The conjugated bilirubin was proportionally more elevated than the unconjugated bilirubin. Coagulopathy was found in a half of the patients; some were very severe.

The other sites for tissue diagnosis of peripheral T-cell proliferative disease/lymphoma are summarized in Table 3. For the histopathologic confirmation of the diagnosis of peripheral T-cell proliferative disease/lymphoma, 10 patients were confirmed from the liver tissue biopsy/necropsy only, 53 patients from multiple-site biopsies, and 7 patients had a complete autopsy performed. Bone marrow involvement of peripheral T-cell lymphoma was demonstrated in 42 of 49 tested cases (85.7%). Twenty-two patients had the lymph node tissues examined; 5 cases showed angioimmunoblastic T-cell lymphoma, 10 cases showed peripheral T-cell lymphoma, unspecified, and 7 cases showed reactive lymphoid hyperplasia. Eight patients had a skin biopsy. Of these, 5 were cutaneous T-cell lymphoma or early lesion of cutaneous T-cell lymphoma, 2 cases were peripheral T-cell lymphoma, unspecified, and 1 was subcutaneous panniculitis-like T-cell lymphoma. Three patients had extranodal NK/T-cell lymphoma, nasal-type, in the sinonasal and upper aerodigestive tract. Peripheral T-cell lymphoma was also found in the gastrointestinal tract of 6 patients, in the brain of 2 patients, and in the lung of 5 patients.

Table 3. Other Sites for Tissue Diagnosis of Peripheral T-cell Lymphoma

Site/Diagnosis	No. of cases				
	Group A (n=30)	Group B (n=14)	Group C (n=13)	Group D (n=13)	All groups (n=70)
Bone marrow involvement of PTCL	16/22	6/6	10/10	10/11	42/49 (85.7%)
Lymph node:					
Angioimmunoblastic T-cell lymphoma	2	1	0	2	5
PTCL, unspecified	2	1	1	6	10
Reactive lymphoid hyperplasia	4	0	2	1	7
Skin:					
Cutaneous T-cell lymphoma	2	3	0	0	5
PTCL, unspecified	1	1	0	0	2
Subcutaneous panniculitis-like T-cell lymphoma	1	0	0	0	1
Sinonasal and upper aerodigestive tract:					
Extranodal NK/T-cell lymphoma, nasal-type	1	1	1	0	3
Gastrointestinal PTCL	2	2	2	0	6
Brain PTCL	0	0	2	0	2
Lung PTCL	1	3	0	1	5

Abbreviation: PTCL, peripheral T-cell lymphoma

Immunohistochemistry and EBV Genomes

Results of the immunohistochemistry, EBV DNA, and *in situ* hybridization for EBV RNA are summarized in Table 4. The majority of phenotypes of the lymphoid cells in the livers were LCA$^+$, CD3$^-$, CD15$^-$, CD16$^-$, CD20$^-$, CD21$^-$, CD30$^-$, CD56$^-$, CD68$^-$, EMA$^-$, TIA-1$^+$, and p53$^-$. Two cases from group C patients and 4 cases from group D patients showed the dual expression of both CD3 and CD20 in the same lymphoid cells, which are markers of T cell and B cell, respectively. For the T-cell subsets study, the majority of cases were CD4$^-$ CD8$^+$ subset. Seven cases were CD4$^+$ CD8$^-$, 11 cases were CD4$^-$ CD8$^+$, and none showed CD4$^+$ CD8$^+$.

The EBV DNA was identified in 43 of 70 cases (61.4%). The presence of EBV RNA (EBER) was detected in 25 of the 70 cases (38.6%); 7 of 30 cases (23.3%) in group A, 5 of 14 cases (35.7%) in group B, 7 of 13 cases (53.8%) in group C, and 6 of 13 cases (46.2%) in group D. All patients with EBV RNA positive also showed EBV DNA positive.

Table 4. Phenotypes and EBV genomes of Lymphocytes in the Livers

Group	Positive cases/Total tested cases																		
	LCA	CD3	CD4	CD8	CD15	CD16	CD20	CD21	CD30	CD45RO	CD56	CD57	CD68	α-1	EMA	G6B	TIA-1	p63	EBV DNA	EBV RNA
A (n=30)	30/30	27/27	2/27	24/26	0/26	0/26	0/30	0/7	0/27	30/30	0/26	0/26	0/26	0/5	0/28	13/27	25/26	0/25	17/30	2/30
B (n=14)	14/14	14/14	3/13	12/13	0/13	0/13	0/14	0/2	0/14	14/14	0/13	0/13	0/13	0/2	0/13	3/13	12/13	0/13	7/14	5/14
C (n=13)	13/13	13/13	3/12	9/12	0/10	0/11	2/13	0/1	0/11	13/13	0/11	1/11	0/10	0/1	0/11	8/10	12/12	0/11	9/13	7/13
D (n=13)	13/13	8/11	0/10	4/10	0/8	0/9	4/13	0/0	0/11	13/13	0/10	1/10	0/11	0/0	0/10	5/8	8/8	1/10	10/13	6/13
All groups (n=70)	70/70 (100%)	62/65 (95.4%)	8/62 (12.9%)	49/61 (80.3%)	0/58 (0%)	0/59 (0%)	6/70 (8.6%)	0/10 (0%)	0/63 (0%)	70/70 (100%)	0/60 (0%)	2/60 (3.3%)	0/60 (0%)	0/8 (0%)	0/62 (0%)	33/58 (56.9%)	57/59 (96.6%)	1/59 (1.7%)	43/70 (61.4%)	25/70 (35.7%)

Clonality Study and TCR Sequence Analysis

Results of the clonality studies of the lymphocytes in the livers are shown in Table 5. By using three sets of oligonucleotide primers for the TCR-β gene, the monoclonal band/s were detected in 50 of the 68 tested cases (73.6%). For the TCR-γ gene, the monoclonal band/s were detected 48 of the 66 tested cases (72.7%). Thirty-nine of the 66 cases (59.1%) showed monoclonal bands in both TCR-β and-γ studies. Monoclonal band/s by either TCR-β or TCR-γ studies were detected in 62 patients (88.6%). Eight cases did not showed the monoclonal band neither by TCR-β nor TCR-γ. Of these, 3 were in group A, 1 was in group C, and 4 were in group D.

Table 5. The Monoclonal Study of Lymphocytes in the Livers

Group	Monoclonal positive cases/Total tested cases		
	TCR-β	TCR-γ	TCR-β and-γ
A (n=30)	24/30	24/30	21/30
B (n=14)	10/13	10/13	7/13
C (n=13)	10/13	8/12	7/12
D (n=13)	6/12	6/11	4/11
All groups (n=70)	50/68 (73.6%)	48/66 (72.7%)	39/66 (59.1%)

Results of the TCR gene-sequence analysis from 33 of the 62 cases which showed the monoclonal band/s of the TCR gene rearrangements are summarized in Table 6. All of these tested cases confirmed the monoclonality. PCR products of both TCR-β and-γ had a very high identity to the human TCR germline gene with the mean homologies of 99.5% and 99.6%, respectively.

Table 6. TCR gene-sequence analyses compared with DNA sequence of the human TCR germline gene

Group	TCR-β gene				TCR-γ gene			
	No. of cases	Identity			No. of cases	Identity		
		No. of bp	Mean (%)	Range (%)		No. of bp	Mean (%)	Range (%)
A	4	27-51	98.8	97-100	5	21-33	100	100
B	3	51-52	100	100	3	126-129	98.3	96-100
C	6	41-53	100	100	5	24-119	99	97-100
D	2	36-48	99.0	98-100	5	24-128	100	100
All groups	15	27-53	99.5±1.0 (mean±SD)	97-100	18	21-129	99.6±1.1 (mean±SD)	99-100

Remark: 12 of 15 cases of the TCR-β, and 15 of 18 cases of the TCR-γ show 100% identity

Histology and Clonality

Except for the bone marrow involvement, 34 of these 70 patients had the simultaneous or subsequent peripheral T-cell lymphoma in other organs. A representative study was conducted in 2 patients, one from group A, and another from group B.

The group A patient developed peripheral T-cell lymphoma, unspecified, in the skin, two months after the liver biopsy, and she died four months later. Photomicrographs of T cells which were infiltrated in the hepatic sinusoids, and peripheral T-cell lymphoma, unspecified, of the skin are shown in Fig. 2. Despite the difference in histopathologic morphology, they exhibited $CD3^+$, $CD4^-$, $CD8^-$, $CD20^-$ and $CD56^-$. The clonality study by the PCR of TCR-γ gene (Fig. 3; lanes a, b, and c), and the DNA-sequence analysis (upper band) showed that T-cells in the liver tissues (biopsy and necropsy), and the neoplastic T-cells in the skin, were the different clones.

The group B patient had angioimmunoblastic T-cell lymphoma in the lymph node, and extranodal NK/T-cell lymphoma, nasal-type, at the base of the tongue. The clonality study (Fig. 3; lanes d, e, and f), and the DNA-sequence analysis (upper band) showed that T cells in the liver, and the neoplastic T cells in the lymph node and the tongue, were from the same clone.

Follow-up and Outcomes

Follow-up data and outcomes of the patients according to the relationship of chemotherapy and the presence of EBV-RNA in the lymphocytes that infiltrated in the liver are summarized in Table 7. Twenty patients (28.6%) received multidrug chemotherapy (CHOP regimen), and 50 patients (71.4%) did not have chemotherapy. Of 20 patients who received chemotherapy, 17 died of disease, and 3 were alive with disease. Of 50 patients who did not have chemotherapy, 36 died of disease, 11 were alive with disease, and 3 had spontaneous regression of disease. One patient in group A who had a spontaneous regression of disease for three years underwent a recurrence of disease. The second liver biopsy showed group A disease which was similar to his previous biopsy. Three months later, the disease regressed spontaneously. Chemotherapy and the presence of EBV RNA in the infiltrated lymphocytes had no association with the outcome of disease. Kaplan-Meier analyses in group A patients and in all groups also showed no significant difference in the survival profile of patients with or without chemotherapy (P values = 0.17 and 0.54, respectively).

Table 7. Outcome of 70 Patients (All Groups) with Respect to Chemotherapy and the Presence of EBV RNA in the Lymphocytes

Outcome	EBV RNA in the lymphocytes			*P* values*
	Positive (no. of cases)	Negative (no. of cases)	Total	
Dead				0.60
With chemotherapy	6	11	17	
Without chemotherapy	13	23	36	
Total	19	34	53	
Alive with disease				0.28
With chemotherapy	2	1	3	
Without chemotherapy	3	8	11	
Total	5	9	14	
Remission				
Spontaneous regression	1	2	3	

*'*P*' values were based on Fisher's exact test

Kaplan-Meier survival estimates of patients in group A, B, C, D, and in all groups are shown in Fig. 4. The median survival time in all groups was 2 months. The median survival time in groups A, B, C and D were 35 months, 1 month, 1 month, and 1 month, respectively. Group A patients had significantly better survival than groups B, C, and D in combined ($P < 0.00001$), but there was no significant difference in survival among groups B, C, and D.

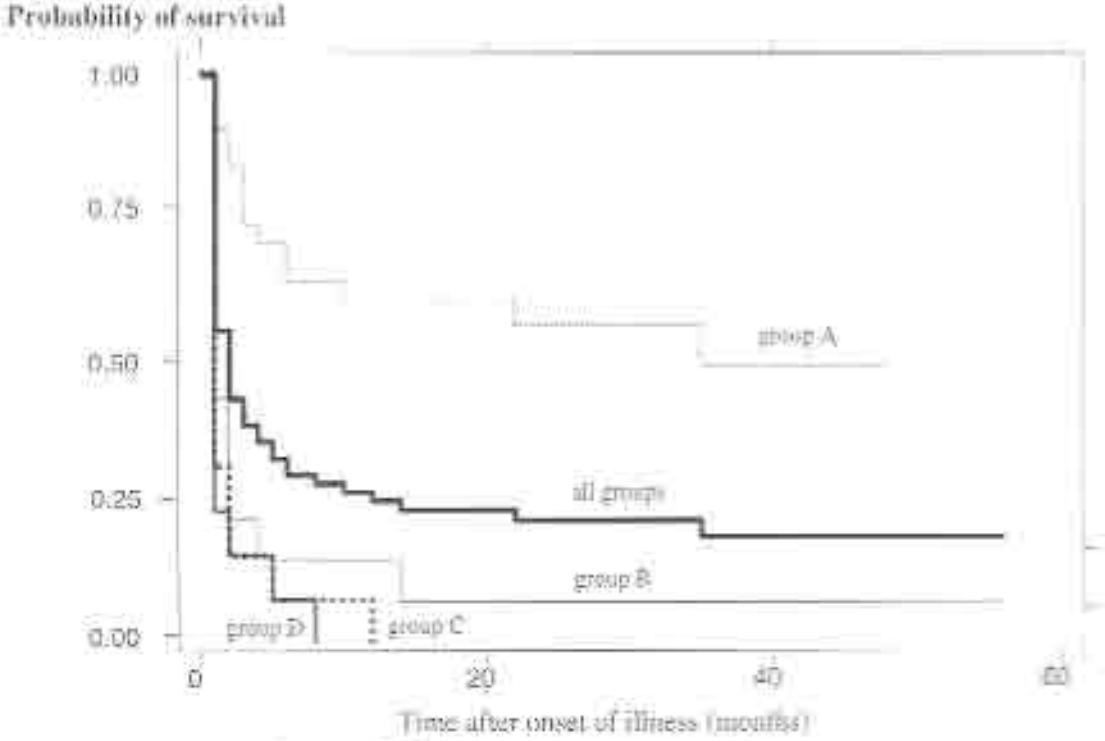

Fig. 4 Survival according to ABCD groups.

DISCUSSION

This group of patients had clinical signs and symptoms, and laboratory findings, similar to the previously described as so-called "malignant histiocytosis".[26] These included fever, weight loss, anemia, jaundice, lymphadenopathy, hepatosplenomegaly, and skin lesions. Laboratory investigation revealed anemia, pancytopenia, abnormal coagulograms, high serum levels of alkaline phosphatase and lactate dehydrogenase. Histopathology of the liver displayed infiltraction of T cells and other inflammatory cells into the hepatic sinusoids and portal tracts. The morphologic spectrum of T cells varied from small mature lymphocytes to the definite malignant lymphoid cells. The majority of the antigenic phenotypes of these T cells were $CD3^+$, $CD4^-$, $CD8^-$, $CD20^-$, $CD21^-$, $CD45RO^+$, $CD56^-$, and $TIA\text{-}1^+$. The liver histopathology was classified into four groups according to the morphology of the T-cell infiltrated. Group A and group B showed mature T cell infiltration. T-cell infiltrates in these two groups might be difficult to distinguish from the chronic inflammatory process. The clues for this were the presence of T cells or mixed cells in the dilated hepatic sinusoids, in combination with clinical signs and symptoms, and the significant laboratory findings. The clonality study and the TCR-sequence analysis confirmed the monoclonal rearrangement of these T-cell infiltrates. Group C showed atypical T cell infiltration, and the clonality study also confirmed the monoclonal rearrangement. In group D, definite malignant lymphoid infiltrates were demonstrated. The monoclonal band/s of TCR gene rearrangements were found in only two-thirds of the patients in this group.

The histopathologic classification of the liver was useful for predicting the outcome of disease. Groups B, C, and D had a poor prognosis; median survival was only 1 month. Nearly all of them died in a short period of time despite receiving multidrug chemotherapy. Group A patients had a better prognosis, with a median survival of 35 months. Spontaneous regression and relapse of disease in some patients were observed in this group. These concurred with previous reports of peripheral T-cell proliferative disease/lymphoma.[1, 2, 43, 44] There was no significant difference in the survival profile of patients with or without chemotherapy, although the early mortality meant that many patients either died before chemotherapy could be started or after only a short period of chemotherapy.

Epstein-Barr virus (EBV) infection is associated with several human malignancies such as Burkitt's lymphoma, Hodgkin's disease, post-transplantation lymphoproliferative disorders, nasopharyngeal carcinoma, gastric carcinoma, smooth muscle tumor in immuno-suppressed patients, thymic carcinoma, and various types of peripheral T-cell proliferative disease/lymphomas.[1, 2, 15-22, 45-51] In this report, we found EBV DNA and EBV RNA in the livers of 61.4% and 38.6% of the patients, respectively. EBV RNA was more commonly found in groups C and D. In a previous report,[11] we have shown that 65% of patients with various types of peripheral T-cell proliferative disease/lymphoma have EBV genomes in their circulating T cells, which is not found in any of the controls.

Those EBV-infected circulating T-cell subsets are $CD4^+$ cells, $CD8^+$ cells, or both $CD4^-$ and $CD8^-$ cells in a single patient. Dual EBV infection (wild-type, and 30bp deletion variant of the latent membrane protein-1 gene) in the peripheral blood $CD3^+$ cells was demonstrated in some cases. In this study, about 50% of the patients had multiple sites of involvement of peripheral T-cell proliferative disease/lymphoma, which were confirmed by the histological examination. Each site of disease in a single patient may had the same or different types of T-cell neoplasm according to the WHO classification on neoplastic diseases of hematopoietic and lymphoid tissues.[20] From the clonality study, we have shown that these tumors might either arise from a single clone, or from multiple clones of T cells. Our findings corresponded to previous reports on EBV-associated T-cell and B-cell neoplasms.[12, 22, 23] For the pathogenic mechanism of the EBV-associated peripheral T-cell proliferative disease/lymphoma, we postulate that the EBV which is dormant in the latently EBV-infected B cells may infect many subsets of T cells. An infected T cell may contribute to the pathogenesis of different types of T-cell neoplasm, or two or more EBV-infected T cells may pass through multistep tumorigenesis and terminate in different types of T-cell neoplasm.

Acknowledgements

This work was supported by the Thailand Research Fund (TRF), and supported in part by The Core University System Exchange Programme under the Japan Society for the Promotion of Science (JSPS), and Ministry of Education, Science, Culture, and Sports, Japan.

LEGENDS FOR FIGURES

Fig. 1 Photomicrographs of the liver biopsies; (A) is from group A, (B) is from group B, (C) is from group C, and (D) is from group D.

Fig. 2 Left: liver biopsy from a patient in group A. Right: skin biopsy from the same patient shows peripheral T-cell lymphoma, unspecified.

Fig. 3 PCR of the T-cell receptor-gamma gene amplification using a set of primers (Vγ I - Vγ III/IV - Jγ 1/2). Lanes a, b, and c are from a patient in group A which is shown in Fig. 2 (a = liver biopsy, b = liver necropsy, c = skin biopsy). Lanes d, e, and f are from another patient in group B (d = liver biopsy, e = lymph node biopsy; angioimmunoblastic T-cell lymphoma, f = base of the tongue biopsy; extranodal NK/T-cell lymphoma, nasal-type). M is a 20bp ladder markers. Monoclonal band's are detected in all specimens. Lane e is a different clone from lanes a and b. Lanes d, e, and f (upper band) are from the same clone.

REFERENCES

1. Mitarnun W, Suwiwat S, Pradutkanchana J, *et al.* Epstein-Barr virus-associated peripheral T-cell and NK-cell proliferative disease/lymphoma: a clinicopathologic, serologic and molecular anaylsis. *Am J Hematol* 2002;70:31-38.
2. Mitarnun W, Kietthubthew S, Suwiwat S. Hepatic peripheral T-cell lymphoma: a spectrum of liver pathology and clinical correlation. *J Med Assoc Thai* 1997;80:219-232.
3. Lukes RJ, Taylor CR, Parker JW, Lincole TL, Pattengale PK, Tindle BH. The morphologic and immunologic surface marker study of 229 cases of non-Hodgkin's lymphomas and related leukemia. *Am J Pathol* 1978;90:461-486.
4. Kadin ME, Berard CW, Namba K, Wakasa H. Lymphoproliferative diseases in Japan and western countries. *Hum Pathol* 1983;14:745-772.
5. Aozasa K, Ohsawa M, Tajima K, *et al.* Nationwide study of lethal midline granuloma in Japan: frequencies of Wegener granulomatosis, polymorphic reticulosis, malignant lymphoma and other related conditions. *Int J Cancer* 1989;44:63-66.
6. Arber DA, Weiss LM, Albujar PF, Chen YY, Jaffe ES. Nasal lymphoma in Peru, high incidence of T-cell immunophenotype and Epstein-Barr virus infection *Am J Surg Pathol* 1993;17:392-399.
7. Navarro-Roman L, Zarate-Osorno A, Meneses A, Kingma DW, Jaffe ES. High grade AIL and Epstein-Barr virus infection in 22 cases from Mexico. *Mod Pathol* 1994;7:117A.
8. Quintanilla-Martinez L, Lome-Maldonado C, Ott G, *et al.* Primary non-Hodgkin's lymphoma of the intestine: high prevalence of Epstein-Barr virus in Mexican lymphomas as compared with European cases. *Blood* 1997;89:644-651.
9. Jaffe ES, Harris NL, Debold J, Muller-Hermelink HK. World Health Organization classification of neoplastic diseases of hematopoietic and lymphoid tissues. A progress report. *Am J Clin Pathol* 1999;111(suppl.1): S8-12.
10. Harris NE, Jaffe ES, Stein H, *et al.* A revised European-American classification of lymphoid neoplasms: a proposal from the international lymphoma study group. *Blood* 1994;84:1361-1392.
11. Kimura H, Nagasaka T, Hoshino Y, *et al.* Severe hepatitis caused by Epstein-Barr virus without infection of hepatocytes. *Hum Pathol* 2001;32:757-762.
12. Nakagawa A, Ito M, Saga S. Fatal cytotoxic T-cell proliferation in chronic active Epstein-Barr virus infection in childhood. *Am J Clin Pathol* 2002;117:283-290.
13. Hauptmann S, Meru N, Schewe C, *et al.* Fatal atypical T-cell proliferation associated with Epstein-Barr virus infection. *Brit J Hemotal* 2001;112:377-380.
14. Quintanilla-Martinez L, Kumar S, Fend F, *et al.* Fulminant EBV+ T-cell lymphoproliferative disorder following acute/chronic EBV infection: a distinct clinicopathologic syndrome. *Blood* 2000;96:443-451.
15. Imai S, Sugiura M, Oikawa O, *et al.* Epstein-Barr vrius (EBV) – carrying and – expressing T-cell lines established from severe chronic active EBV infection. *Blood* 1996;87:1446-1457.
16. Ohga S, Kimura N, Takeda H, *et al.* Restricted diversification of T-cell in chronic active Epstein-Barr virus infection: potential inclination to T-lymphoproliferative disease. *Am J Hematol* 1999;61:26-33.
17. Jaffe ES, Krenacs L, Kumar S, Kingma DW, Raffeld M. Extranodal peripheral T-cell and NK-cell neoplasms. *Am J Clin Pathol* 1999;111(suppl.1): S46-55.

18. Tao Q, Ho FC, Loke SL, Srivastava G. Epstein-Barr virus is localized in the tumor cells of nasal lymphomas of NK, T or B cell type. *Int J Cancer* 1995;60:315-320.

19. Chan ACL, Ho JWY, Chung AKS, Srivastava G. Phenotypic and cytotoxic characteristics of peripheral T-cell and NK-cell lymphomas in relation to Epstein-Barr virus association. *Histopathology* 1999;34:16-24.

20. deBruin PC, Jiwa NM, Oudejans JJ, Radaszkiewicz T, Meijer CJLM. Epstein-Barr virus in primary gastrointestinal T-cell lymphoma: Association with gluten-sensitiveenteropathy, pathologic features, and immunophenotype. *Am J Pathol* 1995;146:861-867.

21. Ohshima K, Haraoka S, Harada N, *et al*. Hepatosplenic γδ T-cell lymphoma: relation to Epstein-Barr virus and activated cytotoxic molecules. *Histopathology* 2000;36:127-135.

22. Arnulf B, Copie-Bergman C, Delfau-Larue MH, *et al*. Non hepatosplenic γδ T-cell lymphoma: a subset of cytotoxic lymphomas with mucosal or skin location. *Blood* 1998;91:1723-1731.

23. Zettl A, Lee SS, Rüdiger T, *et al*. Epstein-Barr virus-associated B-cell lymphoproliferative disorders in angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma, unspecified. *Am J Clin Pathol* 2002;117:368-379.

24. Jaffe BS, Costa J, Fauci AS, Coventon J, Tsokos M. Malignant lymphoma and erythrophagocytosis simulating malignant histiocytosis. *Am J Med* 1983;75:741-749.

25. Cattoretti G, Viita A, Vezzoni P, Giardini R, Lombardi L, Rilke F. Malignant histiocytosis. A phenotypic and genotypic investigation. *Am J Pathol* 1990;136:1009-1019.

26. Warnke RA, Kim H, Dorfman RF. Malignant histiocytosis (histiocytic medullary reticulosis) I. Clinicopathologic study of 29 cases. *Cancer* 1975;35:215-230.

27. Wong KF, Chan JKC, Matutes E, *et al*. Hepatosplenic gamma-delta T-cell lymphoma. A distinctive aggressive lymphoma type. *Am J Surg Pathol* 1995;19:718-726.

28. Marsden PD, Hall MSR, Wilks NE, *et al*. An investigation of tropical splenomegaly at Mulago Hospital, Kampala, Uganda. *Brit Med J* 1965;1:89-92.

29. Bhattacharya DN, Harries JR, Emerson PA. Tropicalsplenomegaly syndrome (T.S.S.) in a European. *Trans R Soc Trop Med Hyg* 1983;77:221-222.

30. Dacie JV, Brain MC, Harrison CV, Lewis SM, Worlledge SM. Non-tropical idiopathic splenomegaly (Primary hypersplenism): a review of ten cases and their relationship to malignant lymphomas. *Brit J Heamatol* 1969;17:317-333.

31. Manoharan A, Bader LV, Finney WR. Non-tropical idiopathic splenomegaly (Dacie's syndrome). Report of 5 cases. *Scand J Haematol* 1982;28:175-179.

32. Blendis LM, Ansell ID, Jones KL, Hamilton E, Williams R. Liver inFelty's syndrome. *Brit Med J* 1970;1:131-135.

33. Schnitzer B. Sinusoidal hepatic infiltrates. *Lancet* 1976;2:258.

34. Miturnun W, Kietthubthew S, Sirwiwat S. Intrahepatocellular erythrocyte inclusions with hepatic sinusoidal infiltrates and splenomegaly. *Arch Pathol Lab Med* 1990;114:148-154.

35. Dhillon AP, Dusheiko GM. Pathology of hepatitis C virus infection. *Histopathology* 1995;26:297-309.

36. Kuno A, Abe M, Yamada M, Murakami K. Clinical and histological features of cytomegalovirus hepatitis in previous healthy adults. *Liver* 1997;17:129-132.

37. Boenisch T. Staining methods. In: Naish SJ, eds. Handbook of immunochemical staining methods. Capinteria, Calif: Dako Corp; 1989:13-18.

38. Kieff E, Leibowitz D. Epstein-Barr virus and its replication. In: Field BN, eds. Virology, 2nd ed. New York: Raven Press; 1990:1889-1920.

39. QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook. QIAGEN, Germany 1999:28-31.

40. Uthara H, Sato Y, Mukai K, *et al.* Detection of Epstein-Barr virus DNA in Reed-Sternberg cells of Hodgkin's disease using the polymerase chain reaction and *in situ* hybridization. *Jpn J Cancer Res* 1990;81:272-278.

41. McCarthy KP, Sloane JP, Kaharowski JHS, Manutes E, Wiedemann LM. The rapid detection of clonal T-cell proliferations in patients with lymphoid disorders. *Am J Pathol* 1991;138:821-828.

42. McCarthy KP, Sloane JP, Kaharowski JHS, Manutes E, Wiedemann LM. A simplified method detecting of clonal rearrangements of the T-cell receptor γ chain gene. *Diagn Mol Pathol* 1992;1:173-179.

43. Chott A, Augustin I, Wrba F, Hauck H, Ohlinger W, Radaszkiewicz T. Peripheral T-cell lymphomas: a clinicopathologic study of 75 cases. *Hum Pathol* 1990;21:117-125.

44. Pinkus GS, O'Hara CJ, Said JW. Peripheral/posthymic T-cell lymphomas: a spectrum of disease. Clinical, pathologic, and immunologic features of 78 cases. *Cancer* 1990;65:971-998.

45. Niedobitek G. Epstein-Barr virus infection in the pathogenesis of nasopharyngeal carcinoma. *J Clin Pathol: Mol Pathol* 2000;53:248-254.

46. Takada K. Epstein-Barr virus and gastric carcinoma. *J Clin Pathol: Mol Pathol* 2000;53:255-261.

47. McClain KT, Leach CT, Jenson HB, *et al.* Association of Epstein-Barr virus with leiomyosarcoma in young people with AIDS. *N Engl J Med* 1995;332:12-18.

48. Levrat H, Henle W, Chahinian AP, *et al.* Association of Epstein-Barr virus with thymic carcinoma. *N Engl J Med* 1985;312:1296-1299.

49. Flavell KJ, Murray PG. Hodgkin's disease and the Epstein-Barr virus. *J Clin Pathol: Mol Pathol* 2000;53:262-269.

50. Zur Hausen H, Schulte-Holthausen H, Klein G, Henle W, Henle G, Clifford P, Santesson L. EBV DNA in biopsies in Burkitt tumors and anaplastic carcinoma of the nasopharynx. *Nature* 1970;228:1056-1058.

51. Craig FE, Gulley ML, Banks PM. Posttransplantation lymphoproliferative disorder. *Am J Clin Pathol* 1993;99:265-271.

52. Martin-Subero JI, Siebert R, Harder L, *et al.* Cytogenetic and molecular characterization of a patient with simutaneous B-cell chronic lymphocytic leukemia and peripheral T-cell lymphoma. *Am J Hematol* 2001;68:276-279.

รายงานฉบับที่ 5

Epstein-Barr Virus-Associated Peripheral T-cell Lymphoma with Gastrointestinal Tract Involvement

บทคัดย่อ

Peripheral T-cell lymphoma (PTCL) เป็นกลุ่มโรคที่พบมากในเอเชียและกลุ่มประเทศในละตินอเมริกากลางและอเมริกาใต้ กลุ่มโรคนี้มีความสัมพันธ์กับการติดเชื้อไวรัสเอ็ปสไตน์-บาร์ (EBV) การศึกษานี้เพื่อประเมินผู้ป่วยที่มีโรค PTCL ในระบบทางเดินอาหาร โดยศึกษาอาการทางคลินิก การดำเนินโรค การเปลี่ยนแปลงทางพยาธิวิทยา ศึกษาในระดับโมเลกุลของการติดเชื้อไวรัส EBV และศึกษาโคลน (clone) ของเซลล์มะเร็ง การศึกษานี้เริ่มตั้งแต่ตุลาคม พ.ศ.2540 จนถึงเดือนธันวาคม พ.ศ.2543 พบผู้ป่วยโรค PTCL ชนิดต่างๆ 129 ราย ในจำนวนนี้ 7 ราย (5.4%) พบรอยโรค PTCL ในระบบทางเดินอาหาร อาการสำคัญของผู้ป่วยที่มีรอยโรค PTCL ในระบบทางเดินอาหารที่ใช้เรื่อรัง น้ำหนักลด ซีด ตับและม้ามโต ต่อมน้ำเหลืองโตมีรอยโรคในพลาสคาวิตี้ และมีเลือดออกในทางเดินอาหาร การตรวจเลือดพบว่ามีการเพิ่มขึ้นของเอ็นซัยม์ alkaline phosphatase และ lactate dehydrogenase และมีความผิดปรกติในระบบการแข็งตัวของเลือด ผู้ป่วย 5 ใน 7 รายเสียชีวิตภายใน 4 เดือนหลังประกฏอาการครั้งแรก ผู้ป่วยอีก 2 ราย หายขาดจากโรคหลังการได้รับยาเคมีบำบัด ผู้วิจัยได้แบ่งลักษณะของเซลล์มะเร็งเป็น 3 ประเภท ประเภทที่มีเซลล์มะเร็งขนาดเล็ก ประเภทที่มีเซลล์มะเร็งขนาดปานกลางผสมกับขนาดใหญ่ และประเภทที่มีเซลล์มะเร็งขนาดใหญ่ ผลการตรวจโปรตีนที่อยู่บนผนังเซลล์ของเซลล์มะเร็งพบว่าเป็นชนิด LCA$^+$, CD3$^-$, CD15$^-$, CD16$^-$, CD30$^+$, CD45RO$^+$, CD57$^-$, CD68$^-$, EMA$^+$, βF1$^-$, granzyme B$^+$, TIA$^+$, และ p53$^+$, สำหรับ CD4, CD8, CD56, CD20 บางรายให้ผลบวกและบางรายให้ผลลบ เซลล์มะเร็งของผู้ป่วยทั้ง 7 รายตรวจพบ EBV RNA ในนิวเคลียสของเซลล์ การตรวจโดยวิธี PCR ของ T-cell receptor (TCR) β และหรือ γ gene rearrangement ปรากฏว่าให้ผลบวกในผู้ป่วยทั้ง 7 ราย และการวิเคราะห์สาย DNA ซึ่งเป็นผลผลิตโดยวิธี PCR ของ TCR ดังกล่าวข้างต้น พบว่าลำดับสาย DNA มีความคล้ายคลึงกับ human TCR germline gene มาก การวิจัยนี้แสดงว่าการเกิด PTCL ในระบบทางเดินอาหาร มีความสัมพันธ์กับการติดเชื้อไวรัส EBV, เซลล์มะเร็งเป็น mature T-cell ที่แสดงโปรตีนของ NK-cell บางชนิดบนผนังเซลล์ กลุ่มเซลล์มะเร็งในผู้ป่วยแต่ละรายมาจากเซลล์เพียงหนึ่งโคลน และเป็นเซลล์ที่มีการ rearrange ของยีน TCR แล้ว

SUMMARY

Aims: Peripheral T-cell lymphoma (PTCL) is a group of diseases which is common in Asia and areas of South and Central America. They are highly associated with the Epstein-Barr virus (EBV) infection. In this study we evaluate patients of gastrointestinal involvement of PTCL with respect to clinical findings and outcome, pathologic features, and molecular analysis for EBV infection and the clonality of tumor cells.

Methods and results: From January 1997 through December 2000, we identified 7 patients with gastrointestinal tract involvement of PTCL. The frequency of gastrointestinal tract involvement in the various types of PTCL was 5.4% (7 of 129 cases). The pertinent clinical features were a prolonged fever, weight loss, anemia, hepatosplenomegaly, lymphadenopathy, multiorgan involvement, and gastrointestinal bleeding. Laboratory results showed a significantly high serum level of alkaline phosphatase and lactate dehydrogenase, and abnormal coagulograms. Five patients died within 4 months after onset of illness, while two were in complete remission after chemotherapy. The tumor cell morphology was classified into three categories: small-sized cells, mixed medium- and large-sized cells, and large-sized cells. The antigenic phenotypes of the tumor cells were LCA+, CD3+, CD15-, CD16-, CD30-, CD45R0+, CD57-, CD68-, EMA-, βF1-, granzyme B+, TIA-1+, and p53+. The expression of CD4, CD8, CD56 and CD20 was variable. EBV-RNA expression by *in situ* hybridization (EBER-ISH) study was positive and T-cell receptor (TCR) beta and/or gamma gene rearrangements were detected in all patients. DNA sequence analysis showed high identity to the human TCR germline gene.

Conclusions: PTCL with gastrointestinal tract involvement is associated with EBV infection. The tumor cells are mature T cells with some NK-cell antigenic expression and all demonstrate TCR gene rearrangements.

INTRODUCTION

Peripheral T-cell proliferative diseases/lymphomas are more commonly found in Asia than in western countries.[1-4] They also have been reported with significant frequency in Central and South America.[5-7] This suggests a racial and/or environmental connection for the disease. Peripheral T-cell proliferative disease/lymphoma exhibits considerable heterogeneity in clinical finding, morphologic pattern, immunology, and prognosis.[4, 8-11] In 1993, the International Lymphoma Study Group proposed a classification for lymphoid neoplasms, entitled, "A Revised European-American Classification of Lymphoid Neoplasms, (REAL classification), by using morphologic, immunologic, and genetic techniques.[12, 13] Recently, the World Health Organization (WHO) classification was proposed.[14] The mature NK/T-cell neoplasm classification in the proposed WHO scheme was largely adapted from the REAL

classification. The WHO classification for predominantly extranodal malignant neoplasms was separated into four prototypes: extranodal NK/T-cell lymphoma, nasal type, enteropathy-type intestinal T-cell lymphoma, subcutaneous panniculitis-like T-cell lymphoma, and hepatosplenic γδ T-cell lymphoma.[12] The involvement of peripheral T-cell lymphomas in the gastrointestinal tract may be found in three categories: enteropathy-type intestinal T-cell lymphoma, extranodal NK/T-cell lymphoma, nasal type, and gastrointestinal γδ T-cell lymphoma.[12-17] Of these, only the enteropathy-type intestinal T-cell lymphoma is considered to be a primary gastrointestinal tract T-cell lymphoma.[14]

Gastrointestinal lymphoma accounts for 4% to 20% of all non-Hodgkin's lymphomas and is the most extranodal site of presentation.[9] The stomach is the major organ involved in gastrointestinal lymphoma. B-cell lymphoma of mucosal-associated lymphoid tissue (MALT) type is the commonest, and gastric MALT lymphoma may be associated with *Helicobacter pylori* and may undergo complete remission following eradication of *H. pylori*.[10] In the western countries, primary gastrointestinal lymphomas account for approximately 10% to 15% of non-Hodgkin's lymphoma cases, and the enteropathy-type intestinal T-cell lymphoma is the most common type.[18-20]

Nineteen cases of gastrointestinal peripheral T-cell lymphoma which were reported from east Asia (Korea, Japan, China), Morocco, and Mexico were 100% positive for Epstein-Barr virus (EBV) RNA by *in situ* hybridization study in most of the tumor cells. Of these, three cases from Mexico were enteropathy-type intestinal T-cell lymphoma, and one case from Morocco was gastrointestinal γδ T-cell lymphoma.[7, 21-23] Sixty-six cases of gastrointestinal T-cell lymphoma which were reported from western Europe, and one case from the United States of America, showed only 16% positive for EBV RNA in the tumor cells.[3, 28-31] The majority of patients in the later series were cases with enteropathy-type intestinal T-cell lymphoma.

Enteropathy-type intestinal T-cell lymphoma is a specific peripheral T-cell lymphoma that occurs in association with celiac disease.[27, 32, 33] Celiac disease is relatively common in Europe. It occurs worldwide, but is quite rare in Asians and black Africans.[33, 34] In one study, about 7% of patients with celiac disease developed enteropathy-type intestinal T-cell lymphoma in a 25-year follow-up.[27] The enteropathy-type intestinal T-cell lymphoma, to our knowledge, has not been reported from Asia.

MATERIALS AND METHODS

The prospective study was conducted in Songklanagarind University Hospital, Songkhla, Thailand, during the 4-year period of January 1997 through December 2000 and follow-up continued until December 2001. There were 129 patients with various types of peripheral T-cell proliferative disease/lymphoma. Seven cases were found to have gastrointestinal tract lesions, and all of them were confirmed by histologic examination.

Immunohistochemistry

Immunohistochemical stainings for leukocyte common antigen (LCA), CD3, CD4, CD8, CD15, CD16, CD20, CD30, CD45RO, CD56, CD57, CD68, T-cell receptor αß (βF1), epithelial membrane antigen (EMA), granzyme B, T-cell intracellular antigen-1 (TIA-1), and p53 protein of tumor tissues were performed on formalin-fixed, paraffin embedded tissue using the following antibodies: monoclonal mouse antihuman LCA (CD45) (Zymed, CA, USA, 1:100), monoclonal mouse antihuman CD3 (Novocastra, UK, 1:120), monoclonal mouse antihuman CD4 (Novocastra, 1:25), monoclonal mouse antihuman CD8 (Dako, Denmark, 1:20), monoclonal rabbit antihuman CD15 (Zymed, 1:50), monoclonal mouse antihuman CD16 (Novocastra, 1:30), monoclonal mouse antihuman CD20 (Dako, 1:70), monoclonal mouse antihuman CD 30 (Dako, 1:20), monoclonal mouse antihuman CD45RO (Zymed, 1:70), monoclonal mouse antihuman CD56 (Novocastra, 1:50); neuroblastoma was used as a positive control, monoclonal mouse antihuman CD57 (Novocastra, 1:50), monoclonal mouse antihuman CD68 (Dako, 1:100), monoclonal mouse antihuman βF1 (Endogen, MA, USA, 1:10), monoclonal mouse antihuman EMA (Dako, 1:20), monoclonal mouse antihuman granzyme B (Novocastra, 1:60), monoclonal mouse antihuman TIA-1 (Coulter, France, 1:30), p53 protein (Dako, 1:250), rabbit antimouse immunoglobulin (Dako, 1:200), swine antirabbit immunoglobulin (Dako 1:25), and horseradish peroxidase-mouse antiperoxidase (Dako, 1:100). The staining procedure was performed as previously described.[28] A tumor was considered p53 positive when more than 50% of the tumor cell nuclei stained intensely with anti-p53.

In situ Hybridization

An *in situ* hybridization (ISH) study for the Epstein-Barr virus (EBV) 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.[36] Appropriate positive and negative controls were run. ISH for EBV RNA was interpreted as positive when the positively stained tumor nuclei were more than 10% of the tumor cell population. The amount of positive cells between 10-25% was considered +1; 26-50%, +2; 51-75%, +3; and more than 75% was considered +4.

DNA Extraction from Tissue for PCR Analysis

DNA extraction was performed using QIAamp DNA Mini Kit (QIAGEN, Germany). Three to five pieces of tissue section (3 to 5 microns) from paraffin blocks were taken, deparaffinized, resuspended in 180 µl of buffer ATL, digested with 50 µl of proteinase K, mixed by vortexing, and incubated at 55°C until the tissue was completely lysed. Further steps were followed as directed by the manufacturer's manual of tissue protocol.[37] The presence of DNA was confirmed by agarose-gel electrophoresis or by amplification of β–globin gene.

Polymerase Chain Reaction (PCR) Analysis

For the clonality study of the tumor cells, a T-cell receptor (TCR)-β chain and TCR-γ chain genes were used. Amplification of the TCR-β chain gene was carried out using the technique described by McCarthy *et al*.[38] We used three separate reactions with the following oligonucleotide primers combinations: Dβ2+Jβ2.3, Dβ2+Jβ2.6, and Dβ2+Jβ2.7. For the monoclonal rearrangement of tumor cells, one or two clearly visible band/s with the expected size range (55 to 100 bp) were identified. Amplification of the TCR-γ chain gene was performed using the PCR technique as described by McCarthy *et al*.[39, 40] We used two separate reactions with the following oligonucleotide primers combinations: Vγ I/Vγ II/IV + Jγ 1/2 (product sizes 70-95 bp), and Vγ II+Jγ1/2 (product sizes approximately 150-180 bp). For the monoclonal rearrangement, one or two cleary visible band/s were identified. DNA extracted from Raji and U8312 (a human T-cell lymphotropic virus type-1 positive cell line) were used as controls.

DNA sequence analysis of TCR genes

The purified PCR products of TCR-β chain and TCR-γ chain genes were used as a template for automated sequencing analysis by using a ABI PRISM Dye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase, FS (ABI PRISM™ Ready Reaction, Applied Biosystems Inc., CA, USA). The purified template (approximately 500 ng) was mixed with 8 µl of Terminator premix, 3.2 pmole of a sequencing primer and deionized water

to a total volume of 20 μl. The mixture was overlaid with one drop of mineral oil and placed in the thermal cycler preheated to 96° C. Thermal cycling was performed on a Perkin-Elmer Cetus thermal cycler model 480 under the following conditions: 96° C denaturation for 15 sec, 60° C annealing for 15 sec and 60° C extension for 4 min with 25 total cycles. After the last cycle, the extension product was precipitated and redissolved in 4 μl of loading buffer containing 5:1 (vol/vol) of deionized formamide with 50 mM EDTA, pH 8.0 and dextrane blue. The reaction tube was heated at 90° C for 2 min before loading onto a 6% polyacrylamide sequencing gel for analysis in the Applied Biosystems Automated DNA sequencer, model 373A. The sequence obtained from each run was analysed with the assistance of SeqEd, a software program (Applied Biosystems Inc.) and compared with the sequence in Genbank using the Blast program.

RESULTS

Clinical Features

Clinical data, pathologic findings, therapeutic interventions and outcomes of the patients are summarized in Table 1, and the pertinent laboratory findings are summarized in Table 2. Of the 129 patients with various types of peripheral T-cell proliferative disease/lymphoma, seven (5.4%) patients were found to have gastrointestinal tract lesions (4 males and 3 females, ranging in age from 23-71 years). All of these seven patients showed a prolonged fever which had lasted from one to six months. Five patients had hepatosplenomegaly; of these, four (case nos. 2, 3, 4, 5) were histologically confirmed to have peripheral T-cell lymphoma involving the liver. Three patients had peripheral T-cell lymphoma in the lymph nodes. Bone marrow involvement was found in three patients (case nos. 2, 4, 5). Case no. 7 had an extranodal NK/T-cell lymphoma, nasal type, in the nasopharynx two years before the gastrointestinal symptoms developed. Case no. 3 had peripheral T-cell lymphoma at the base of the tongue. An autopsy from case no. 5 revealed multiorgan involvement of peripheral T-cell lymphoma which included the liver, spleen, brain, and both adrenal glands.

The hemograms revealed anemia in all cases. The numbers of white blood cells and platelets varied. Most of these patients had high serum levels of alkaline phosphatase and lactate dehydrogenase, and abnormal prolonged coagulograms.

Of three patients who received multidrug chemotherapy, two (case nos. 2, 6) achieved a complete remission in the 46-month and 42-month follow-up, and one of them (case no. 7) died. In four patients who did not have chemotherapy, the disease followed a rapidly progressive course and all of them died within one to four months after the first onset of illness.

Table 1. Clinical and histologic findings

Patient no	Age(yr)/ sex	Presenting symptom and sign	Operation site	Gross and size	Histologic type	Histologic finding in other site	Postoperative therapy	Follow-up/ outcome
1	28/M	High-grade fever 2 mos, hepatosplenomegaly, lymphadenopathy, ascites, bowel perforation	Terminal ileum and right half colon resection	Bulky tumor at ileum and caecum 3x4x8 cm, caecal perforation	SC	PTCL in lymph node	NA	DOD, 4 mos
2	23/F	Low-grade fever and bloody diarrhea 1 mo, hepatosplenomegaly	Duodenum and ileum biopsy	Edema of small bowel mucosa	SC	PTCL in liver; RH in lymph node	CHOP	CR, 46 mos
3	71/F	High-grade fever 1 mo, weight loss 10 kg, bloody diarrhea, hepatosplenomegaly	Terminal ileum and right half colon resection	Multiple ulcers at ileum upto 5x2 cm, ileal perforation	SC	PTCL in liver; ABLD in lymph node; PTCL of the tongue	NA	DOD, 2 mos
4	28/M	High-grade fever 1 mo, bloody diarrhea 1 week, hepatosplenomegaly	Jejunum, ileum and right half colon resection	Multiple infiltrative lesions and ulcers (up to 3 cm) in jejunum, ileum and colon	MLC	PTCL in liver	NA	DOD, 2 mos
5	27/M	High-grade fever 1 mo, weight loss 5 kg, bloody diarrhea, hepatosplenomegaly	Terminal ileum and total colon resection	Multiple small infiltrative lesions and ulcers in entire colon	MLC	Autopsy showed PTCL in stomach and small bowel, liver, lymph nodes, spleen, bone marrow, brain and adrenal glands	NA	DOD, 1 mo
6	34/F	Fever and abdominal pain 6 mos, weight loss 7 kg	Duodenum and portion of jejunum resection	An ulcer in duodenum, 5x3 cm	LC		CHOP	CR, 42 mos
7	51/M	High-grade fever 1 mo, maculopapular rash and skin ulcers on body and face, status post radiation therapy for NK/T in nasopharynx 2 yrs ago, bloody diarrhea 1 day	Left half colectomy; biopsy from duodenum and jejunum	Multiple small infiltrative lesions and ulcers (0.7-1.2 cm) in colon	LC	Cutaneous T-cell lymphoma; RH in lymph node	Vincristin, Endoxan	DOD, 2 mos

Abbreviations: DOD = dead of disease, CR = complete remission after chemotherapy, NA = not applicable, SC = small-sized cell, MLC = mixed medium- and large-sized cells, LC = large-sized cell, PTCL = peripheral T-cell lymphoma, RH = reactive hyperplasia, ABLD = angioimmunoblastic T-cell lymphoma, NK/T = extranodal NK/T-cell lymphoma, nasal type.

Table 2. Laboratory findings

Test	Case No.						
	1	2	3	4	5	6	7
Hemoglobin, g/L	85	82	83	103	81	81	88
White blood cell count, $x10^9$/L	2.5	24.6	1.2	9.5	5.7	4.7	10.5
Platelets, $x10^9$/L	167	574	262	26	66	305	330
Total protein, g/L	45	48	50	30	46	61	57
Albumin, g/L	21	24	29	19	13	30	33
Total bilirubin, μmol/L	23.8	8.5	22.1	251.7	132.6	6.8	10.8
Conjugated bilirubin, μmol/L	19.2	3.4	13.7	161.5	107.2	5.4	3.4
Aspartate aminotransferase, U/L (0-35)	68	46	54	1970	15	15	38
Alanine aminotransferase, U/L (0-35)	27	43	27	161	14	9	35
Alkaline phosphatase, U/L (30-120)	287	218	235	266	1003	80	55
Lactate dehydrogenase, U/L (230-460)	512	280	504	ND	356	491	ND
Activated PTT, s (28-31)	69	34	ND	64.6	54	26.5	37.6
Prothrombin time, s (13-15)	25.5	13	ND	21.3	38.5	13.3	16.7
Anti HIV (by ELISA)	ND	neg	neg	neg	neg	neg	neg

Abbreviations : ND, not done; neg, negative; PTT, partial thromboplastin time

Pathological Features

Gross pathology The major tumor sites in the gastrointestinal tract were the ileocaecal region and colon. (Fig.1). Lesions in the stomach, duodenum, and jejunum were also found in some cases. In most cases, multicentric lesions involving different areas of infiltrative and ulcerative lesions were the major feature. However, two patients (case nos. 1, 6) had a single lesion. Perforation of the bowel was seen in cases 1 and 3.

Histopathology There was a wide spectrum of cytologic morphology of tumor cells ranging from small-sized to large atypical and hyperchromatic cells. There was also an admixture of these types of cells. Some may be difficult to distinguish from an inflammatory process. Infiltrations with eosinophils, monocytes, and plasma cells were variable. We classified the tumor morphology into three categories: small-sized cells, mixed medium- and large-sized cells, and large-sized cells.

Small-sized cells (Fig. 2): the infiltrate was composed of small round lymphocytes, but there was no marginal zone pattern. Necrosis or angiocentric pattern were rarely observed. Infiltration with eosinophils and plasma cells varied from mild to moderate. The lesions were confined mainly to the mucosa and upper part of the muscular wall.

Mixed medium- and large-sized cells (Fig. 3): the infiltrate was composed of medium-sized cells, large atypical hyperchromatic cells, and a few small-sized cells. An angiocentric pattern and coagulative necrosis were prominent features. Infiltration with eosinophils, monocytes and plasma cells varied from mild to massive. The lesions were located mainly in the mucosa and occasionally extended to the muscular wall and adventitia.

Large-sized cells (Fig. 4): The infiltrate was mostly composed of large-size cells. The cells were pleomorphic, and had hyperchromatic nuclei and abundant mitoses. An angiocentric pattern and coagulative necrosis were very prominent features. Apoptotic cells were seen in large numbers. Infiltration of monocytes with massive hemophagocytosis was observed in the tumor areas, and in the lumen of nearby vessels. Infiltration with eosinophils and plasma varied from minimal to absent.

Cases 1, 2 and 3 were classified as small-sized cells, cases 4 and 5 as mixed medium- and large-sized cells, and cases 6 and 7 as large-sized cells. In the group of small-sized cells, none showed an angiocentric pattern and only case 1 showed coagulative necrosis. An angiocentric pattern and coagulative necrosis were found in all patients with the histologic types of mixed medium- and large-sized cells, and large-sized cells. The adjacent mucosal tissue and/or the uninvolved small bowel mucosa in all cases revealed no evidence of celiac disease.

Immunohistohemistry Results of the immunohistochemistry are summarized in Table 3. The phenotypes of tumor cells in these cases were LCA+, CD3+ (membrane pattern), CD15-, CD16-, CD30-, CD45RO+, CD57-, CD68-, βF1-, EMA-, granzyme B+, TIA-1+, and p53+. The expression of CD4, CD8, CD56 and CD20 was variable. One case expressed CD4,

Table 3. Phenotypes and EBV RNA (EBER) of tumor cells in 7 patients

Case No.	Phenotypes																	EBV RNA
	LCA	CD3	CD4	CD8	CD15	CD16	CD20	CD30	CD45RO	CD56	CD57	CD68	βF1	EMA	GrB	TIA-1	p53	
1	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−2
2	−	−	ND	−	−	ND	−	−	−	ND	−	−	ND	ND	ND	ND	ND	−2
3	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−3
4	+	−	+	+	−	−	+	−	−	−	−	−	−	−	−	+	+	+4
5	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+4
6	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+4
7	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+4

Abbreviations: LCA, leukocyte common antigen; GrB, granzyme B; ND, not done.

three cases expressed CD8, and one case expressed CD56. Case no. 4 expressed both CD20 and CD3, which are the markers of B cell and T cell, respectively.

In situ hybridization. The presence of EBV RNA (EBER) in tumor cells was detected in all cases, ranging from +2 to +4 (Table 3). Tumors of the mixed medium- and large-sized cells, and the large-sized cells morphologies, showed a strongly positive reaction (Fig. 4, inset).

Clonality study. Results of the clonality studies of these tumor cells are shown in Figures 5 and 6. By using three sets of oligonucleotide primers for TCR-β gene, the monoclonal band was detected in four cases; case nos. 1, 2, 3 and 6. (Fig. 5). For the TCR-γ gene, the monoclonal band/s were detected in five cases; case nos. 1, 4, 5, 6 and 7. (Fig. 6).

TCR sequence analysis. Results of the TCR gene- sequence analysis (sense strand in case nos. 1, 2, 3 and anti-sense strand in case nos. 4, 5, 6, 7) are summarized in Table 4. All of these cases confirmed the monoclonality. PCR products had a very high identity to the human TCR germline gene.

DISCUSSION

Peripheral T-cell proliferative disease/lymphoma has been recognized as a common group of diseases in Southern Thailand.[4,41-43] In our institute, a 700-bed hospital, we found 129 cases in our 4-year prospective study. Of these, 7 had gastrointestinal tract involvement. The patients presented with prolonged fever, weight loss, anemia, hepatosplenomegaly, abnormal prolonged coagulograms, and significantly high serum levels of alkaline phosphatase and lactate dehydrogenase. All four patients with liver involvement of peripheral T-cell lymphoma showed high serum levels of alkaline phosphatase. The pertinent gastrointestinal symptom was bloody diarrhea, and some patients had bowel perforation.

Gastrointestinal non-Hodgkin's lymphoma accounts for 4% to 20% of all non-Hodgkin's lymphoma cases, and is the most common extranodal site of presentation.[44] Intestinal B-cell lymphomas appear as polypoid masses or annular lesion in the distal and terminal ileum.[20] Enteropathy-type intestinal T-cell lymphomas in western countries are mainly found in the proximal small intestine, in contrast to intestinal T-cell lymphomas in Asia and in the presented study, which tend to be located in the terminal ileum and the large intestine.[20,21]

Extranodal peripheral T-cells and NK-cell neoplasms are classified into 4 major categories: nasal, intestinal, subcutaneous panniculitis-like, and hepatosplenic γδ.[23] EBV-positive extranodal NK/T-cell lymphoma, nasal type, which frequently involves the intestine as a secondary site, is almost always CD56+ (70% to 100%), CD3 (membrane)-, CD3 (cytoplasm)+, high prevalence of p53 overexpression, and lacks features associated with the clonal rearrangement of TCR-β and TCR-γ. CD16 expression varies from 10% to 60%.[13, 45-50] Based on the morphology, the disease is characterized by a broad cytologic spectrum, ranging from cytologically benign, which cannot be distinguished from an inflammatory process, to the definite malignant morphology.[13] An angiocentric pattern on histologic examination is a common feature, and necrosis is seen in most cases.[45]

Table 4. Results of TCR gene-sequence analysis

Case	Primers	PCR product (bp)	Part of sequencing product (bp)	Homology site of TCR germline gene	Identity bp (percent)
1	Vγ1 + VγII/IV + Jγ 1/2	70-95	43	gi/5566238/gb/AF 159056.1 (Vγ1 regions)	34/34 (100%)
2	D2 + Jβ2.3	80-90	59	gi/1197256/emb/x86129.1 (71-116)	46/46 (100%)
3	D2 + Jβ2.6	80-90	68	gi/338852/gb/M 14159.1 (2099-2148)	46/50 (92%)
4	Vγ1 + Jγ 1/2	160-180	140	gi/5566238/gb/AF 159056.1 (56793-56911)	117/119 (98%)
5	Vγ1 + Jγ 1/2	160-180	141	gi/5566238/gb/AF 159056.1 (56793-56924)	131/132 (99%)
6 (upper band)	Vγ1 + VγII/IV + Jγ 1/2	80-90	62	gi/3047019/gb/AF 057177.1 (Vγ1 regions)	30/30 (100%)
6 (lower band)	Vγ1 + VγII/IV + Jγ 1/2	70-80	53	gi/3047019/gb/AF 057177.1 (Vγ1 regions)	29/29 (100%)
7	Vγ1 + VγII/IV + Jγ 1/2	70-95	43	gi/5566238/gb/AF 159056.1 (Vγ1 regions)	19/19 (100%)

In gastrointestinal tract involvement of peripheral T-cell lymphomas, granzyme B-positive cells are mainly associated with enteropathy-type intestinal T-cell lymphoma. A total of 77% of enteropathy-type intestinal T-cell lymphoma cases versus 14% of the non-enteropathy-type intestinal T-cell lymphoma cases are granzyme B positive in the tumor cells.[9] Although all of our six cases studied were positive for granzyme B and TIA-1, the clinical history and the adjacent mucosal tissue and/or the uninvolved small intestine mucosa in all cases failed to reveal evidence of celiac disease, which is the preceding lesion of the enteropathy-type intestinal T-cell lymphoma.[12,13]

The antigenic phenotype of this reported series showed T-cell markers with some NK-cell antigenic expression. The NK-cell-associated marker CD56, which has been considered as a universally positive marker in the extranodal NK/T-cell lymphoma, nasal type, was negative in 5 of 6 cases in our series. The neuroblastoma positive control and the internal positive control (neurons in tissue section) gave a strongly positive reaction. The tumor cells showed no expression of CD16, whereas the activated macrophages which infiltrated in the tumor area showed intense membrane staining. The intracellular cytotoxic molecules (granzyme B, TIA-1), and the p53 expression, were detected in all tested cases. In this study, the cases were all positive for the EBV genome, similar to the previous reports of extranodal NK/T-cell lymphoma, nasal type, but there were evidences against the diagnosis in that they all demonstrated T-cell receptor gene rearrangements, and a majority of cases failed to express CD56. The findings suggest that these cases were not extranodal NK/T-cell lymphoma, nasal type, eventhough case no.1 had a preceding extranodal NK/T-cell lymphoma, nasal type, in the nasopharynx. Another important finding was the expression of CD20 in case no. 4. This concurred with our previous report on a high-grade hepatic peripheral T-cell lymphoma which showed expression of both CD45RO and CD20 in 3 of 8 cases,[4] and a recent case report of peripheral T-cell lymphoma with aberrant expression of CD20 and CD79a.[14]

All of our seven cases demonstrated either TCR-β or TCR-γ gene rearrangements. Cases 2 and 3 had only the rearrangement of the TCR-β locus, and cases 4, 5 and 7 had only the rearrangement of the TCR-γ locus. This finding was slightly different from a previous report of peripheral T-cell lymphomas where the TCR-β locus was found to be rearranged in 78% of cases versus 96% of the TCR-γ locus.[15] The TCR sequence analysis in all cases confirmed the monoclonality and had a high identity to the human TCR germline gene. This indicated the neoplastic nature instead of an inflammatory process of peripheral (mature) T cell in our cases.

ACKNOWLEDGEMENTS

This study was supported by the Thailand Research Fund (TRF), grant no. BRG/7/2542, and was supported in part by the Core University System Exchange Programme under the Japan Society for the Promotion of Science (JSPS), coordinated by the University of Tokyo Graduate School of Medicine, Japan, and Prince of Songkla University, Faculty of Medicine, Thailand.

LEGEND FOR FIGURES

Fig. 1 Case 5. Macroscopic view of the entire colon shows multiple small infiltrative lesions with areas of hemorrhage and ulceration.

Fig. 2 Small-sized cells tumor. The majority of tumor cells are small with round to only slightly irregular nuclei and have a homogeneous pattern.

Fig. 3 Mixed medium- and large-sized cells tumor. The tumor cells are medium-sized and large-sized cells with atypical hyperchromatic nuclei. Note the angiocentric pattern.

Fig. 4 Large-sized cells tumor. Angiocentric pattern is a prominent feature. The tumor cells are pleomorphic, and have hyperchromatic nuclei and abundant mitoses. Inset, the *in situ* hybridization showing EBV-RNA in the nuclei of the tumor cells

Fig.5 PCR T-cell receptor-beta gene amplification using three pairs of primers: D2+Jβ2.3 (a), D2+J β2.6 (b), and D2+ J β2.7 (c). Lanes 1 through 7 are cases 1 to 7 respectively. Lane R is Raji control, Lane N is negative control, M is ladder markers. The monoclonal band is detected in cases 1, 2, 3, and 6, (10% acrylamide gel).

Fig. 6 PCR T-cell receptor-gamma gene amplification using two sets of primers: Vγ I+Vγ III/IV+Jγ1/2 (a), and Vγ II+Jγ1/2 (b). Lanes 2 through 7 are cases 2 to 7 respectively. Lane U is U8312 positive control, Lane N is negative control, M is ladder markers. The monoclonal band is detected in cases 4, 5, 6, and 7, (case 1 not amplified, 10% acrylamide gel).

REFERENCES

1. Lukes RJ, Taylor CR, Parker JW, Lincoln TL, Pattengale PK, Tindle BH. The morphologic and immunologic surface marker study of 329 cases of non-Hodgkin's lymphomas and related leukemias. *Am J Pathol* 1978;90:461-486.
2. Kadin ME, Berard CW, Namba K, Wakasa H. Lymphoproliferative diseases in Japan and western countries. *Human Pathol* 1983;14:745-772.
3. Aozasa K, Ohsawa M, Tajima K, *et al*. Nationwide study of lethal midline granuloma in Japan: frequencies of Wegener granulomatosis, polymorphic reticulosis, malignant lymphoma and other related conditions. *Int J Cancer* 1989;44:63-66.
4. Mitarnun W, Kietthubthew S, Suwiwat S. Hepatic peripheral T-cell lymphoma: a spectrum of liver pathology and clinical correlation. *J Med Assoc Thai* 1997;80:219-233.
5. Arber DA, Weiss LM, Albujar PF, Chen YY, Jaffe ES. Nasal lymphoma in Peru, high incidence of T-cell immunophenotype and Epstein-Barr virus infection. *Am J Surg Pathol* 1993;17:392-399.
6. Navarro-Roman L, Zarate-Osorno A, Meneses A, Kingma DW, Jaffe ES. High grade AIL and Epstein-Barr virus infection in 22 cases from Mexico. *Mod Pathol* 1994;7:117A.
7. Quintanilla-Martinez L, Lome-Maldonado C, Ott G, *et al*. Primary non-Hodgkin's lymphoma of the intestine: high prevalence of Epstein-Barr virus in Mexican lymphomas as compared with European cases. *Blood* 1997;89:644-651.
8. Pinkus GS, O'Hara CJ, Said JW. Peripheral/postthymic T-cell lymphomas: a spectrum of disease. Clinical, pathologic, and immunologic features of 78 cases. *Cancer* 1990;65:971-998.
9. Weiss LM, Crabtree GS, Rouse RV, Warnke R. Morphologic and immunologic characterization of 50 peripheral T-cell lymphomas. *Am J Pathol* 1985;118:316-324.
10. Weisenburger DD, Astorino RN, Glassy EJ, Miller CH, Mackenzie MR, Caggiano V. Peripheral T cell lymphoma. A clinicopathologic study of a morphologically diverse entity. *Cancer* 1985;56:2061-2068.
11. Chott A, Augustin I, Wrba F, *et al*. Peripheral T-cell lymphomas: a clinicopathologic study of 75 cases. *Hum Pathol* 1990;21:1117-1125.
12. Harris NE, Jaffe ES, Stein H, *et al*. A revised European-American classification of lymphoid neoplasms: a proposal from international lymphoma study group. *Blood* 1994;84:1361-1392.
13. Chan JKC, Banks PM, Cleary ML, *et al*. A revised European-American classification of lymphoid neoplasms proposed by the international lymphoma study group. A summary version. *Am J Clin Pathol* 1995;103:543-560.
14. Jaffe ES, Harris NL, Diebold J, Muller-Hermelink HK. World Health Organization classification of neoplastic diseases of hematopoietic and lymphoid tissues. A progress report. *Am J Clin Pathol* 1999;111(suppl.1): S8-12.
15. Jaffe ES, Krenacs L, Kumar S, Kingma DW, Raffeld M. Extranodal peripheral T-cell and NK-cell neoplasms. *Am J Clin Pathol* 1999;111(suppl.1) :S46-55.
16. Jaffe ES, Chan JKC, Su IJ, *et al*. Report of workshop on nasal and related extranodal angiocentric T/natural killer cell lymphomas. Definitions, differential diagnosis, andepidemiology. *Am J Surg Pathol* 1996;20:103-111.

17. de Wolf-Peeters C, Achten R. γδ T-cell lymphoma: a homogeneous entity? *Histopathol* 2000;36:294-305.

18. d' Amore F, Christensen BE, Brincker H, *et al.* Clinicopathological features and prognostic factors in extranodal non-Hodgkin's lymphomas. *Eur J Cancer* 1991;27:1201-1208.

19. Isaacson PG. Gastrointestinal lymphomas of T- andB-cell types. *Mod Pathol* 1991;12:151-158.

20. Crump M, Gospodarowicz M, Shepard FA. Lymphoma of the gastrointestinal tract. *Semin Oncol* 1999;26:324-337.

21. Huh J, Cho K, Heo DS, Kim JE, Kim CW. Detection of Epstein-Barr virus in Korean peripheral T-cell lymphoma. *Am J Hematol* 1999;60:205-214.

22. Lee SS, Jang JJ, Cho KJ, Khang SK, Kim CW. Epstein-Barr virus-associated primary gastrointestinal lymphoma in non-immunocompromised patients in Korea. *Histopathol* 1997;30:234-242.

23. Chan JKC, Sin VC, Wong KF, *et al.* Nonnasal lymphoma expressing the natural killer cell marker CD56: a clinicopathologic study of 49 cases of an uncommon aggressive neoplasm. *Blood* 1997;89:4501-4513.

24. Abe Y, Muta K, Ohshima K, *et al.* Cytotoxic T-cell lymphoma diffusely involving the entire gastrointestinal tract associated with Epstein-Barr virus and tubercle bacilli infection. *Int J Hematol* 2000;71:379-384.

25. Lavergne A, Brocheriou I, Delfau MH, Copie-Bergman C, Houdart R, Gaulard PH. Primary intestinal gamma-delta T-cell lymphoma with evidence of Epstein-Barr virus. *Histopathol* 1998;32:271-276.

26. de Bruin PC, Jiwa NM, Oudejans JJ, Radaszkiewicz T, Meijer CJLM. Epstein-Barr virus in primary gastrointestinal T-cell lymphoma: Association with gluten-sensitiveenteropathy, pathologic features, and immunophenotype. *Am J Pathol* 1995;146:861-867.

27. Ilyas M, Niedobitek G, Agathanggelou A, *et al.* Non-Hodgkin's lymphoma, coeliac disease, and Epstein-Barr virus: a study of 13 cases of enteropathy-associated T-and B- cell lymphoma. *J Pathol* 1995;177:115-122.

28. de Bruin PC, Connolly CE, Oudejans JJ, *et al.* Enteropathy-associated T-cell lymphomas have a cytotoxic T-cell phenotype. *Histopathol* 1997;31:313-317.

29. Arnulf B, Copie-Bergman C, Delfau-Larue MH, *et al.* Non hepatosplenic γδ T-cell lymphoma: a subset of cytotoxic lymphomas withmucosal or skin location. *Blood* 1998;91:1723-1731.

30. Ott MM, Ott G, Klinker H, Trunk MJF, Katzenberger T, Muller-Hermelink HK. Abdominal T-cell non-Hodgkin's lymphoma of the gamma/delta type in a patient with selective immunoglobulin A deficiency. *Am J Surg Pathol* 1998;22:500-506.

31. Ranheim EA, Jones C, Zehnder JL, Warnke R, Yuen A. Spontaneous relapsing clonal, mucosal cytotoxic T-cell lymphoproliferative disorder: Case report and review of the literature. *Am J Surg Pathol* 2000;24:296-301.

32. Holmes GKT, Prior P, Lane MR, Pope D, Allan RN. Malignancy incoeliac disease-effect of a gluten free diet. *Gut* 1989;30:333-338.

33. Gale J, Simmonds PD, Mead GM, Sweetenham JW, Wright DH. Enteropathy-type intestinal T-cell lymphoma: clinical features and treatment of 31 patients in a single center. *J Clin Oncol* 2000;18:795-803.

34. Kagnoff MF. Celiac disease. *Gastroenterol Clin N Am* 1993;21:405-425.

35. Boenisch T. Staining methods. In:Naish SJ, eds. Handbook of immunochemical staining methods. Capinteria, Calif: Dako Corp. 1989:13-18.

36. Kieff E, Leibowitz D. Epstein-Barr virus and its replication. In: Field BN, eds. Virology. 2nd ed. New York: Raven Press; 1990:1889-1920.

37. QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook. QIAGEN, Germany 1999:28-31.

38. McCarthy KP, Sloane JP, Kabarowski JHS, Matutes E, Wiedemann LM. The rapid detection of clonal T-cell proliferations in patients with lymphoid disorders. *Am J Pathol* 1991;138:821-828.

39. McCarthy KP, Sloane JP, Kabarowski JHS, Matutes E, Wiedemann LM. A simplified method detecting of clonal rearrangements of the T-cell receptor γ chain gene. *Diagn Mol Pathol* 1992;1:173-179.

40. Dass TC, Watts M, Pan LX, Burke M, Linch D, Isaacson PG. The polymerase chain reaction in the demonstration of monoclonality in T cell lymphomas. *J Clin Pathol* 1995;48:1045-1050.

41. Mitarnun W, Suwiwat S, Pradutkanchana J, *et al*. Epstein-Barr virus-associated peripheral T-cell and NK-cell proliferative disease/lymphoma: a clinicopathologic, serologic and molecular analysis. *Am J Hematol* (in press).

42. Mitarnun W, Kietthubthew S, Suwiwat S. Intrahepatocellular erythrocyte inclusions with hepatic sinusoidal infiltrates and splenomegaly. *Arch Pathol Lab Med* 1998;114:148-154.

43. Mitarnun W. Granulomatous reaction in peripheral T-cell proliferative disease: a case report. *J Med Assoc Thai* 1997;80:795-798.

44. Nakamura S, Suchi T, Koshikawa T, *et al*. Clinicopathologic study of CD56 (NCAM) - positive angiocentric lymphoma occurring in sites other than upper and lower respiratory tract. *Am J Surg Pathol* 1995;19:284-296.

45. Gorp JV, Weiping L, Jacobus K, *et al*. Epstein-Barr virus in nasal T-cell lymphomas, polymorphic reticulosis/midline malignant reticulosis in western China. *J Pathol* 1994;173:81-87.

46. Ng CS, Chan JKC, Lo STH. Expression of natural killer cell markers in non-Hodgkin's lymphomas. *Hum Pathol* 1987;18:1257-1262.

47. Kanavaros P, Lescs MC, Briere J, *et al*. Nasal T-cell lymphoma: a clinicopathologic entity associated with peculiar phenotype and with Epstein-Barr virus. *Blood* 1993;81:2688-2695.

48. de Bruin PC, Kummer JA, van de Valk P, *et al*. Granzyme B-expressing peripheral T-cell lymphoma, neoplastic equivalents of activated cytotoxic T-cell with preference for mucosa-associated lymphoid tissue localization. *Blood* 1994;84:3785-3791.

49. Ilsal K, Sun NCJ, Hernandez AM, Arber DA. Sinonasal NK/T-cell lymphomas in United States. *Am J Surg Pathol* 2000;24:1511-1517.

50. Quintanilla-Martinez L, Franklin JL, Guerrero I, *et al*. Histological and immunophenotypic profile of nasal NK/T cell lymphoma from Peru: high prevalence of p53 overexpression. *Hum Pathol* 1999;30:849-855.

51. Yao X, Teruya-Feldstein J, Raffeld M, Sorbara L, Jaffe ES. Peripheral T-cell lymphoma with aberrant expression of CD79a and CD20: a diagnostic pitfall. *Mod Pathol* 2001;14:105-110.

52. Theodorou I, Raphael M, Bigorgne C, *et al*. Recombination pattern of the TCR γ locus in human peripheral T-cell lymphomas. *J Pathol* 1994;174:233-242.

รายงานฉบับที่ 6

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

บทคัดย่อ

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 และผู้ป่วยโรคอื่นๆ จำนวน 21 ราย ผู้ป่วยได้รับการตรวจหาแอนติบอดีต่อการติดเชื้อของไวรัส 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 การตรวจหาแอนติบอดีต่อการติดเชื้อ EBV พบว่า แอนติบอดีชนิด anti-viral capsid antigen-IgG ในกลุ่มผู้ป่วยโรคเลือด (NHL-B, HD, และ acute leukemia) ที่ตรวจพายีนของ 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

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, we 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 the 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 wods: EBV, NHL-B, Hodgkin's disease, SLE, Acute leukemia

INTRODUCTION

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], we reported on 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% of the patients, which was not found in any of the controls. We also found 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. We 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) as 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. We postulated that the EBV which was dormant in non-T cells might infect T cell and contributed to the pathogenesis of the disease in a selected 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 were informed. Blood samples for the EBV serologic study and the molecular study were available for all patients. The 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, Bethesda, MD) supplemented with 10% heat inactivated fetal calf serum (Gibco) were treated with 4mM n-butyric acid and 20 ng/ml 12-tetradecanoylphorbor-13-acetate (TPA) (Sigma, St. Louis, MO) 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) 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, Uppsala, 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, Oslo, Norway), cells were fractionated into non-T cells ($CD3^-$ cells), and T cells ($CD3^+$ cells). The steps of isolation were followed by the manufacturer's protocol. 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 I. 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.

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

Diseases	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

Results of the serologic tests for the EBV antibodies are summarized in Table II. 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 was not detected in any case. There was a significant 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.

Table II. 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
1280	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2560	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

The EBV genomic study (IR-1) of the peripheral blood lymphocytes is summarized in Table III. 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% to 92.9%. 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 IV).

Table III. 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 IV. 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
1280	1	0	0	0
2560	1	0	0	0

*p-value of anti-VCA-IgG by Mann-Whitney test = 0.037;

**p-value of anti-EBNA by Mann-Whitney test = 0.7415

Table V. 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.

Table V. *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.

Diseases	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%)

DISCUSSION

In this report we describe the serologic findings, and the EBV genomic studies of 14 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 a 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 30% 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 was not associated with the presence of EBV genome in the peripheral blood T cells.

In our previous study of patients with T-cell and NK-cell proliferative disease/lymphomas, we found that 65% of them showed EBV genomes in the circulating $CD3^-$ cells[31]. In this study we also found the presence of the EBV genome in circulating T cells in some patients with NHL-B, Hodgkin's disease, acute leukemia, and SLE. 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 is detected in about 20-25% of Hodgkin disease tumor specimens [14]. The EBV-RNA expression in Reed-Sternberg cells appears to be less common in developed countries, at between 20% and 50%, in contrast to underdeveloped countries which have much higher rates [15-22]. The EBV-DNA in plasma was detected in about 50% of Hodgkin's disease patients, whereas none was detected in the controls [23]. In our Hodgkin's disease patients, 60% showed EBV-RNA in tumors, and 37.5% showed EBV-DNA in the circulating T cells.

The EBV genome (EBER) positivity is found in about 7% 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 our NHL-B patients, EBV-RNA expression was detected in 4 of 13 tested cases (31%). The EBV genome in peripheral blood T cells was detected in 71% 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]. Our study also confirmed this. About 40% of our SLE patients showed EBV-DNA in the peripheral blood T cells.

In our previous study [11], the EBV-DNA was not 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 this 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.

ACKNOWLEDGEMENTS

This study was supported by the Thailand Research Fund (TRF), grant no. BRG/7/2542, and was supported in part by the Core University System Exchange Programme under the Japan Society for the Promotion of Science (JSPS), coordinated by the University of Tokyo Graduate School of Medicine, Japan, and Prince of Songkla University, Faculty of Medicine, Thailand.

REFERENCES

1. Straus SE, Cohen JI, Tosato G, Meier J. Epstein-Barr virus infection: biology, pathogenesis and management. *Ann Int Med* 1993;118:45-58.
2. Okano M, Gross TG. Epstein-Barr virus-associated hemophagocytic syndrome and fatal infectious mononucleosis. *Am J Hematol* 1996;53:111-5.
3. Niedobitek G. Epstein-Barr virus infection in the pathogenesis of nasopharyngeal carcinoma. *J Clin Pathol: Mol Pathol* 2000;53:248-54.
4. Takada K. Epstein-Barr virus and gastric carcinoma. *J Clin Pathol: Mol Pathol* 2000;53:225-61.
5. McClain KL, Leach CT, Jenson HB, *et al*. Association of Epstein-Barr virus with leiomyosarcoma in young people with AIDS. *N Engl J Med* 1995;332:12-8.
6. Levyraz H, Henle W, Chahinian AP, *et al*. Association of Epstein-Barr virus with thymic carcinoma. *N Engl J Med* 1985;312:1296-9.
7. Flavell KJ, Murray PG. Hodgkin's disease and the Epstein-Barr virus. *J Clin Pathol: Mol Pathol* 2000;53:262-9.
8. Kumar S, Fend F, Quintanilla-Martinez L, *et al*. Epstein-Barr virus-positive primary gastrointestinal Hodgkin's disease. Association with inflammatory bowel disease and immunosuppression. *Am J Surg Pathol* 2000;24:66-73.
9. Zur Hausen H, Schulte-Holthausen H, Klein G, *et al*. EBV DNA in biopsies in Burkitt tumors and anaplastic carcinoma of the nasopharynx. *Nature* 1970;228:1056-8.
10. Craig FE, Gully ML, Banks PM. Posttransplantation lymphoproliferative disorders. *Am J Clin Pathol* 1993;99:265-71.
11. Maurnun W, Suwiwat S, Pradutkanchana J, *et al*. Epstein-Barr virus associated peripheral T-cell and NK-cell proliferative disease/lymphoma: a clinicopathologic, serologic and molecular analysis. *Am J Hematol* 2002;70:31-38.
12. Ohara H, Sato Y, Mukai K, *et al*. Detection of Epstein-Barr virus DNA in Reed-Sternberg cells of Hodgkin's disease using the polymerase chain reaction and *in situ* hybridization. *Jpn J Cancer Res* 1990;81:272-8.
13. Evans AS, Gutensohn NM. A population based case control study of EBV and other viral antibodies among persons with Hodgkin's disease and their siblings. *Int J Cancer* 1984;34:149-57.
14. Weiss LM, Strickler JG, Warnke RA, *et al*. Epstein-Barr virus DNA in tissues of Hodgkin's disease. *Am J pathol* 1987;129:86-91.
15. Weiss LM, Chen Y-Y, Liu X-F, *et al*. Epstein-Barr virus and Hodgkin's disease: a correlative *in situ* hybridization and polymerase chain reaction study. *Am J Pathol* 1991;139:1259-65.
16. Herbst H, Steinbrecher E, Niedobitek G, *et al*. Distribution and phenotype of Epstein-Barr virus-harboring cells in Hodgkin's disease. *Blood* 1992;80:484-91.
17. Hummel M, Anagnostopoulos I, Dallenbach F, *et al*. EBV infection patterns in Hodgkin's disease and normal lymphoid tissue: expression and cellular localization of gene products. *Br J Haematol* 1992;82:689-94.
18. Zhou X-G, Hamilton-Dutoit SJ, Yan Q-H, *et al*. The association between Epstein-Barr virus and Chinese Hodgkin's disease. *Int J Cancer* 1993;55:359-63.

19. Chang KL, Albujar PF, Chen Y-Y, *et al*. High prevalence of Epstein-Barr virus in the Reed-Sternberg cells of Hodgkin's disease occurring in Peru. *Blood* 1995;85:486-501.
20. Leoncini L, Spina D, Nyong'o A, *et al*. Neoplastic cells of Hodgkin's disease show differences in EBV expression between Kenya and Italy. *Int J Cancer* 1996;65:781-4.
21. Weinreb M, Day PJR, Niggli F, *et al*. The consistent association between Epstein-Barr virus and Hodgkin's disease in children in Kenya. *Blood* 1996;87:3828-36.
22. Weinreb M, Day PJR, Niggli F, *et al*. The role of Epstein-Barr virus in Hodgkin's disease from different geographical areas. *Arch Dis Child* 1996;74:27-31.
23. Wagner HJ, Schlager F, Clavier A, Bucsky P. Detection of Epstein-Barr virus DNA in peripheral blood of paediatric patients with Hodgkin's disease by real-time polymerase chain reaction. *Eur J Cancer* 2001;37:1853-7.
24. d'Amore F, Johansen P, Houmistad A, *et al*. Epstein-Barr virus genome in non-Hodgkin's lymphoma occurring in immunocompetent patients: Highest prevalence in nonlymphoblastic T-cell lymphoma and correlation with a poor prognosis. *Blood* 1996;87:1045-55.
25. James JA, Neas BR, Moser KL, *et al*. Systemic lupus erythematosus in adults is associated with previous Epstein-Barr virus exposure. *Arthritis Rheum* 2001;44:1122-6.
26. Katz BZ, Salimi B, Kim S, Nsiah-Kumi P, Wagner-Weiner L. Epstein-Barr virus burden in adolescents with systemic lupus erythematosus. *Pediatr Infect Dis J* 2001;20:148-53.
27. Stephan JL, Jeannoel P, Chanoz J, Gentil-Perret A. Epstein-Barr virus-associated Kikuchi disease in two children. *Am J Pediatr Hematol Oncol* 2001;23:240-3.
28. Thongpuksuri P, Kayasut K. Histiocytic necrotizing lymphadenitis (Kikuchi's disease): clinicopathologic characteristics of 23 cases and literature review. *J Med Assoc Thai* 1999;82:813-8.

การประยุกต์การใช้ผลงานวิจัย

เชิงพาณิชย์: การสร้าง kit เพื่อตรวจหา free viral DNA ของไวรัส EBV ที่อยู่ในพลาสม่าหรือซีรั่ม อาจเป็นการตรวจโดยวิธี PCR หรือโดยวิธี dot-blot, ผลบวกของการตรวจจะช่วยยืนยันว่าผู้ป่วยกำลังมีโรคและอาการของกลุ่มโรค PTPD นี้อยู่ ถึงแม้ว่าการตรวจโดยวิธีนี้ไม่ได้จำเพาะสำหรับกลุ่มโรค PTPD เพียงอย่างเดียว (มีรายงานว่าสามารถพบ free EBV DNA ได้ในโรค EBV-associated อื่นๆ เช่น nasopharyngeal carcinoma, infectious mononucleosis, EBV-associated hepatitis) แต่ผลของการตรวจจะช่วยสนับสนุนว่าเป็นโรค และ/หรือยังมีการดำเนินของโรคของกลุ่มโรค PTPD นี้อยู่

เชิงนโยบาย: เนื่องจากกลุ่มโรค PTPD พบบ่อยในประเทศไทย แพทย์ส่วนใหญ่เข้าใจโรคนี้น้อยมาก กระทรวงสาธารณสุขขาดความรู้ของโรคนี้และไม่ให้ความสนใจเท่าที่ควร โดยมักจะระบุว่าผู้ป่วยเป็นไข้เรื้อรังไม่ทราบสาเหตุ ไม่มีการวิจัยในแนวกว้าง เมื่อเร็วๆนี้ กระทรวงสาธารณสุขได้มีโครงการวิจัยขนาดใหญ่ที่เกี่ยวกับการหาสาเหตุของไข้เรื้อรัง ในการวิจัยนี้ไม่ได้รวมเอากลุ่มโรค PTPD ไว้ด้วย สกว. ควรต้องส่งรายงานฉบับนี้ไปยังกระทรวงสาธารณสุข และกระตุ้นให้เกิดความสนใจด้วย

เชิงสาธารณะ: คณะผู้วิจัยได้ศึกษากลุ่มโรคนี้ในหลายแนวทาง ได้แก่ ลักษณะอาการทางคลินิก การตรวจทางห้องปฏิบัติการ สาเหตุและวิธีการเกิดโรค และการดำเนินของโรค คณะผู้วิจัยพบว่าการให้ยาแคมีภาบำบัด (chemotherapy, CHOP regimen) ไม่มีผลต่อการรักษาโรค จากข้อสังเกตพบว่า การให้ยาในกลุ่มสเตียรอยด์สามารถยืดอายุผู้ป่วยได้และทำให้คุณภาพชีวิตของผู้ป่วยดีขึ้น ในอนาคต ต้องมีการร่วมมือกันในหลายสถาบันการศึกษา เพื่อหาแนวทางในการรักษาโรค และ/หรือทำให้คุณภาพชีวิตของผู้ป่วยดีขึ้น สกว. ควรเป็นผู้ประสานงานวิจัยเกี่ยวกับการรักษาโดยอาจจัดให้มีการประชุมร่วมกันระหว่างแพทย์ผู้เกี่ยวข้องจากสถาบันต่างๆ อนึ่ง คณะผู้วิจัยเห็นว่าควรต้องมีการวิจัยเกี่ยวกับการสร้างวัคซีนต่อต้านไวรัส EBV ทั้งนี้เพราะไวรัสชนิดนี้เป็นไวรัสก่อมะเร็งที่น่ากลัวมาก โดยเฉพาะคนไทยและกลุ่มคนเอเชีย

เชิงวิชาการ: ความรู้ที่ได้จากผลงานวิจัยครั้งนี้ ควรกำหนดไว้ในหลักสูตรแพทยศาสตรบัณฑิต ซึ่งเป็นโรคที่นักศึกษาแพทย์และแพทย์ทั่วไปจะต้องทราบ กระทรวงสาธารณสุขต้องยอมรับว่าเป็นปัญหาระดับชาติ และมีการถ่ายทอดความรู้ให้แก่บุคลากรทางสาธารณสุขทุกระดับ ทั้งนี้กลุ่มโรค PTPD มีแนวทางวิจัยสำหรับนักวิจัยใหม่และนักวิจัยอาวุโสตลอดจนคำถามเกี่ยวกับกลุ่มโรคนี้มีอีกมากมาย คณะผู้วิจัยยินดีให้ข้อมูลในแนวลึกและแนะแนววิจัยแก่นักวิจัยทุกท่านที่สนใจ

ผลงานที่ได้จากงานวิจัย

รายงานฉบับที่ 1	ตีพิมพ์แล้วใน Am J Hematol 2002;70:31-38
รายงานฉบับที่ 2	ส่งตีพิมพ์ในวารสาร Br J Hematol
รายงานฉบับที่ 3	กำลังจะส่งตีพิมพ์
รายงานฉบับที่ 4	กำลังจะส่งตีพิมพ์
รายงานฉบับที่ 5	ส่งตีพิมพ์ในวารสาร Histopathology
รายงานฉบับที่ 6	ได้รับการยอมรับตีพิมพ์แล้วใน J Med Assoc Thai

ภาคผนวก

American Journal of Hematology 70:31–38 (2002)

Epstein-Barr Virus-Associated Peripheral T-Cell and NK-Cell Proliferative Disease/Lymphoma: Clinicopathologic, Serologic, and Molecular Analysis

Winyou Mitarnun,[1]* Supaporn Suwiwat,[1] Jintana Pradutkanchana,[1] Vannarat Saechan,[1] Takafumi Ishida,[2] Satomi Takao,[2] and Atsumi Mori[2]

[1]Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat-Yai, Thailand
[2]Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo, Japan

Peripheral T-cell proliferative disease/lymphoma is a group of diseases which exhibits heterogeneity in clinical manifestations, pathological findings and outcomes. They are highly associated with the Epstein-Barr virus (EBV) infection. It is likely that EBV plays an important role in the tumorigenesis. From January 1997 through April 2000, we identified 100 patients. One hundred healthy age- and sex- matched controls were selected. Serologic tests for the EBV infection and the study of EBV genomes in circulating non-T cells ($CD3^-$ cells), T cells ($CD3^+$ cells), and T-cell subsets ($CD4^+$ and $CD8^+$ cells) were performed. The main features were prolonged fever, weight loss, hepatosplenomegaly, lymphadenopathy, multiorgan involvement, anemia, and high serum alkaline phosphatase and lactate dehydrogenase. Fifty-one patients had an aggressive course and died; median survival was 21 months. Chemotherapy was not effective in improving survival. Anti-viral capsid antigen-IgG and anti-early antigen-IgG were significantly elevated, whereas there was no significant difference in anti-EBV nuclear antigen. EBV internal repeat-1 region (IR-1) in the peripheral blood $CD3^+$ cells was detected in 65% of the patients but in none of the controls. For the $CD3^+$ cells, EBV IR-1 was detected in 88% of the patients and 50% of the controls. Among twenty-five patients whose $CD3^+$ cells were positive for EBV IR-1, 6 (24%) showed EBV IR-1 in only $CD4^+$ cells, 6 (24%) in only $CD8^+$ cells, and 13 (52%) in both $CD4^+$ and $CD8^+$ cells. The 30-bp deletion variant of the EBV latent membrane protein-1 gene was significantly higher in the patients than in the controls. These data support the chronic infective process. The EBV which is dormant in non-T cells may infect T cells and contribute to the pathogenesis of disease in a select group of patients. Am. J. Hematol. 70:31–38, 2002.

Key words: Epstein-Barr virus; peripheral T-cell lymphoma; EBV viral capsid antigen; EBV early antigen; EBV nuclear antigen; EBV internal repeat-1; EBV latent membrane protein-1

INTRODUCTION

Epstein-Barr virus (EBV) is an etiologic agent of infectious mononucleosis, which is a benign self-limited disease and results in a polyclonal B-cell proliferation secondary to EBV infection [1]. A fatal form of infectious mononucleosis 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 [2]. Infection with EBV has also been implicated in the development of a variety of malignancies including nasopharyngeal carcinoma, gastric carcinoma, smooth muscle tumor in immunosuppressed patients, thymic carcinoma, Hodgkin's disease, Burkitt's lymphoma, post-transplantation lymphoproliferative disorder, and various types of peripheral T-cell proliferative diseases/lymphomas [3–9].

Contract grant sponsor: The Thailand Research Fund (TRF); Contract grant number: BRG/7/2547; Contract grant sponsor: The Core University System Exchange Programme under the Japan Society for the Promotion of Science (JSPS), and Ministry of Education, Science, Culture, and Sports, Japan.

*Correspondence to: Winyou Mitarnun, M.D., Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat-Yai, Thailand 90110. E-mail: mwinyou@medicine.psu.ac.th

Received for publication 23 May 2001; Accepted 14 December 2001

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ajh.10074

The EBV-associated lymphoproliferative disorders of T cell and NK cell include fulminant EBV-positive T-cell lymphoproliferative disorder following acute and chronic EBV infection [10–12], hepatic peripheral T-cell lymphoma [13], extranodal NK/T-cell lymphoma, nasal type [14–16], enteropathy-type intestinal T-cell lymphoma [17], NK-cell proliferation following EBV infection in subcutaneous tissue terminating in aggressive subcutaneous NK-cell lymphoma [18], hepatosplenic γδ T-cell lymphoma [19], and nonhepatosplenic γδ T-cell lymphomas of various sites [20]. T-cell neoplasms in Asia and areas of South and Central America are highly associated with EBV, and it is likely that EBV plays an important role in the tumorigenesis which was confirmed by the EBV clonality study [14,15,21–23]. The susceptibility to EBV and the mode of entry in T cell are unclear.

In this study, we report 100 patients with various types of peripheral T-cell proliferative disease/lymphoma. All cases were studied with respect to clinical findings and outcome, serologic study for EBV infection, and EBV genomic study in the circulating peripheral blood lymphocytes; 100 healthy controls were also included in this study.

MATERIALS AND METHODS

Study Population

A case–control study of patients with peripheral T-cell proliferative disease/lymphoma was conducted in Songklanagarind University Hospital, a 700-bed hospital in Songkhla Province, Thailand. Patients were recruited from January 1997 through April 2000, and follow-up continued until December 2000. There were 100 patients included in the study. The pathological diagnoses were confirmed by histologic examination. T-cell neoplasms were mainly classified according to the WHO classification [24]. HIV positive patients were excluded from the study. One hundred healthy volunteers, residents of Songkhla Province, Thailand, were recruited as a control group. They were selected based on matching with the patients according to sex and age (±5 years). Volunteers with a previous history of serious illness, hematologic diseases, or fever within 1 month prior to the study were excluded from the study. This study was approved by the Hospital Ethics Committee, and all of the patients and controls were informed.

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, after the following treatment. Cells cultured in RPMI 1640 (Gibco, Bethesda, MD) supplemented with 10% heat inactivated fetal calf serum (Gibco) were treated with 4 mM n-butyric acid and 20 ng/mL 12-tetradecanoylphorbol-13-acetate (TPA) (Sigma, St. Louis, MO) for 48 hr. Cells were smeared on a slide. After air-drying, they were fixed with acetone for 3 min at room temperature. The cell smears were first treated with diluted (1:10 in PBS) plasma at 37°C for 30 min and then washed with PBS. They were then incubated with FITC-labeled anti-human IgG, IgA, and IgM rabbit serum (Dako, Denmark) at 37°C for 30 min. After being rewashed, they were mounted with glycerine buffer and examined for specific antibodies to EBV-VCA and -EA under a fluorescence microscope. When a positive reaction was detected at this dilution (1:10), the sample was then serially diluted and the titer of antibody determined.

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

White Blood Cell Preparation and DNA Extraction

Twenty milliliters of heparinized peripheral blood samples were obtained from the 100 healthy controls and 100 patients. Lymphocytes were isolated by centrifugation using Ficoll solution (Ficoll-Paque Plus, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Isolated lymphocytes were washed with saline and medium, and then suspended in 0.5–1.0 mL fetal calf serum supplemented medium. By using anti-human CD3 antibody conjugated magnetic beads (Dynabeads M-450 CD3, Dynal AS, Oslo, Norway), cells were fractionated into non-T cells ($CD3^-$ cells) and T cells ($CD3^+$ cells). Peripheral blood samples from 25 patients whose $CD3^+$ cells were positive for EBV genome were harvested for $CD4^+$ cells and $CD8^+$ cells, using anti-human CD4 and CD8 antibodies conjugated magnetic beads (Dynabeads M-450 CD4, CD8). Cells were fractionated into T-cell subsets: $CD4^+$ cells and $CD8^+$ cells. The steps of isolation followed the manufacturer's protocol.

DNA extraction was performed using Qiagen RNA/DNA Mini Kit (QIAGEN, Germany). The $CD3^+$ cells, $CD3^+$ cells, $CD4^+$ cells, and $CD8^+$ cells were homogenized in QRL 1 buffer by passing the lysate through a 20-gauge needle. The further steps followed the manufacturer's manual of animal cell protocols.

TABLE I. Clinical and Laboratory Findings of 100 Patients

Findings	No. of cases
Prolonged fever	84
Weight loss	57
Enlarged lymph nodes	56
Hepatomegaly	55
Splenomegaly	34
Skin lesions	41
Sinonasal/upper aerodigestive tract lesions	8
Arthritis	3
Hemoglobin less than 10.0 g/dL	72
White blood cell count less than 5,000/mm^3	31
Platelets count less than 10^5/mm^3	28
Pancytopenia	21
Presence of atypical lymphocytes in peripheral blood	68
Jaundice	29
Increase in serum transaminases	68
Increase in serum alkaline phosphatase	80
Increase in serum lactate dehydrogenase	65
Prolonged coagulograms	34

TABLE II. Sites for Tissue Diagnosis of Peripheral T-Cell Proliferative Disease/Lymphoma in 100 Patients

Sites	No. of cases
Liver	39
Bone marrow	53
Skin	36
Lymph node (angioimmunoblastic T-cell lymphoma)	10
Lymph node (peripheral T-cell lymphoma)	11
Sinonasal/upper aerodigestive tract	8
Gastrointestinal tract and omentum	4
Lungs and pleura	5
Brain	4
Spleen	2
Adrenal gland	1

Polymerase Chain Reaction (PCR) Analysis

DNA which was extracted from $CD3^-$, $CD3^+$, $CD4^+$, and $CD8^+$ cells was analyzed for the presence of the EBV genome by using internal repeat-1 region (IR-1) as a marker. PCR for this EBV DNA, 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 [25]. The amplified fragment length was 129 bp.

PCR analysis for the EBV latent membrane protein-1 (LMP-1) gene was performed on DNA extracts from those $CD3^-$ cells and $CD3^+$ cells which showed EBV IR-1. A pair of oligonucleotide primers for the PCR was used as described elsewhere [26]. The wild-type of LMP-1 gene yielded a 233-bp product, whereas the 30-bp deletion variant yielded a 203-bp product. DNA from Raji cells was used as a wild-type control.

RESULTS

Clinical and Laboratory Findings

The patients were 49 males and 51 females, ranging in age from 10 months to 84 years. Twelve cases were pediatric patients of under 15 years. The sex-matched controls ranged in age from 2 to 86 years. The mean (± SD) ages of the patients and the controls were 43.4 ± 20.7 years and 43.0 ± 17.1 years, respectively. The clinical features and laboratory findings of the 100 patients are summarized in Table I. Characteristic findings were the frequent constitutional signs and symptoms such as a prolonged fever, loss of body weight between 3 and 20 kg, neck or systemic lymphadenopathy, hepatosplenomegaly, presence of various types of skin lesion, and sinonasal/upper aerodigestive tract lesions. Other manifestations included pulmonary lesions, gastrointestinal symptoms, and central nervous system symptoms. A majority of the patients had more than one lesion; 27 patients had skin lesions only.

Hemograms revealed anemia in 72 patients. The average hemoglobin was 8.9 g/dL (range 4.3–15.0 g/dL). Atypical lymphocytes from peripheral blood were observed in 68 patients, ranging from 1% to 40% of total white blood cell count. The pertinent laboratory findings of these patients were the high serum levels of alkaline phosphatase and/or lactate dehydrogenase. The levels of alkaline phosphatase ranged from minimal elevation to more than 10 times the upper normal value (average 4.8 times) and the levels of lactate dehydrogenase ranged from minimal elevation to more than 10 times the upper normal value (average 2.5 times). Twenty-nine patients had jaundice. The conjugated bilirubin was proportionally more elevated than the unconjugated bilirubin. Coagulopathy was found in one-third of the patients; some were very severe.

Histopathological Findings

The sites of tissue biopsy and autopsy of these 100 patients are summarized in Table II. For the histopathologic confirmation of the diagnosis of peripheral T-cell proliferative disease/lymphoma, 46 patients were from a single site biopsy, 51 patients were from multiple-site biopsies, and 3 patients had a complete autopsy performed.

Hepatic involvement of peripheral T-cell lymphoma was demonstrated in 39 patients. Fifty-three patients were found to have bone marrow involvement. Thirty-six patients had a skin biopsy. Of these, 19 were mycosis fungoides or early lesions of mycosis fungoides, 8 were extranodal NK/T-cell lymphoma, nasal type, and 9 were subcutaneous panniculitis-like

TABLE III. Serologic Tests for Anti-VCA in 100 Controls and 100 Patients

	No. of cases					
	Controls			Patients		
Titers	IgG	IgA	IgM	IgG	IgA	IgM
<10	3	100	100	1	88	99
10	10	0	0	3	3	1
20	19	0	0	4	3	1
40	41	0	0	21	2	0
80	16	0	0	28	0	0
160	11	0	0	20	0	0
320	0	0	0	8	0	0
640	0	0	0	6	0	0
1,280	0	0	0	5	0	0
2,560	0	0	0	3	0	0
5,120	0	0	0	2	0	0

T-cell lymphoma. Ten patients were diagnosed to have angioimmunoblastic T-cell lymphoma in the lymph nodes, and 11 patients showed peripheral T-cell lymphoma. Eight patients had sinonasal or upper aerodigestive tract lesions. Of these, 7 patients had extranodal NK/T-cell lymphoma, nasal type in the sinonasal regions, and 1 patient had a similar lesion at the base of the tongue.

Three patients had extranodal NK/T-cell lymphoma, nasal type in the gastrointestinal tract and one patient had peripheral T-cell lymphoma in the omentum. Peripheral T-cell lymphoma was also found in the lungs and pleura of 5 patients, in the brain of 4 patients, in the spleen of 2 patients, and in both adrenal glands of 1 patient.

Serologic Tests for EBV Antibodies

Results of the serologic tests for the EBV antibodies of the 100 controls and 100 patients are summarized in Tables III–V. Among the controls, anti-VCA-IgG was detected in 97 cases (97%) with the highest titer of 1:160; none of them had elevated anti-VCA-IgA or anti-VCA-IgM. Among the patients, the anti-VCA-IgG was found in 99 patients (99%) with the highest titer of 1:5,120. There was a significant difference in the titer of anti-VCA-IgG between the controls and the patients using the Wilcoxon rank-sum test ($P < 0.0001$). Twelve patients showed anti-VCA-IgA with titers of 1:10 to 1:40, and two patients showed anti-VCA-IgM with titers of 1:10 to 1:20. Of the 15 patients who had anti-VCA-IgG titer of more than 1:160, none of them showed anti-VCA-IgM, but 5 of them had the anti-VCA-IgA with titers of 1:10 to 1:20. Two patients with anti-VCA-IgM titers of 1:10 and 1:20 showed the anti-VCA-IgG titers of 1:80 and 1:160, respectively.

The presence of anti-EA-IgG in the controls and the patients was significantly different using the Wilcoxon rank-sum test ($P = 0.0004$). None in the control group had elevated anti-EA-IgA and anti-EA-IgM. Four patients had elevated anti-EA-IgA, and three patients had elevated anti-EA-IgM with the highest titer of 1:20. For the anti-EBNA, there was no significant difference between the control and the patient groups.

Molecular Studies

The EBV genomic studies (IR-1, LMP-1) in the peripheral blood lymphocytes of the 100 controls and 100 patients are summarized in Table VI. Among the control group, EBV genome (IR-1) in the peripheral blood $CD3^+$ cells was not detected in any subject, whereas 50 controls (50%) contained the EBV genome in the peripheral blood $CD3^-$ cells. In contrast to the controls, 65 patients (65%) showed the EBV genome in the peripheral blood $CD3^+$ cells, and 88 patients (88%) showed this EBV genome in the peripheral blood $CD3^-$ cells. All patients with $CD3^+$ cells positive also showed $CD3^-$ cells positive. Twenty-five patients who had the EBV genome in the peripheral blood $CD3^+$ cells were studied for the presence of the EBV genome in their peripheral blood $CD4^+$ and $CD8^+$ cells; 6 patients (24%) had the EBV genome in the peripheral blood $CD4^+$ cells, 6 patients (24%) had EBV genome in the peripheral blood $CD8^+$ cells, and 13 patients (52%) had this EBV genome in both $CD4^+$ and $CD8^+$ cells. The chi-square test showed no significant difference between the patients with and without the EBV genome in the peripheral blood $CD3^+$ cells with respect to anti-VCA-IgG titer level when using the cut-off titer of 1:160. Ten of the eleven patients with anti-VCA-IgG titers

TABLE IV. Serologic Tests for Anti-EA in 100 Controls and 100 Patients

	No. of cases					
	Controls			Patients		
Titers	IgG	IgA	IgM	IgG	IgA	IgM
<10	90	100	100	75	86	97
10	8	0	0	5	2	2
20	1	0	0	9	1	1
40	1	0	0	8	1	0
80	0	0	0	2	0	0
160	0	0	0	2	0	0
320	0	0	0	1	0	0

TABLE V. Serologic Test for Anti-EBNA in 100 Controls and 100 Patients

	No. of cases	
Titers	Controls	Patients
<10	11	23
10	37	19
20	33	15
40	10	23
80	7	7
160	0	3

between 1:1,280 and 1:5,120 showed the presence the EBV genome in their peripheral blood $CD3^+$ cells.

The 30-bp deletion variant of the EBV LMP-1 gene was more common in the patients than in the controls. According to the peripheral blood $CD3^+$ cells, the 30-bp deletion variant was found in 9 of 50 (18%) of the controls versus 38 of 83 (45.3%) of the patients. The chi-square test showed a significantly higher proportion in the patients than in the controls ($P < 0.002$). For the peripheral blood $CD3^+$ cells in the patient group, this 30-bp deletion variant was detected in 31 of 62 cases (50%).

Twelve of the 65 patients with the EBV genome (IR-1) in their peripheral blood $CD3^+$ cells repeated this genomic study during an interval of 2 months to 2 years. In ten patients the positive test persisted, while in two patients the test returned to negative. Of three patients who had a spontaneous regression of disease, two showed negative results and another patient still showed positive results in the follow-up study.

Follow-up and Outcomes

Follow-up data and outcome of the 100 patients according to the relationship of the EBV genome in the peripheral blood $CD3^+$ cells, and the Kaplan–Meier survival estimates of patients with and without chemotherapy, are summarized in Table VII and Fig. 1, respectively. Forty-seven patients received multidrug chemotherapy (CHOP regimen), 50 patients did not have chemotherapy, and 3 patients were lost to follow-up. Of 47 patients who received chemotherapy, 25 died of disease, 14 were alive with disease, and 8 were in a complete remission after chemotherapy in the 8- to 24-month follow-up. Of fifty patients who did not have chemotherapy, 26 died of disease, 18 were alive with disease, and 6 had spontaneous regression of disease. The median survival time was 21 months. Twenty-seven percent died within 2 months after the onset of illness. Chemotherapy and the presence of EBV genomes in the peripheral blood $CD3^+$ cells had no association with the outcome of disease. Kaplan–Meier

TABLE VI. EBV Genomic Study in Peripheral Blood Lymphocytes of 100 Controls and 100 Patients*

	Controls (no. of cases)				Patients (no. of cases)			
		LMP-1				LMP-1		
Type of cells	IR-1	WT	del	WT + del	IR-1	WT	del	WT + del
$CD3^+$ cells	65/100 (65%)	ND	ND	ND	85/100 (85%)	31/62 (50%)	26/62 (41.9%)	5/62 (8.1%)
$CD3^+$ cells	50/100 (50%)	41/50 (82%)	2/50 (4.0%)	7/50 (14.0%)	88/100 (88%)	45/83 (54.2%)	31/83 (37.3%)	7/83 (8.5%)
$CD4^+$ cells only	ND	ND	ND	ND	6/23 (26%)	ND	ND	ND
$CD8^+$ cells only	ND	ND	ND	ND	6/23 (26%)	ND	ND	ND
$CD4^+$ and $CD8^+$ cells	ND	ND	ND	ND	12/23 (52%)	ND	ND	ND

*WT, wild-type of LMP-1 gene; del, 30-bp deletion of LMP-1 gene; ND, not done.

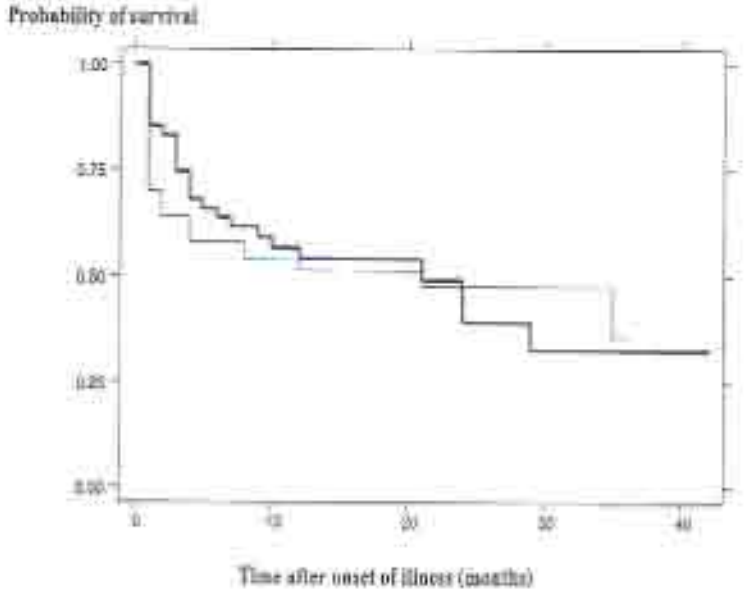

Fig. 1. Kaplan-Meier survival estimates of 47 patients who had chemotherapy (solid line) and 50 patients who had no chemotherapy (dotted line).

TABLE VII. Outcome of 100 Patients With Respect to Chemotherapy and The Presence of EBV Genome in the Peripheral Blood $CD3^+$ Cells

	(R-1 in $CD3^+$ cells			
Outcome	Positive (no. of cases)	Negative (no. of cases)	Total	*P* value*
Dead				1.00
With chemotherapy	19	7	26	
Without chemotherapy	19	8	28	
Total	38	15	54	
Alive with disease				0.17
With chemotherapy	10	4	14	
Without chemotherapy	8	10	18	
Total	18	14	32	
Remission				1.00
Complete remission	3	5	8	
Spontaneous regression	4	2	6	
Total	7	7	14	
Lost follow-up	2	1	3	

*P values were based on Fisher's exact test.

analysis showed no significant difference in the survival profile of patients with or without chemotherapy (Fig. 1), although the early mortality meant that many patients died either before chemotherapy could be started or after only a short period of chemotherapy. Similarly, no significant survival difference was found between the patients with wild-type and those with 30-bp deletion variant of the EBV LMP-1 gene in the $CD3^+$ cells.

DISCUSSION

In this report we describe the clinicopathologic features, serologic findings, and the EBV genomic studies of 100 patients with various types of peripheral T-cell proliferative disease/lymphoma. One hundred healthy controls were included for comparison. The notable findings of these patients in the present study were the significant elevation of the anti-VCA-IgG and anti-EA-IgG titers, and the presence of EBV genomes in the peripheral blood T cells in 65% of the patients, which was not found in any of the controls.

From our study of the healthy control population, we found that at least 97% showed a previous EBV infection, as indicated by the elevated titer of anti-VCA-IgG. In half of these controls, the EBV genomes were detected in the circulating non-T cells. T cells and NK cells do not bear CD21 (EBV-receptor molecule) on their cell surfaces and they are seldom infected with EBV. In an in vitro study, transfection

with EBV genome into cord blood T cells has been reported to induce T-cell immortalization [27]. EBV is found to infect peripheral blood T cells ($CD4^+$ and $CD8^+$ cells) and NK cells in a few patients with fatal infectious mononucleosis, EBV-positive T-cell lymphoma and fulminant EBV-positive T-cell lymphoproliferative disorder following chronic active EBV infection [28–32]. To our knowledge, this report examines the largest series of patients showing the EBV genome in the peripheral blood T cells.

EBV LMP-1 is considered a viral oncogene because of its ability to transform rodent fibroblasts in vitro and render them tumorigenic in nude mice [33]. The 30-bp deletion variant of the LMP-1 is considered to have a higher tumorigenic potential for the lymphoid neoplasm, nasopharyngeal carcinoma, gastric carcinoma, and other types of carcinoma [26,34,35]. In our data, the 30-bp deletion variant of the LMP-1 gene was significantly more common among patients than among controls, but there was no evidence of any difference in survival of patients between those with wild-type and those with the 30-bp deletion variant. The presence of dual infection (wild-type and the 30-bp deletion variant) in the peripheral blood $CD3^+$ cells of some patients, and the presence of the EBV IR-1 in both $CD4^+$ and $CD8^+$ cells in a single patient, indicated that these infected circulating T cells might not be neoplastic cells or might reflect oligoclonal malignancy. Previous reports on EBV-associated T-cell neoplasms, to our knowledge, have never shown such dual infection [26,29,34,36]. Kanegane et al. [29] reported two patients who had evidence of EBV infection in the peripheral blood $CD4^+$ T cell 3 months and 19 months before the diagnosis of EBV-positive T-cell lymphoma. This confirms the association between circulating T-cell EBV infection and the EBV-associated T-cell neoplasm.

The serologic response of patients with EBV-associated diseases is a well-known subject and has been published elsewhere [1,10,12,37–39]. Infectious mononucleosis shows a rise of anti-VCA-IgM, anti-VCA-IgG, and anti-EA-IgG with a subsequent fall, and a life-long persistence of anti-VCA-IgG and anti-EBNA. Burkitt's lymphoma and nasopharyngeal carcinoma show high titers of anti-VCA-IgG and anti-EA-IgG. The serologic test for post-transplantation lymphoproliferative disorder, T-cell lymphoproliferative disease in chronic active EBV infection, and angiocentric lymphoma also show very high titers of anti-VCA-IgG and anti-EA-IgG and mild to moderately high titers of anti-VCA-IgA. Anti-VCA-IgM is rarely positive, and anti-EBNA titer is not significantly increased. In this report we showed the significantly high titers of anti-VCA-IgG and anti-EA-IgG among the patients as compared to the controls, even though the titers of the two groups overlapped to a considerable extent. The serologic study in this report supported the chronic infective process by EBV, which is quite similar to the previous reports on EBV-associated T-cell neoplasms.

Peripheral T-cell proliferative disease/lymphoma is a group of diseases which exhibits considerable heterogeneity in clinical findings, morphologic pattern (ranging from benign morphology to malignant histology), phenotypic expression of tumor cells, therapeutic response and prognosis [13,40,41]. In some patients, the disease pursues a highly aggressive course and death results within a few months. Spontaneous regression in some patients has been reported [13,42, 43]. Our reported cases also showed variation in the course of disease and prognosis. Outcome did not have any apparent relation to the presence of the EBV genome in the peripheral blood $CD3^+$ cells. Chemotherapy was not effective for the improvement of survival.

REFERENCES

1. Straus SE, Cohen JI, Tosato G, Meier J. Epstein-Barr virus infections: biology, pathogenesis and management. Ann Int Med 1993;118:45–58.
2. Okano M, Gross TG. Epstein-Barr virus-associated hemophagocytic syndrome and fatal infectious mononucleosis. Am J Hematol 1996;51:111–115.
3. Nicholls JM. Epstein-Barr virus infection in the pathogenesis of nasopharyngeal carcinoma. J Clin Pathol Mol Pathol 2000;53:348–354.
4. Takada K. Epstein-Barr virus and gastric carcinoma. J Clin Pathol Mol Pathol 2000;53:255–261.
5. McClain KL, Leach CT, Jenson HB, Joshi VV, Pollock BH, Prieditis RG, DeLeon EJ, Chadwick EG, Murphy SB. Association of Epstein-Barr virus with leiomyosarcoma in young people with AIDS. N Engl J Med 1995;332:12–18.
6. Leyvraz JL, Henle W, Chabrerie AP, Pedrinaci C, Klein G, Gordon RY, Rosenheim M, Holland JF. Association of Epstein-Barr virus with thymic carcinoma. N Engl J Med 1983;312:1296–1299.
7. Harrell KE, Murray PG. Hodgkin's disease and the Epstein-Barr virus. J Clin Pathol Mol Pathol 2000;53:262–269.
8. Zur Hausen H, Schulte-Holthausen H, Klein G, Henle W, Henle G, Clifford P, Santesson L. EBV DNA in biopsies in Burkitt tumors and anaplastic carcinoma of the nasopharynx. Nature 1970;228:1056–1058.
9. Craig FE, Gulley ML, Banks PM. Posttransplantation lymphoproliferative disorders. Am J Clin Pathol 1993;99:265–276.
10. Quintanilla-Martinez L, Kumar S, Fend F, Reyes E, Tavera-Felddkin J, Kimura DW, Serbana L, Raffeld M, Straus SE, Jaffe ES. Fulminant EBV$^+$ T-cell lymphoproliferative disorder following acute chronic EBV infection: a distinct clinicopathologic syndrome. Blood 2000;96:443–451.
11. Imai S, Nagata M, Okuno Y, Kuzushima S, Hirao M, Kimura H, Hayashibara H, Terai N, Tsurumi H, Oda T, Chiba S, Osato T. Epstein-Barr virus (EBV)-carrying and -expressing T-cell lines established from severe chronic active EBV infection. Blood 1996;87:1446–1457.

12. Ohga S, Kimura N, Takeda H, Nakano M, Ochiai K, Nomura A, Morioka K, Take H, Yamamori S, Hara T. Restricted diversification of T cells in chronic active Epstein-Barr virus infection: potential initiation to T-lymphoproliferative disease. Am J Hematol 1999;61:26–33.

13. Mitamun W, Kanthabhiben S, Suwanwela S. Hepatic peripheral T-cell lymphoma: a spectrum of liver pathology and clinical correlation. J Med Assoc Thai 1997;80:229–237.

14. Jaffe ES, Krenacs L, Kumar S, Kingma DW, Raffeld M. Extranodal peripheral T-cell and NK-cell neoplasms. Am J Clin Pathol 1999;111(Suppl 1):S40–S55.

15. Tao Q, Ho FC, Loke SL, Srivastava G. Epstein-Barr virus is localized in the tumor cells of nasal lymphomas of NK, T or B cell type. Int J Cancer 1995;60:315–320.

16. Chan ACL, Ho JWY, Chuang AKS, Srivastava G. Phenotypic and cytotoxic characteristics of peripheral T-cell and NK-cell lymphomas in relation to Epstein-Barr virus association. Histopathology 1999;34:16–24.

17. deBruin PC, Jiwa NM, Oudejans JJ, Radaszkiewicz T, Meijer CJLM. Epstein-Barr virus in primary gastrointestinal T-cell lymphoma: Association with gluten-sensitive enteropathy, pathologic features, and immunophenotype. Am J Pathol 1993;146:861–867.

18. Abe Y, Muta K, Chihama K, Yamamoto S, Matsushima M, Syito R, Tsujita J, Shibata T, Furui M, Kikuchi M, Nishimura J, Nawata H. Subcutaneous panniculitis by Epstein-Barr virus-restricted natural killer (NK) cell proliferation terminating in aggressive subcutaneous NK cell lymphoma. Am J Hematol 2000;64:221–225.

19. Ohshima K, Hamasaki S, Harada N, Kanazawa T, Suzuyama T, Kanda M, Kawasaki C, Sugihara M, Kikuchi M. Hepatosplenic γδ T-cell lymphoma: relation to Epstein-Barr virus and activated cytotoxic molecules. Histopathology 2000;36:127–135.

20. Arnulf B, Copie-Bergman C, Delfau-Larue MH, Lavergne-Slove A, Bologna S, Wechsler J, Wassef M, Malamuth-Samsky C, Epardaud B, Stern M, Bagot M, Reyes F, Gaulard P. Non-hepatosplenic γδ T-cell lymphoma: a subset of cytotoxic lymphomas with mucosal or skin location. Blood 1998;91:1723–1731.

21. Gong JY, Weiping L, Jianbin R, Yong-Hua L, Feng-Yuan L, de Wolgie RA, Li G. Epstein-Barr virus in nasal T-cell lymphomas (polymorphic reticulosis/midline malignant reticulosis) in western China. J Pathol 1994;173:81–87.

22. Huh J, Cho K, Heo DS, Kim H, Kim CW. Detection of Epstein-Barr virus in Korean peripheral T-cell lymphoma. Am J Hematol 1999;60:203–208.

23. Quintanilla-Martinez L, Lome-Maldonado C, Ott G, Gesk-enwetter A, Gredler E, Reyes E, Angeles-Angeles A, Fend F. Primary non-Hodgkin's lymphoma of the intestine: high prevalence of Epstein-Barr virus in Mexican lymphomas as compared with European cases. Blood 1997;89:644–653.

24. Jaffe ES, Harris NL, Diebold J, Muller-Hermelink H-K. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. Am J Clin Pathol 1999;111(Suppl 1):S8–S12.

25. Uhara H, Sato Y, Mukai K, Akao I, Matsuno Y, Furuya S, Hoshikawa T, Shirooshi Y, Saida T. Detection of Epstein-Barr virus DNA in Reed-Sternberg cells of Hodgkin's disease using the polymerase chain reaction and in situ hybridization. Jpn J Cancer Res 1990;81:272–278.

26. Leung SY, Yuen ST, Chung LP, Chan ASY, Wong MP. Prevalence of mutations and 30-bp deletion in the C terminal region of Epstein-Barr virus latent membrane protein-1 oncogene in reactive lymphoid tissue and non nasopharyngeal EBV-associated carcinomas in Hong Kong Chinese. Int J Cancer 1997;72:225–230.

27. Steinman M, Volsky B, Hadenskog M, Volsky DJ. Immortalization of human T lymphocytes after transfection of Epstein-Barr virus DNA. Science 1986;233:960–964.

28. Mori M, Kuroiwa H, Arizgi K, Tanaka Y, Imai S, Osato T. Monoclonal proliferation of T cells containing Epstein-Barr virus in fatal mononucleosis. N Engl J Med 1992;327–358.

29. Kanegane H, Ohota K, Gutierrez M, Kaneda H, Wada T, Yachie A, Seki H, Arai T, Kagimoto S, Okazaki M, Ohshiki T, Moghaddam A, Wang F, Tosato G. A syndrome of peripheral T-cell infection with Epstein-Barr virus (EBV) followed by EBV-positive T-cell lymphoma. Blood 1998;91:2085–2091.

30. Ishihara S, Tawa A, Yamura-Yaki K, Murata M, Hara J, Yabuuchi H, Imai K, Kawa-Ha K. Clonal T-cell proliferation containing Epstein-Barr (EB) virus DNA in a patient with chronic active EB virus infection. Jpn J Cancer Res 1988;80:99–101.

31. Kikuta H, Taguchi Y, Tomizawa K, Kajiwa K, Kawamura N, Iwanaka A, Sakiyama Y, Matsumoto S, Imai S, Kinoshita T. Epstein-Barr virus genome-positive T lymphocytes in a boy with chronic active EBV infection associated with Kawasaki-like disease. Nature 1988;333:455–457.

32. Kanegane H, Wada T, Yamagata K, Seki H, Taniguchi N, Tosato G. Chronic persistent Epstein-Barr virus infection in natural killer cells and B cells associated with granular lymphocytes expansion. Br J Haematol 1996;95:116–122.

33. Wang D, Liebowitz D, Kieff E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cell. Cell 1985;43:831–840.

34. Kingma DW, Weiss WR, Jaffe ES, Kumar S, Frekko K, Raffeld M, Epstein-Barr virus latent membrane protein-1 oncogene deletions: correlation with malignancy in Epstein-Barr virus-associated lymphoproliferative disorders and malignant lymphomas. Blood 1996;88:242–251.

35. Cheung ST, Lo KW, Leung SF, Chan WY, Chan PH, Johnson PJ, Lee JC, Huang DP. Prevalence of LMP-1 deletion variant of Epstein-Barr virus in nasopharyngeal carcinoma and gastric tumors in Hong Kong. Int J Cancer 1999;66:713–722.

36. Dirnhofer S, Angeles-Angeles A, Ortiz-Hidalgo C, Reyes E, Gerdes J, Kingma J, Fend F, Quintanilla-Martinez L. High prevalence of a 30-base pair deletion in the Epstein-Barr virus (EBV) latent membrane protein 1 gene and of strain type B EBV in Mexican classical Hodgkin's disease and reactive lymphoid tissue. Hum Pathol 1999;30:781–787.

37. Okano M, Thiele GM, Davis JR, Grierson HL, Purtilo DT. Epstein-Barr virus and human disease: recent advances in diagnosis. Clin Microbiol Rev 1988;1:300–312.

38. Jones JF, Shurin S, Abramowsky C, Tubbs RR, Sciotto CG, Wahl R, Sands J, Gottman D, Katz BZ, Sklar J. T-cell lymphoma containing Epstein-Barr viral DNA in patients with chronic Epstein-Barr virus infections. N Engl J Med 1988;318:733–741.

39. Harabuchi Y, Yamanaka N, Kataura A, Imai S, Kinoshita T, Murata I, Osato T. Epstein-Barr virus in nasal T-cell lymphomas in patients with lethal midline granuloma. Lancet 1990;335:128–130.

40. Choi A, Augustin L, Wiho F, Hanak H, Ohlinger W, Radaszkiewicz T. Peripheral T-cell lymphomas: a clinicopathologic study of 75 cases. Hum Pathol 1990;21:117–125.

41. Pirker GS, Offner CI, Said JW. Peripheral/post-thymic T-cell lymphomas: a spectrum of disease. Clinical, pathologic, and immunologic features of 70 cases. Cancer 1990;65:971–988.

42. Rambeau EA, Jones C, Zehnder JL, Warnke R, Yunis A. Spontaneous relapsing clonal, cytotoxic T-cell lymphoproliferative disorder. Case report and review of the literature. Am J Surg Pathol 2000;24:296–301.

43. Nakamura S, Suchi T, Koshikawa T, Kitoh K, Koike K, Kamiura H, Ida S, Kagami Y, Ogura M, Kaleb E, Kurita S, Suzuki H, Kobashi Y, Yamabe H, Hirabayashi N, Ueda R, Takahashi T. Clinicopathologic study of CD56 (NCAM)-positive angiocentric lymphoma occurring in sites other than upper and lower respiratory tract. Am J Surg Pathol 1995;19:284–296.