases of LIP were described to be EBV-positive (23). By PCR amplification using the IR3 region of EBV, 3 of the 6 cases of LIP were reported to be EBV-positive (24). LMP1 was described to be detected in a case of LIP (25) and in 10 of 13 cases of LIP in another study (26). Tao and Kahn reported a case of MALT lymphoma, which progressed from low-grade to high-grade, showing monoclonality of EBV by Southern blot hybridization and PCR (27). In this case, EBV was detected at first examination and the fact coincided with our results. On the contrary, Tamura *et al* reported that all 12

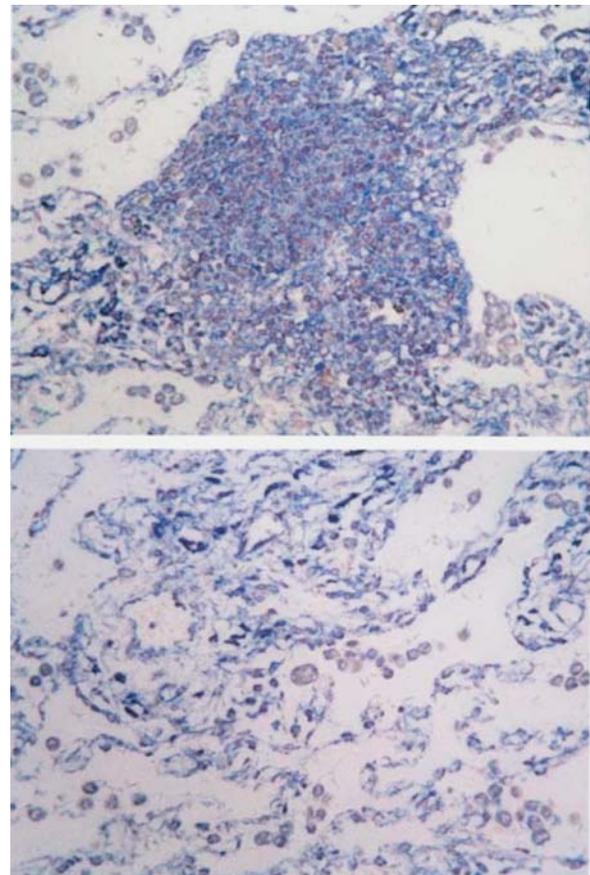


Figure 4. Double staining with *BamHIW* mRNA *in situ* hybridization and CD68. Large macrophages in (upper figure) and around (lower figure) the lymphoma and lymphoproliferative region were double stained. EBV mRNA was stained in purple-blue and CD68 was stained in brown. Upper, case 1; lower, case 4.

cases of MALT lymphoma showed no signal of EBV using *in situ* hybridization (28). Our experiments of EBV1 *in situ* hybridization also showed weak signals. Therefore, this discrepancy could depend on the method used for EBV detection. One possible reason could be the different sensitivity of the used probes. Our *BamHIW* probes did not contain an 'Alu-family'-like sequence, and so the reaction was considered to be specific to EBV. The antisense probe did not react with the EBV-negative cells. As the *BamHIW* fragment of EBV contains a 10-fold redundant sequence (19), this probe is very sensitive in detecting EBV. Furthermore, the *BamHIW* fragment is a leader sequence of the mRNAs of EBNA2 (29), which are suspected to be oncogenes of EBV. Therefore, the *BamHIW* probe is important for the detection of EBV transformation. Moreover, we confirmed the expression of the oncogenic proteins, EBNA2 and LMP1, in this study. Our results with *in situ* hybridization and immunofluorescence suggest an oncogenic and tumor progressive role of EBV in lymphoproliferative disorders of the lung.

Hybridization signals and fluorescence were observed in all these experiments of lymphoproliferative diseases in the lung specimens. This fact showed that EBV infected cells of these disorders at early stages and that EBV infection did not occur accidentally in the long-term course of progression.

We have shown EBV expression in macrophages (30) and Langerhans' cells (12,31). EBV expressed not only the transforming genes but also the replication protein in the macrophages (30). Also in this study, we confirmed the EBV expression in alveolar macrophages. The co-culture of several B-cell lymphoma lines with human macrophages revealed an enhancement of soft agar colony formation and growth in low serum concentration in a cell line of Namalwa, which was derived from Burkitt's lymphoma, but not in other cell lines (our results, data not shown). We suspect that macrophages have some roles in the transformation or progression of EBV-associated lymphoproliferative disorders of the lung. More study is necessary to clarify the function of macrophages. Inflammation and cancer has been discussed for many years (32,33). MALT lymphomas are thought to originate from cells that are generated in response to various types of chronic inflammation (34). EBV-transformed lymphocytes and macrophages must be studied in this viewpoint.

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