Epstein-Barr Virus-Associated Non-Hodgkin's Lymphoma of B-cell Origin, Hodgkin's Disease, Acute Leukemia, and Systemic Lupus Erythematosus : A Serologic and Molecular Analysis

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Abstract

Parallel studies of (a) patients with Epstein-Barr virus (EBV)-associated peripheral T-cell proliferative disease/lymphomas and (b) a group of patients with a prolonged fever from other causes were conducted at Songklanagarind University Hospital from 1997 through 2000. (Reports on EBV-associated peripheral T-cell and NK-cell proliferative disease/lymphomas have been published elsewhere) In this study, the authors identified 58 patients; 14 were non-Hodgkin's lymphoma of B-cell origin (NHL-B), 8 were Hodgkin's disease, 6 were acute leukemia, 9 were systemic lupus erythematosus (SLE), and 21 were patients with other diseases. Serologic tests for the EBV infection, the study of EBV genome in circulating non-T-cells (CD3-cells) and T-cells (CD3+ cells), and the EBV-RNA study in the tumor cells were performed. EBV internal repeat-1 region (IR-1) in peripheral blood CD3+ cells was detected in 10 of 14 patients (71.5%) with NHL-B, 3 of 8 patients (37.5%) with Hodgkin's disease, 1 of 6 patients (16.7%) with acute leukemia, 4 of 9 patients (44.5%) with SLE, and was not detected in any of the 21 patients with other diseases. Anti-viral capsid antigen-IgG was significantly elevated in hematologic malignancy patients with EBV IR-1 genome in the peripheral blood CD3+ cells when compared to hematologic malignancy patients with a negative result, whereas there was no significant difference in anti-EBV nuclear antigen among these two groups. EBV-RNA expression in tumor cells by *in situ* hybridization was detected in 4 of 13 patients (31%) with NHL-B (all showed EBV IR-1 genome in peripheral blood CD3+ cells), and 3 of 5 patients (60%) with Hodgkin's disease (only two showed EBV IR-1 genome in peripheral blood CD3+ cells). These data support the theory that chronic EBV infection is often found in association with cases of NHL-B, Hodgkin's disease, acute leukemia, and SLE.

Key word : Epstein-Barr Virus, Non-Hodgkin's Lymphoma, Hodgkin's Disease, Acute Leukemia, Systemic Lupus Erythematosus, CD3+ Cell

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Epstein-Barr virus (EBV) is a lymphocryptovirus belonging to the subfamily of gammaherpesvirinae. It is an etiologic agent of infectious mononucleosis, which is a self-limiting disease, and a fatal form is an uncommon presentation of primary infection and is characterized by an uncontrolled B-cell proliferation due to a defect in T-cell-mediated immune regulation[1,2]. EBV infection has also been implicated in the development of a variety of malignancies, including nasopharyngeal carcinoma, gastric carcinoma, smooth muscle tumor in immunocompromised patients, thymic lymphoepithelial carcinoma, Hodgkin's disease, and non-Hodgkin's lymphoma of B-cell and T-cell (NHL-B, NHL-T) origins[3-10].

In a previous study[11], the authors reported 100 patients with various types of peripheral T-cell and NK-cell proliferative disease/lymphomas and 100 healthy age- and sex- matched controls. Results in the study showed the EBV genome in the peripheral T-cells in 65 per cent of the patients, which was not found in any of the controls. Dual infections (wild-type and the 30-bp deletion variant) of EBV in the peripheral blood CD3+ cells of some patients, and the presence of the EBV genome in both CD4+ and CD8+ cells in a single patient were also found. It is believed that these infected circulating T-cells might not be neoplastic cells or might reflect oligoclonal malignancy. There was a significant elevation of the anti-viral capsid antigen-IgG (anti-VCA-IgG) and anti-early antigen-IgG (anti-EA-IgG) compared to the controls, whereas there was no significant difference in the anti-EBV nuclear antigen (anti-EBNA) and the IgM or IgA classes antibody. The serologic study supported the chronic infective process. The authors postulated that the EBV which was dormant in non-T-cells might infect the T-cell and contribute to the pathogenesis of disease in a select group of patients.

MATERIAL AND METHOD

A prospective study was conducted in Songklanagarind University Hospital, Songkhla, Thailand, during the 4-year period from January 1997 through December 2000. This was performed parallel to a study of EBV-associated peripheral T-cell and NK-cell proliferative disease/lymphomas which has been published elsewhere[11]. The patients in this study presented with prolonged fever. The diagnoses were later confirmed by clinical findings, serologic tests, and pathological examination. HIV positive patients were excluded from the study. The study was approved by the Hospital Ethics Committee, and all of the patients gave informed consent. Blood samples for the EBV serologic study and the molecular study were available for all patients. Paraffin-embedded tissues in NHL-B and Hodgkin's diseases for the EBV-RNA study were available in some cases.

Serologic study for EBV antibodies

P3HR-1 cells and Raji cells were prepared as target antigens of EBV viral capsid antigen (VCA) and EBV early antigen (EA), respectively, using the following treatment. Cells cultured in RPMI 1640 (Gibco, USA) supplemented with 10 per cent heat inactivated fetal calf serum (Gibco, USA) were treated with 4mM n-butyric acid and 20 ng/ml 12-tetradecanoylphorbor-13-acetate (TPA) (Sigma, USA) for 48 hours. Cells were smeared on a slide. After air-drying, they were fixed with acetone for 3 minutes at room temperature. The cell smears were first treated with diluted (1:10 in PBS) plasma at 37°C for 30 minutes, and then washed with PBS. They were then incubated with FITC-labeled anti-human IgG, IgM, and IgA rabbit serum (Dako, Denmark) at 37°C for 30 minutes. After rewashing, they were mounted with glycerine buffer and examined for specific antibodies to EBV-VCA and -EA under a fluorescent microscope. When a positive reaction was detected at this dilution (1:10), the sample was then serially diluted and titer of antibody was determined.

For the detection of anti-EBV nuclear antigen (anti-EBNA), untreated Raji cells were used as antigen. The cells after smearing were treated as above, except with FITC-labeled anti-human C3 rabbit serum (Dako, Denmark) instead of anti-human immunoglobulins.

White blood cell preparation and DNA extraction

Twenty milliliters of heparinized peripheral blood samples were obtained from 58 patients. Lymphocytes were isolated by centrifugation using Ficoll solution (Ficoll-Paque Plus, Amersham Pharmacia Biotech AB, Sweden). Isolated lymphocytes were washed with saline and medium, and then suspended in fetal calf serum supplemented 0.5-1.0 ml of medium. By using anti-human CD3 antibody conjugated magnet beads (Dynabeads M-450 CD3, Dynal AS, Norway), cells were fractionated into non-T-cells (CD3- cells), and T-cells (CD3+ cells). The steps of isolation followed the manufacturer's pro-

tocol. High molecular weight DNA samples were then extracted from the fractionated cells for PCR analysis.

DNA extraction was performed by using the Qiagen RNA/DNA Mini Kit (QIAGEN, Germany). The CD3- cells, CD3+ cells, were homogenized in QRL 1 buffer by passing the lysate through a 20-gauge needle. Further steps were followed according to the manufacturer's manual of animal cell protocols. The presence of DNA was confirmed by agarose gel electrophoresis.

Polymerase chain reaction (PCR) analysis

The DNA which was extracted from the CD3-, and CD3+ cells was analyzed for the presence of the EBV internal repeat-1 (IR-1). PCR for this EBV-DNA by using the oligonucleotide primers for amplification of IR-1, E1:3′ (1087-1106), E2:3′ (1196-1215), was performed as described previously, using DNA from Raji cells as a positive control[12]. The amplified fragment length was 129 base pairs.

In situ Hybridization

An *in situ* hybridization (ISH) study for the Epstein-Barr virus genomes was performed on formalin-fixed, paraffin embedded tissue using the fluorescein-conjugated EBV (EBER) oligonucleotides, complementary to nuclear RNA portions of the EBER genes that are actively transcribed in latently infected cells. Briefly, tissue sections of 5 microns were deparaffinized with xylene, rehydrated in graded water-ethanol solutions, and digested with proteinase K (3 mg/L in 0.05 M Tris/HCl, pH 7.6) for 30 minutes at 37°C. After dehydration and air-drying, the fluorescein-conjugated (FITC) EBER oligonucleotide probes (Y 0017, Dako) were applied to the sections for 2 hours at 37°C. The following immunohistochemical detection system (K 046, Dako) was used: rabbit F(ab′) anti-FITC/AP for 30 minutes and a solution containing 5-bromo-4-chloro-3-indolyphosphate (BCIP) and nitroblue tetrazolium (NBT) for 30-60 minutes. Then the slides were washed in running tap water and mounted in glycergel. Appropriate positive and negative controls were run.

RESULTS

The clinical data of 58 patients are summarized in Table 1. There were 32 males and 26 females ranging in age from 2 years to 76 years. All of the patients had had a prolonged fever lasting from 3 weeks to 3 months. Hematologic malignancies were confirmed in 28 patients; 14 cases were non-Hodgkin's lymphoma of B-cell origin (NHL-B), 8 cases were Hodgkin's disease, and 6 cases were acute leukemia (5 were acute myeloid leukemia, and 1 was acute lymphoblastic leukemia). Nine patients were confirmed to have the systemic lupus erythematosus (SLE) by using the 1982 American Rheumatism Association revised criteria. The other patients were 5 cases of Kikuchi-Fujimoto disease, 4 cases of tuberculosis, 5 cases of erythema nodosum, 2 cases of polyarteritis nodosa, 2 cases of drug allergy, 2 cases of bacterial infection, and 1 case of viral infection.

Results of the serologic tests for the EBV antibodies are summarized in Table 2. Among the patients with hematologic malignancies, the anti-VCA-IgG was detected in all cases with the highest titer of 1:2560, the anti-VCA-IgA was detected in 2 cases (titers 1:40 and 1:80), and the anti-VCA-IgM wasn't detected in any case. There was a significant

Table 1. Clinical data of 58 patients with prolonged fever.

	No. of patients			Age (years)	
	Male	Female	Total	Mean	Range
NHL-B	11	3	14	46.9	2-74
Hodgkin's disease	6	2	8	44.6	12-70
Acute leukemia	4	2	6	32.7	11-43
SLE	2	7	9	33.9	8-46
Others	9	12	21	38.2	16-76
Total	32	26	58		

NHL-B = non-Hodgkin's lymphoma of B-cell origin, SLE = systemic lupus erythematosus
Others : Kikuchi-Fujimoto disease = 5, tuberculosis = 4, erythema nodosum = 5, polyarteritis nodosa = 2, drug allergy = 2, bacterial infection = 2, viral infection = 1

difference in the titer of anti-VCA-IgG between the patients with hematologic malignancies and the 100 healthy controls from our previous study(11) ($p< 0.01$). Two patients in this group had a low titer of anti-EA-IgG, and none had elevated anti-EA-IgA and anti-EA-IgM. For the anti-EBNA, there was no significant difference between the 100 healthy controls (11) and the group of patients with hematologic malignancies in this study.

The EBV genomic study (IR-1) of the peripheral blood lymphocytes is summarized in Table 3. The EBV genome in the peripheral blood CD3+ cells was detected in 10 of 14 patients (71.5%) with NHL-B, 3 of 8 patients (37.5%) of Hodgkin's disease, 1 of 6 patients (16.7%) of acute leukemia (acute myeloid leukemia, M5), 4 of 9 patients (44.5%) of SLE, and none was detected in other patients. CD3- cells positive for the EBV genome varied from 47.6 per cent to 92.9 per cent. All patients with CD3+ cells positive always showed CD3- cells positive. Hematologic malignancy patients who had the EBV genome in the peripheral blood CD3+ cells showed a significantly high titer of the anti-VCA-IgG compared to the CD3+ cells negative patients ($p=0.037$), but there was no significant difference for the anti-EBNA ($p=0.7415$, Table 4).

Table 5 shows the results of the *in situ* hybridization study for the EBV-RNA (EBER) which was performed on 13 cases of NHL-B and 5 cases of Hodgkin's disease. EBV-RNA in tumor cells was detected in 4 cases of NHL-B (1 case of Burkitt's lymphoma, 1 case of T-cell-rich B-cell lymphoma, and 2 cases of diffuse large cell) and 3 cases of Hodgkin's disease (1 case was lymphocytic depletion, 2 cases were mixed cellular). All of the EBV-RNA in tumor cell positive cases of NHL-B were also EBV-DNA positive in the peripheral blood CD3+ cells. In contrast to the Hodgkin's disease, only 2 of 3 of the EBV-RNA positive cases showed positive EBV-DNA in the peripheral blood CD3+ cells.

DISCUSSION

In this report the authors describe the serologic findings, and the EBV genomic studies of 14

Table 2. Serologic tests for anti-VCA-IgG, anti-EA-IgG, and anti-EBNA in 55 of 58 patients.

Titers	No. of cases														
	NHL-B (n=12)			Hodgkin's disease (n=8)			Acute leukemia (n=6)			SLE (n=8)			Others (n=21)		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Non-specific	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
<10	0	11	2	0	7	0	0	5	2	0	7	3	2	18	6
10	1	0	4	0	0	3	0	0	0	1	0	2	0	2	2
20	1	0	6	4	1	2	2	0	2	1	1	3	1	0	7
40	3	1	0	0	0	2	1	1	2	1	0	0	12	1	5
80	5	0	0	1	0	1	2	0	0	0	0	0	4	0	1
160	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0
320	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
1,280	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2,560	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

A = anti-VCA-IgG, B = anti-EA-IgG, C = anti-EBNA

Table 3. EBV genomic study (IR-1) in peripheral blood lymphocytes of 58 patients.

Type of cells	No. of cases with IR-1 positive									
	NHL-B (n=14)		Hodgkin's disease (n=8)		Acute leukemia (n=6)		SLE (n=9)		Others (n=21)	
		%		%		%		%		%
CD3+ cells	10	71.5	3	37.5	1	16.7	4	44.5	0	0
CD3- cells	13	92.9	6	75	4	66.7	8	88.9	10	47.6

n = total number

Table 4. Serologic tests for anti-VCA-IgG and anti-EBNA with respect to the presence of EBV genome (IR-1) in peripheral blood CD3+ cells of 26 patients with hematologic malignancies.

Titers	EBV genome in peripheral blood CD3+ cells			
	Positive (n=13)		Negative (n=13)	
	anti-VCA-IgG*	anti-EBNA**	anti-VCA-IgG*	anti-EBNA**
<10	0	1	0	3
10	0	5	1	2
20	1	5	6	5
40	3	1	1	3
80	5	1	3	0
160	1	0	2	0
320	1	0	0	0
1,280	1	0	0	0
2,560	1	0	0	0

* p-value of anti-VCA-IgG by Kruskal-Wallis test = 0.037
** p-value of anti-EBNA by Kruskal-Wallis test = 0.7415

Table 5. *In situ* hybridization (ISH) for EBV-RNA (EBER) in tumor cells and the presence of EBV genome (IR-1) in the peripheral blood CD3+ cells.

	EBV-ISH positivity			
	Positive IR-1 in CD3+ cells		Negative IR-1 in CD3+ cells	
		%		%
NHL-B (n=13)	4/10	40	0/3	0
Hodgkin's disease (n=5)	2/3	66.7	1/2	50
Total	6/13	46.1	1/5	20

patients with NHL-B, 8 patients with Hodgkin's disease, 6 patients with acute leukemia, 9 patients with SLE, and 21 patients with various diseases presented as prolonged fever. The notable findings were the presence of EBV genome in the peripheral blood T-cells in patients with NHL-B, Hodgkin's disease, acute leukemia, and SLE, which were not found in any of the other diseases, and the significant elevation of anti-VCA-IgG in hematologic malignancy patients who had the EBV genome in their peripheral blood T-cells. EBV-RNA in tumor cells was detected in 31 per cent of NHL-B and all were associated with the presence of EBV genome in the peripheral blood T-cells, whereas 3 of 5 patients (60%) was detected in Hodgkin's disease; one patient wasn't associated with the presence of EBV genome in the peripheral blood T-cells.

In a previous study by the authors of patients with T-cell and NK-cell proliferative disease/lymphomas, it was found that 65 per cent of them showed EBV genomes in the circulating CD3+ cells (11). In this study, the presence of the EBV genome in circulating T-cells in some patients with NHL-B, Hodgkin's disease, acute leukemia, and SLE was also found. To our knowledge, this report examines the largest series of patients of the above diseases showing the EBV genome in the circulating T-cells. The serologic study also confirmed the chronic infective process, even though 3 of 8 patients with SLE had autoantibodies that interfered with the EBV serologic test.

The evidence of EBV infection and the pathogenesis of Hodgkin's disease was reviewed by Flavell KJ et al(7). EBV could either play a direct or indirect role in the pathogenesis, possibly by triggering the pathogenic mechanism, or by reflecting the presence of an inherited or acquired depression of immunoregulation, which is a prelude both to the

malignancy and to the reactivation of EBV[13]. EBV-DNA has been detected in about 20-25 per cent of Hodgkin's disease tumor specimens[14]. The EBV-RNA expression in Reed-Sternberg cells appears to be less common in developed countries, at between 20 per cent and 50 per cent, in contrast to under-developed countries which have much higher rates [15-22]. The EBV-DNA in plasma was detected in about 50 per cent of Hodgkin's disease patients, whereas none was detected in the controls[23]. Of the Hodgkin's disease patients in the present study, 60 per cent showed EBV-RNA in tumors, and 37.5 per cent showed EBV-DNA in the circulating T-cells.

The EBV genome (EBER) positivity was found in about 7 per cent of NHL-B cases[24]. A high percentage of positivity is usually found in T-cell-rich B-cell lymphoma, Burkitt's lymphoma, posttransplantation lymphoproliferative disease, and diffuse mixed cell NHL[9,10,24]. In NHL-B patients in the present study, EBV-RNA expression was detected in 4 of 13 tested cases (31%). The EBV genome in peripheral blood T-cells was detected in 71 per cent of NHL-B patients, which was a slightly higher rate than our previous report on peripheral T-cell and NK-cell proliferative disease/lymphomas [11]. The findings confirmed the high association of chronic EBV infection, and the pathogenesis of NHL-B.

Recently, many investigations have shown that EBV might somehow be involved in the etiology and/or pathogenesis of SLE[25,26]. The present study also confirmed this, as about 40 per cent of the SLE patients showed EBV-DNA in the peripheral blood T-cells.

In the authors' previous study[11], the EBV-DNA wasn't detected in the peripheral blood T-cells of any of the healthy controls. This concurred with the other diseases in the present study. Kikuchi-Fujimoto disease is believed to be associated with viral infection or postviral hyperimmune reaction. These viruses include EBV, cytomegalovirus, HHV-6, HIV, and Parvovirus B19[27,28]. Five cases of Kikuchi-Fujimoto disease in the present series did not show evidence of EBV infection. None showed EBV genome in the peripheral blood T-cells, and none showed significant elevation of the EBV antibodies.

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ความสัมพันธ์ของการติดเชื้อไวรัส เอ๊ปสไตน์-บาร์ กับการเกิดโรคนอน-ฮอดจกิ้นส์ ลิมโฟม่า, โรคฮอดจกิ้นส์, มะเร็งเม็ดเลือดขาวชนิดเฉียบพลัน และโรคซิสเตมิก ลูปัส อีรีเทมาโตซัส : การศึกษาทางเซอโรโลยี่และการศึกษาในระดับโมเลกุล

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คณะผู้รายงานได้ศึกษาผู้ป่วยที่มีไข้เรื้อรังซึ่งไม่ใช่โรคของ T-cell จำนวน 58 ราย ที่ได้รับการรักษาในโรงพยาบาลสงขลานครินทร์ หาดใหญ่ สงขลา ระหว่างปี พ.ศ. 2539-2543 ผู้ป่วยดังกล่าวเป็น non-Hodgkin's lymphoma ชนิด B-cell (NHL-B) จำนวน 14 ราย ผู้ป่วย Hodgkin's disease (HD) จำนวน 8 ราย ผู้ป่วย acute leukemia จำนวน 6 ราย ผู้ป่วย systemic lupus erythematosus (SLE) จำนวน 9 ราย และผู้ป่วยโรคอื่น ๆ จำนวน 21 ราย ผู้ป่วยได้รับการตรวจหาแอนติบอดีย์ต่อการติดเชื้อของไวรัส Epstein-Barr (EBV), ศึกษาหายีนของ EBV ใน T-cell (CD3+ cell) และ non-T-cell (CD3-cell) ที่ไหลเวียนในกระแสเลือด และศึกษาหา EBV-RNA ในเซลล์มะเร็งของกลุ่มผู้ป่วย NHL-B และ HD ผลการศึกษาพบยีนของ EBV (internal repeat-1 region, IR-1) ใน CD3+ cell 10 ใน 14 ราย (71.5%) ของผู้ป่วย NHL-B, 3 ใน 8 ราย (37.5%) ของผู้ป่วย HD, 1 ใน 6 รายของผู้ป่วย acute leukemia, 4 ใน 9 ราย (44.5%) ของผู้ป่วย SLE, และตรวจไม่พบเลยในผู้ป่วย 21 ราย ซึ่งเป็นไข้เรื้อรังจากสาเหตุอื่น การตรวจหาแอนติบอดีย์ต่อการติดเชื้อ EBV พบว่าแอนติบอดีย์ชนิด anti-viral capsid antigen-IgG ในกลุ่มผู้ป่วยโรคเลือด (NHL-B, HD, และ acute leukemia) ที่ตรวจพบยีนของ EBV ใน CD3+ cell มีค่าสูงกว่าอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มผู้ป่วยโรคเลือดที่ตรวจไม่พบยีนของ EBV ใน CD3+ cell, 4 ใน 13 ราย (31%) ของผู้ป่วย HNL-B และ 3 ใน 5 ราย (60%) ของผู้ป่วย HD ตรวจพบ EBV-RNA ในเซลล์มะเร็ง การศึกษานี้ช่วยสนับสนุนว่า การติดเชื้อเรื้อรังของ EBV ในกระแสเลือดมีความสัมพันธ์กับการเกิดโรคในผู้ป่วยบางรายของ NHL-B, HD, acute leukemia และ SLE

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