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☐期中進度報告

（計畫名稱）

與病毒有關之凋亡作用和血球細胞吞噬作用 - 臺灣兒童感染紅血球病毒 B 十九型或 Epstein-Barr 病毒之研究

Virus-associated apoptosis and hemaphagocytosis – Childhood infections of erythrovirus B19 and Epstein-Barr virus in Taiwan

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計畫主持人：彭慶添

共同主持人：周寬基 (t6218@hpd.cmch.org.tw)

計畫參與人員：戴玉滿

陳怡嘉

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中文摘要

血球細胞吞噬現象在病毒感染或發炎的病人中偶爾會發現，在具有血球細胞吞噬症候群的病人則時常發現；這種病人身上更常發現有病毒感染。本研究針對血球吞噬細胞與病毒感染做進一步深入了解：病毒的存在與否以核酸原位雜交、聚合　鏈反應及免疫細胞染色法偵測。研究計畫執行的一年當中患有血球細胞吞噬症候群的病人發現有六位，其中四位偵測到 EB 病毒，一位偵測到 HHV8，另一位則偵測到 PVB19；此六位病人皆有病毒血的現象，而細胞型態與免疫細胞染色的結果顯示，四位病人骨髓中具有吞噬能力的細胞主要是吞噬血球 (macrophage)；另兩位病人則是天然殺手細胞。與 PVB19 有關之血球吞噬症候，所吞噬之細胞主要是紅血球系列；但是罹患與 EB 病毒有關之天然殺手細胞淋巴瘤患者，癌細胞所吞噬之細胞則包含各血球系列。細胞吞噬過程中所引起之凋亡作用是在細胞內執行。

關鍵字：血球細胞吞噬作用、病毒感染、凋亡作用

Abstract

Hemophagocytosis is a clinicopathologic condition that could be occasionally recognized in patients with inflammation, or it could be readily identified in patients with hemophagocytic syndrome. Virus infection has been frequently detected. In this study we prospectively investigated the hemocytophagic cells and their relationship to Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus 8 (HHV8), and parvovirus B19 (PVB19). The presence of virus was detected by in situ hybridization, polymerase chain reaction, and immunocytochemistry. Six patients had severe hemocytophagia. Among these six patients, four patients were EBV-related, one was PVB19-related, and one was HHV8-related. All six patients had viremia. Morphological and immunocytochemical features showed that phagocytes in four patients were macrophages, and two patients were natural killer cells. In PVB19-related HS, the phagocytes ingest mostly erythroid cells. However, in EBV-related NK cell lymphoma, the malignant cells engulfed the blood cells of all lineages indifferently. HS-associated apoptosis was proceeded inside the phagocytes.

Key Words: Hemophagocytosis, virus infection, HTLV-I, EBV, CMV, HHV8, PVB19

The abbreviations used are: HS, hemophagocytic syndrome; EBV, Epstein-Barr virus; CMV, cytomegalovirus; HHV8, human herpesvirus 8; PVB19, parvovirus B19; HTLV-I, type I human T-cell lymphotropic virus

目　　錄

Introduction

Phagocytosis is an important cellular mechanism for defending the host against microbial invasion, and for regulating the hematopoiesis as well as the normal immune function by engulfing the extraneous macromolecules or cell debris.[1,2] Pathophysiological dysfunction of phagocytes could be easily identified cytologically by the abnormal morphological changes.[2] Interestingly, these peculiar morphological characteristics usually reflect the impairment of microbicidal capability, the loss of chemotactic orientation, or the defect of enzymatic function.[3-11] An aberrant increase of phagocyte number (e.g., Langerhans cell histiocytosis), and phagocytic activity (e.g., malignant histiocytosis and HS), however, would trigger adverse outcome for the patient.[12-15]

Histologically, four types of phagocytotic disorders have been identified: familial hemophagocytic lymphohistiocytosis, Rosai-Dorfmann's disease, malignant histiocytosis, and virus-associated hemophagocytic syndrome (VAHS).[16] Although, respectively, these four types represent different clinicopathological entities, they share some similar appearances, a high percentage of hemophagocytosis. In particular, in patients with VAHS, the disease could deteriorate fast and become fatal if it was compounded with malignant T-cell lymphoma, which in the Orient is highly correlated with EBV infection.[14,15]

Many viruses, including EBV,[14,15,17-19] CMV,[20,21] HHV8,[22] and PVB19,[21,23] are associated with HS. Although most of the patients were presented with the similar symptoms, the underlying pathogenetic mechanism and the morphological characteristics responding to the virus infection may vary from patient to patient.[14-23] Therefore, in this study we conducted a prospective investigation to analyze the morphological differences of hemocytophagic cells and to examine the relationship of EBV, CMV, HHV8, HTLV-I, and PVB19 to the disease.

Materials and Methods

Patients and tissue samples

One hundred and eighty nine patients with biopsy-confirmed leukemia and lymphoma, or unexplained pancytopenia were collected in China Medical College Hospital, Taiwan from October 1997 to January 2001. Bone marrow aspiration, bone marrow biopsy, lymph node biopsy, or laparoscopic biopsy was routinely scheduled after the admission when the procedure was required for the affirmative diagnosis. Peripheral blood was taken every other day to determine the presence of viremia when the virus signal was detected in the biopsies. A written informed consent was obtained from each patient before biopsy. Clinical record was reviewed and each section was stained with hematoxylin-eosin for histopathology. Imprints that were made from bone marrow aspirate and excised lymph node were examined with Riu staining method or Wright-Giemsa staining. Myeloperoxidase was stained by Hanker's method.[24]

Phenotype determination by immunocytochemistry and flow cytometry

Using labeled streptavidin-biotin complex method (DAKO, LSAB2 System, Carpenteria, CA), immunophenotype was studied with a panel of monoclonal antibodies, such as CD1a, CD2, CD3, CD4, CD8, CD10, CD14, CD15, CD16, CD19, CD20, CD30, CD34, CD45RO, CD56, CD68, and lysozyme (DAKO, Copenhagen, Denmark).[25] The chromogen was aminoethyl carbazole (AEC). Slide was counterstained with hematoxylin or methyl green. Positive staining was recognized under a microscope as crimson granules. To detect the gene product of virus, antibodies to latent membrane protein 1 (LMP1), early antigen diffused (EA-D), EBV BZLF-1 replication activator (ZEBRA), and EBV-associated nuclear protein 2 (EBNA-2), or to the VP2 capsid protein of PVB19 (DAKO, Copenhagen, Denmark), or to the late gene of CMV (Novocastra, Newcastle, UK) was performed. Antibodies to ZEBRA, EBNA-2, and EA-D were used to determine if EBV infection was lytic, and antibodies to LMP1 were used to determine if EBV infection was latent. Immunophenotyping of leukemia/lymphoma cells from bone marrow

aspirate and peripheral blood was further confirmed by a flow cytometric analysis (FACSCalibur, Benton-Dickinson, San Jose, CA).

Virus detection by in situ hybridization and signal amplification

The RNA ISH procedure was described previously.[25] Briefly, four-μm section taken from paraffin-embedded tissue was deparaffinized in xylene, dehydrated, and predigested with 0.1 mg/ml nuclease-free proteinase K (Boehringer Mannheim, Germany) at room temperature for 15 minutes. The slide was then washed with distilled water, rinsed with 70% ethanol, and air-dried. One drop of fluorescein isothiocyanate (FITC)-conjugated antisense probe (250 ng/ml in 50% formamide, 6× SSC, and 0.25% dry milk) to EBERs, CMV early gene (Novocastra, Newcastle, UK), PVB19 NS/VP genes or HHV8 ORF26/ORF K7/nut-1 RNAs was placed over each tissue section. The probes were synthesized by BRL (Bethesda, MD), and the respective probe sequences are listed in Table 1.

The sample was denatured at 75°C on a thermal plate for 5 minutes and then moved to a moist chamber at 37°C for 2 hours. Following extensive wash with 2 × SSC at 45°C, hybridization product was detected using anti-FITC antibodies conjugated with alkaline phosphatase (Amersham, Buckinghamshire, UK). The chromogenic development was processed in 4-nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolphosphate (BCIP) mixture. Slide was counterstained with nuclear fast red or methyl green. Positive staining was recognized under the microscope as brownish purple granules. Specimens of nasopharyngeal carcinoma (NPC) with positive serology for EBV and specimens of Kaposi's sarcoma (KS) with positive serology to HHV8 were used as positive controls. Paraffin sections of normal tonsil and normal appendix were used as negative controls. For detecting proviral DNA of HTLV-I, the procedure was similar to that described above except that: (1) the biotinylated HTLV-I pX probe was used; (2) denaturing temperature was set at 100°C; and (3) HTLV-I-containing MT-2 cells were used as positive control.[26] For the morphological characterization of phagocytotic cells and the comparison of their correlation to the viruses, archival specimens of twelve cases of histiocytic necrotizing lymphadenitis (HNL),[25] ten cases of KS, one case of Rosai-Dorfman's disease, one case of LCH,[12] two cases of adult T-cell lymphotropic lymphoma (ATLL),[26] fifty-six cases of T-cell lymphoma, seventy cases of HD,[27] one hundred and thirty-four cases of NPC, and two cases of Burkitt's lymphoma (BL) were studied at the same time.

The detailed PCR procedure for specimen on tissue section was described previously.[25,26,28] Briefly, tissue section positive for viral signal was marked on the glass slide under a microscope. The positive area was scraped into a 0.5 ml microfuge tube containing PCR mixture with a surgical blade. The reaction mixture was then subjected to 35 cycles of amplification using standard procedure: denaturing DNA at 94°C for 1 min, annealing at 56°C for 40 seconds, and elongating at 72°C for 1.2 min. Primer sequences and length of the amplified DNA fragments for the individual virus are listed in Table 2. The amplified product was resolved in a 2% agarose gel, and the presence of the specific virus was indicated by the appearance of the specific DNA fragment. Specificity of the amplified products was confirmed respectively by DNA sequencing (ABI Prism, Perkin-Elmer, Foster City, CA). The positive control DNA for HTLV-I was prepared from MT-2 cells,[28] and those for EBV and HHV8 were prepared from BC-2 cells (CRL-2231, American Type Culture Collection, Manassas, VA). The positive control DNA for CMV and PVB19 was prepared from patients with viremia. β-globin gene was used to determine the integrity of DNA in the paraffin material.

Results

Different features of hemocytophagic cells in bone marrow smears

Among the 189 patients, following the examination of bone marrow smears, bone marrow biopsy, lymph node aspirates, and laparoscopic biopsy, twenty-three patients had indication of hemophagocytosis.[18] Presence of hemophagocytosis was identified by hematologists. In 17

patients, the frequency of hemophagocytosis in bone marrow was less than 2%. PVB19 was detected in 15 cases, CMV in one case, and EBV in two cases. One patient had both CMV and PVB19 infections. These patients did not have evident bacterial infection. However, six patients had hemocytophagia (presence of hemophagocytosis more than 2%),[18] and clinical indication of HS. Among these six patients, four patients were EBV-related, one was PVB19-related, and one was HHV8-related. All six patients had persistent viremia for more than six days, and all except one patient with HHV8 viremia and severe hemolytic anemia died of disseminated intravascular coagulation (DIC) and concomitant sepsis within two to three weeks after admission. Results of immunostaining and the presence of virus detected respectively by ISH and PCR are summarized in Table 3. Also listed in Table 3 are two controls (patients 7 and 8) that had abundant histiocytes or virus-involved bone marrow without symptom of hemophagocytosis.

Interestingly, morphological and immunocytochemical features showed that phagocytes accounting for hemocytophagia were macrophages in five patients (patients 3-6, Table 3), and natural killer (NK) cells in two patient (patients 1 and 2). In PVB19-associated HS (patients 5), the phagocytes ingested mostly erythroid cells (Image 1A), and these phagocytes were positive for CD68 and lysozyme (Image 1B). Erythroid cells were presented by morphologically well-defined normoblasts and red blood cells. In one HHV8- and two EBV-related hemocytophagia (patients 3, 4 and 6), macrophages ingested mostly lymphoid cells (Image 1C). Immunologically, CD3, CD20 and CD45RO staining could easily identify lymphocytes. It is worth noting that in patient with EBV-associated NK cell lymphoma (patients 1 and 2), it is the malignant NK cells that indifferently ingest blood cells of all lineages (Image 1D), including peroxidase-positive myelocytes (Image 1E). The NK cells were positive for CD16 and CD56 (Image 1F). Interestingly, these hemocytophagic cells were negative for CD68, a marker for the activated macrophage (Table 3). Among the remaining 17 patients, in whom frequency of hemophagocytosis was less than 2%, and bone marrow cells that were positive for CD13, CD56 or CD68 were less than 1-8%, the hemocytophagic cells, however, were $CD68^+$ macrophages.

Association of virus with hemocytophagic cells

Except patients 1, no other patient had lymph node involvement. However, unlike normal lymph node, ratio of $CD45RO^+$ cell number was more than 45% of the total mononuclear cells in area containing patchy karyorrhexic bodies in lymph node from patients 1. Among $CD45RO^+$ cells, most of the $EBER^+$ cells were large cells (Image 2). Nonetheless, EBV signal was not identified in the nuclei of normal lymphocytes or within the karyorrhexic debris.[25] Also, unlike patients with HNL or BL, in which the relative abundance of $CD68^+$ cells varied from one case to another, expression of CD68 was not identified in this patient. The presence of EBV genome was confirmed by PCR, and expressions of ZEBRA and EA-D were detected in this case. LMP1, EBNA-2, CMV late gene or VP2, however, was not detected (data not shown). Interestingly, in bone marrow smears from the other two patients with EBV infection (patient 3 and 4), only expressions of ZEBRA and EA-D were detected in lymphoid cells. On the other hand, in patient with PVB19 infection (patient 5), expression of VP2 was identified in erythroid cells. In contrast to two reference cases of ATLL, neither PCR nor ISH could detect HTLV-I sequence in patients with hemocytophagia.

Discussion

Virus infection has been a major subject in the disease development of hemophagocytic syndrome.[14,15,17-23] Although the detailed mechanism is yet to be determined, the current evidence suggested that, independent of direct interaction with the target cells, the hemophagocytosis should include two steps to recognize and to ingest the blood cells.[1,2] A variety of data indicated that receptors, such as CR1, CR3, Fc, carbohydrate and fimbrae, are responsible for recognition and should be normally present on phagocytes in patients. The present study sheds further light on the morphological characteristics of hemocytophagic cell types during disease progression and that may not be completely major histocompatibility complex-restricted.[29] The presence of active phagocytes and sometimes a high percentage of lymphocytes when compared to the normal bone

marrow smears, laparoscopic biopsies, and lymph node imprints are consistent with the observation of the others that hemophagocytic syndrome could be a combination resulting from the chronic inflammation and the reactivation of virus infection, in particular, EBV infection.[1,5,14,15,17-23,30] Nonetheless, the status of virus infection, either latent or active, could not be determined by a one-time-point examination. Neither were the essence of phagocyte origin and that of the engulfed cell types investigated in detail in these latter studies.

It is worth noting that, in addition to macrophages and histiocytes, tumor cells were shown having phagocytic activities.[20,29-37] In many cases of hemocytophagia, cytologically atypical cells that were frequently detected did not express either lymphocytic or histiocytic markers.[19,29,30,33-35] Pathologic features further indicated that these cells are NK cells or peripheral T cell lymphoma (PTCL) cells. Our data confirmed their findings with identification of hemocytophagic $CD16^+/CD56^+$ cells immunocytochemically. Moreover, our results showed that unlike PVB19-, HHV8- and other EBV-related hemocytophagia, in which hemocytophagic cells were $CD68^+$ macrophages, and the ingested cells were mostly erythroid cells or lymphoid cells, $CD56^+$ lymphoma cells, nonetheless, engulfed the blood cells of all lineages indifferently. More interestingly, these hemocytophagic cells were $EBER^+$ cells. Elegant studies by Tsutsumi *et al*[19] and Abe *et al*[36] have shown that EBV infection could be intimately associated with NK-cell malignancies, and supported the concept proposed by Su *et al*[30] that EBV-positive T cell lymphoma could mimic other phagocytotic disorder, malignant histiocytosis. Upon examination of various EBV-related gene expressions, however, except replication-associated genes, no latent gene expression was detected in the malignant cells.[19,36]

As mentioned previously, massive hemophagocytosis and virus infection are two imperative characteristics for patients with hemocytophagia.[15-17,22,23,29-36] Although EBV, CMV, HHV8, and PVB19, all have been detected, similar symptoms appeared in most of the patients with hemocytophagia indicated a common pathogenetic mechanism that in turn would determine the features of the engulfed cell types and the corresponding phagocytes. The disparity of target cell recognition by the different phagocytes would then suggest a subtle interaction between virus infection and the host response. Prior studies have shown the association of EBV with an array of malignancies, such as HD, NPC, BL, and PTCL.[27,38-41] Despite the presence of viral DNA, only a limited number of viral genes were expressed. At present, it is unclear what host factor(s) might be involved with the viral gene expression and how virus infection might interact with the host factor(s), which would determine the state of disease to be transient or progressive.[25-28,38-42] The major difference between the six patients with hemocytophagia and the remaining 17 patients with slightly discernible hemophagocytosis was the persistence of viremia for more than 5-7 days.

Okano and Gross had suggested that VAHS and fatal infectious mononucleosis be resulted from the imbalanced cytokine responses and the activation of T cells directed against EBV infection.[42] However, the disease progression of hemocytophagia, the immunologic differences of phagocytes, and the low frequency of hemophagocytosis detected in the other virus-associated diseases, e.g., KS, HD, hepatoma, HNL, BL, and NPC, would implicate a more sophisticated interaction.[5,15-17,22,23,33,34] In particular, detection of EBER signal in the karryorrhexic debris (e.g., HNL and BL) or in the phagocytes (NK cell lymphoma) indicated that the presence of EBV in these three different diseases may play different pathologic roles.

Conclusion

At present, there is no clear explanation for the relationship between the virus infection and the increased activity of histiocytes and that between the virus infection and NK cell–associated hemocytophagia. Although expressions of EA-D and ZEBRA in NK cells, which were different from those detected in patients with BL, HD, NPC and HNL, indicated a correlation between the early gene products of EBV and the disease progression of NK-cell lymphoma, the prospective role of EBV in the different EBV-associated malignancies remains to be determined.

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Legends

Figure 1 Different features of virus-associated hemocytophagia. In PVB19-associated HS, the phagocytes ingested mostly erythroid cells (A), and these phagocytes were positive for CD68 and lysozyme (B). Erythroid cells were presented by morphologically well-defined normoblasts and red blood cells. (C) In one HHV8- and three EBV-related HS, phagocytes ingested typically lymphoid cells. In patient with EBV-associated NK cell lymphoma, it is the malignant cells that indifferently ingest the other blood cells (D), including peroxidase-positive myelocytes (E). The NK cells were positive for CD16 and CD56, which were detected by an immunoperoxidase method (F). (Original magnification, ×1000)

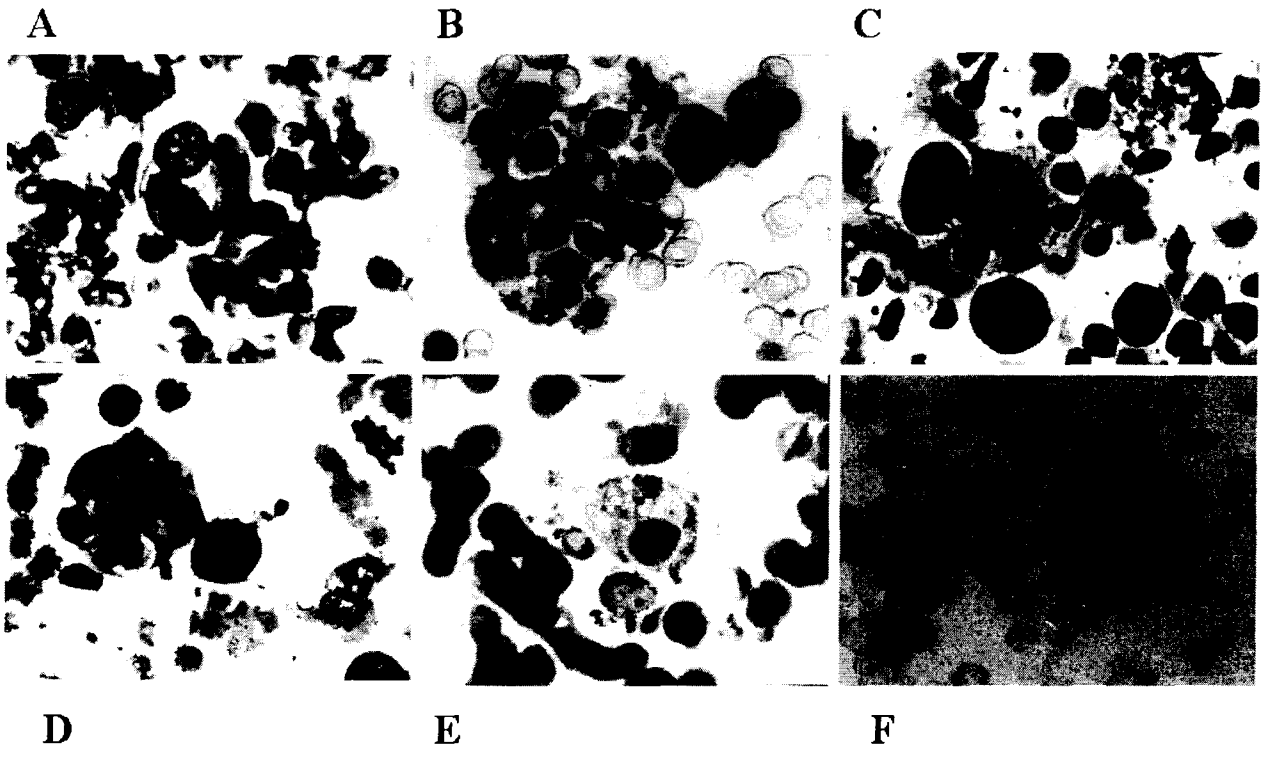

Figure 2 Expression of EBERs in hemocytophagic NK cells. Unlike normal lymph node, ratio of NK cell number was more than 45% of the total mononuclear cells in area containing patchy karyorrhectic bodies, which were $EBER^-$; most of the $EBER^+$ cells were large NK lymphoma cells.

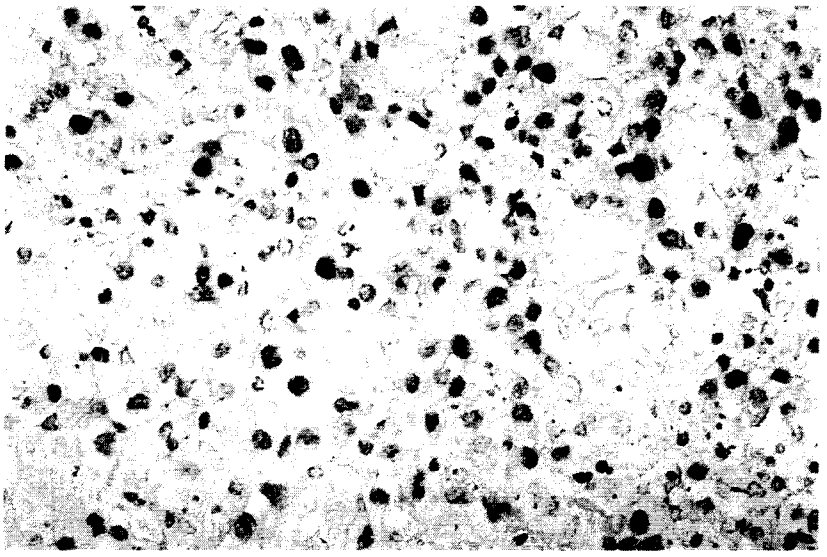

Table 1 Probe sequences used for in situ hybridization to detect the different viruses

Probe sequences for EBER1 and EBER2 (GenBank/J02078)
5' FITC-ATGCTCTAGGCGGGAAGCCTCTCTTCTCCT-3' (nts 612 to 583)
5' FITC-CGGTAGCACCGCACTGAGCGTTGGCGGTGT-3' (nts 560 to 531)
5' FITC-CTGGTACTTGACCGAAGACGGCAGAAAGCAGA-3' (nts 321 to 290)
5' FITC-CACGTCTCCTCCCTAGCAAAA-CCTCTAGGG-3' (nts 216 to 187)
Probe sequences for HHV8 ORF 26, ORF K7 and nut-1 RNAs (GenBank/ KSU75698)
5' FITC-TGCAGCAGCTGTTGGTGTACCACATCTACT-3' (nts 47378 to 47407)
5' FITC-ATATTGCCAAAAGCGACGCAATCAACCCACAAT-3' (nts 28796 to 28764)
5' FITC-GTAGTGCACCACTGTTCTGATACACCAGTGGGC-3' (nts 29104 to 29072)
5' FITC-TTGTCGCCTATGTCATTCAAATCGACTTGCTTAC-3' (nts 29350 to 29317)
Probe sequences for PVB19 NS and VP2 regions (GenBank/AF113323)
5' FITC-TATCCCATTATGGGACTAATGG-3' (nts 2948 to 2927)
5' FITC-CTAAAGTATCCTGACCTTGCCCTAAC-3' (nts 3211 to 3186)
5' FITC-GCACCAGTGCTGGCTTCTGCAGAA-3' (nts 2738 to 2715)
5' FITC-TTTGCCACTTTCTTTACTCATAATCTAC-3' (nts 2040 to 2013)
5' FITC-GATTCTCCTGAACTGGTCCC-3' (nts 1669 to 1649)
5' FITC-AGGTAAACCCCTTACTCCGTCCCACACA-3' (nts 1805 to 1778)

Table 2 Primer sequences and the expected length of the amplified DNA fragments for the different viruses

Primer sequences for the different viruses	Length of DNA fragment (bp)
Primer sequences for EBV BamH1 W region	
AA: 5'-GCCAGAGGTAAGTGGACTTT-3' (GenBank/M15973, nts 1400 to 1419)	241
AZ: 5'-TGGAGAGGTCAGGTTACTTA-3' (GenBank/M15973, nts 1640 to 1621)	
Primer sequences for CMV	
M1: 5'-CACCACGCAGCGGCCCTTGATGTTT-3' (GenBank/X17403, nts 118878 to 118902)	400
M2: 5'-CACCTGTCACCGCTGCTATATTTGC-3' (GenBank/X17403, nts 119277 to 119253)	
Primer sequences for PVB19 NS and VP2 regions	
NS1: 5'-CAGAGGTTGTGCCATTTAAT-3' (GenBank/AF162273, nts 1213 to 1232)	810
NS2: 5'-TGTGCATTACACCATGTAAGCCACTGTTGTAC-3' (GenBank/AF162273, nts 2023 to 1992);	
S1: 5'-ATAAATCCATATACTCATTGGA-3' (GenBank/AF162273, nts 3115 to 3136)	699
S2: 5'-CTAAAGTATCCTGACCTTG-3' (GenBank/AF162273, nts 3814 to 3796)	
Primer sequences for HTLV-I pX region	
pX1: 5'-CGGATACCCAGTCTACGTGT-3' (GenBank/L03562, nts 7338 to 7356)	326
pX2: 5'-GGTTCCATGTATCCATTTCG-3' (GenBank/L03562, nts 7663 to 7644)	
Primer sequences for HHV8 ORF 26 region	
KS1: 5'-TGCAGCAGCTGTTGGTGTACCACATCTACT-3' (GenBank/ KSU75698, nts 47378 to 47407)	201
KS2: 5'-CTGATAGGATACAAAGGTACATGGACAG-3' (GenBank/KSU75698, nts 47578 to 47551)	

Table 3 The representative results of immunostaining, ISH, and PCR in patients with HS, KS, LCH and AIDS

	Patient number							
Categories	1	2	3	4	5	6	7	8
Age	12	2	2	19	64	56	2	26
Sex	M	F	F	F	F	M	M	M
Diagnosis	NKL	NKL	M2	M4	AA	KS	LCH	AIDS
Phagocyte Markers								
CD13	20.31	85.12	68.25	69.17		7.33		
CD16	73.46							
CD56	39.16	56.62	0.34	0.21	0.52	1.40	0.47	0.72
CD68	0.02	32.76	12.5	68.74	36.84	24.3	7.6	1.21
Detection of virus								
CMV	-	-	-	-	-	-	-	+*/-
EBERs	+	+	+	+	-	-	-	-
HHV8	-	-	-	-	-	+	-	-
HTLV-I	-	-	-	-	-	-	-	-
PVB19	-	-	-	-	+	-	-	+
Serum EBV	+	+	+	+	-	-	-	-
Serum HHV8	-	-	-	-	-	+	-	-
Serum PVB19	-	-	-	-	+	-	-	+
Hemocytophagia	Yes	Yes	Yes	Yes	Yes	Yes	No	No
DIC	Yes	Yes	Yes	Yes	Yes	No	No	No

NKL: NK cell leukemia/lymphoma; M4: acute myelogenous leukemia, type IV; M2: acute myelogenous leukemia, type II; AA: aplastic anemia; KS: Kaposi's sarcoma; LCH: Langerhan's cell histiocytosis; AIDS: acquired immunodeficiency syndrome.

*Signals of both PVB19 and CMV were only detected in a patient with AIDS.