

Characterization of Epstein–Barr virus (EBV)-infected natural killer (NK) cell proliferation in patients with severe mosquito allergy; establishment of an IL-2-dependent NK-like cell line

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(Accepted for publication 19 November 1998)

SUMMARY

The clinical evidence of a relationship between severe hypersensitivity to mosquito bite (HMB) and clonal expansion of EBV-infected NK cells has been accumulated. In order to clarify the mechanism of EBV-induced NK cell proliferation and its relationship with high incidence of leukaemias or lymphomas in HMB patients, we studied clonally expanded NK cells from three HMB patients and succeeded in establishing an EBV-infected NK-like cell line designated KAI3. Immunoblotting and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses revealed that KAI3 cells as well as infected NK cells exhibited an EBV latent infection type II, where EBV gene expression was limited to EBNA 1 and LMP1. As KAI3 was established by culture with IL-2, IL-2 responsiveness of peripheral blood NK cells from patients was examined. The results represented markedly augmented IL-2-induced IL-2R α expression in NK cells. This characteristic property may contribute to the persistent expansion of infected NK cells. However, KAI3 cells as well as the NK cells from patients were not protected from apoptosis induced by either an anti-Fas antibody or NK-sensitive K562 cells. Preserved sensitivity to apoptosis might explain the relatively regulated NK cell numbers in the peripheral blood of the patients. To our knowledge, KAI3 is the first reported NK-like cell line established from patients of severe chronic active EBV infection (SCAEBV) before the onset of leukaemias or lymphomas. KAI3 cells will contribute to the study of EBV persistency in the NK cell environment and its relationship with high incidence of leukaemias or lymphomas in HMB patients.

Keywords chronic active EBV infection mosquito allergy NK cell line

INTRODUCTION

EBV, a widespread human herpes virus, infects >90% of the population by adulthood, and persists in B cells and epithelial cells in the oropharynx, where reactivation and viral replication may intermittently occur [1,2]. EBV has the capacity to transform B cells into lymphoblastoid cells *in vitro*. Clinically, it causes infectious mononucleosis, a benign lymphoproliferative syndrome induced by virus-infected B cell expansion, and B lymphomas in conjunction with immunodeficiencies [3]. The virus has also been linked to various B cell and non-B cell neoplasms, such as endemic Burkitt's lymphoma and nasopharyngeal carcinoma [4].

Severe chronic active EBV infection (SCAEBV) is another life-threatening disease associated with unusual EBV activation [5–10]. The major clinical symptoms include prolonged fever,

hepatosplenomegaly, and lymphadenopathy persisting over months to several years. One characteristic point of SCAEBV is its frequent association with a number of potentially fatal complications, including lymphoid neoplasia, virus-associated haemophagocytotic syndrome, severe liver dysfunction, systemic bleeding, and coronary aneurysms. High levels of the EBV genome and expression of antigens in affected tissues together with abnormal immunological responses to the virus have been reported. Among heterogeneous SCAEBV, accumulating evidence has shown that a latent EBV infection of T cells can also lead to SCAEBV [7].

Recently, another type of SCAEBV, characterized by clonal expansion of EBV-infected NK cells and severe hypersensitivity to mosquito bites (HMB), has been reported [11,12]. In patients with severe HMB, a single mosquito bite has produced intense local skin symptoms including a bulla that subsequently develops into necrosis or an ulcer, and general symptoms of high fever and liver dysfunction [13]. Furthermore, it has become apparent that about

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Table 1. Profiles of patients

Patient no.	Age (years)/sex	Major clinical manifestations	Serum immunoglobulin		Serum EBV antibody titres				
			IgG (mg/dl)	IgE (U/ml)	VCA		EA-DR		EBNA
					IgG	IgM	IgG	IgA	
1	12/F	HMB, thrombocytopenia, CH, hepatosplenomegaly	1221	1357	2560	10 >	640	40	40
2	10/F	HMB, PH, aneurysm*, CH, hepatosplenomegaly	2270	1287	1280	10 >	2560	160	160
3	13/M	HMB, food allergy, CH, hepatosplenomegaly	1295	7686	80	ND	40	ND	20

CH, Chronic hepatitis; PH, pulmonary hypertension.

* Aortic aneurysm and left coronary artery aneurysm.

half of these patients die of malignant histiocytosis, which could now be reassessed as haemophagocytotic syndrome or leukaemias/lymphomas [12]. Thus, clinical evidence of a relationship between HMB and clonal expansion of EBV-infected NK cells has been accumulated, but the mechanism of NK cell proliferation induced by EBV has not been determined.

Here, we characterized NK cell expansion in three HMB patients using virologic, haematologic and immunological techniques. In addition, we established an EBV carrying NK-like cell line from one of the patients. This NK-like cell line will contribute to the study of EBV persistency in an NK cell environment and the pathogenesis of leukaemias or lymphomas associated with HMB.

SUBJECTS AND METHODS

Subjects

Three Japanese HMB patients were studied. The clinical profiles are summarized in Table 1. Although detailed clinical features have been reported elsewhere [14], intense local skin symptoms and high fever lasting for 2–3 days following mosquito bites were common to three patients. Patient 1 is a 12-year-old girl, and had suffered from severe HMB since the age of 2 years. Hepatosplenomegaly, thrombocytopenia and liver dysfunction persisted since the age of 3 years. Patient 2 is a 10-year-old girl. She developed an HMB, accompanied by liver dysfunction and elevated serum levels of IgE, since the age of 5 years. When 9 years old she complained of general fatigue, and pulmonary hypertension, aortic aneurysm, and left coronary artery aneurysm were detected. Patient 3 is a

13-year-old boy who had suffered from HMB, hepatosplenomegaly, asymptomatic liver dysfunction and marked elevation of serum levels of IgE since the age of 1 year. It is noteworthy that SCAEBV patients with NK cell proliferation do not always exhibit severe HMB (data not shown).

Cell preparations and culture conditions

EBV-transformed B lymphoblastoid cell lines (LCL) were established from peripheral blood mononuclear cells (PBMC) by incubation with the B95-8 culture supernatant as described [15]. To enrich for NK cells, monocytes were depleted by incubation on plastic dishes for 1 h. The resulting lymphocytes were further depleted of T cells and B cells by addition of magnetic beads coated with anti-CD3 and anti-CD19 antibodies (Dynal, Oslo, Norway) following exposure to a magnetic field. The purity of NK cells was >90%. To obtain highly purified NK cells for *in situ* hybridization experiments, PBMC from patients were stained with FITC-conjugated anti-CD16 and PE-conjugated anti-CD56 (Coulter, Hialeah, FL) and sorted into CD16⁺CD56⁺ and CD16⁺CD56[−] cells by EPICS Elite (Coulter). The final purity of both populations was >98%.

Activation of NK cells were achieved by culture with IL-2 (rIL-2; Shionogi, Osaka, Japan; 100 U/ml) in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS). For the purpose of establishing EBV-infected NK cell lines, limiting dilution using irradiated autologous LCL as feeder cells was performed. An established NK-like cell line designated KAI3 has been growing stably for >16 months in the presence of IL-2.

Table 2. Characterization of lymphocytes from patients

Patient no.	Leucocytes	Lymphocytes (%)	Lymphocyte subset							EBER1 ⁺ cells in NK cells (%)
			CD2	CD3	CD4	CD8	CD20	CD16	CD56	
Patient 1	5200*	3170* (61%)	2940* (92.8%)	558* (17.6%)	364* (11.5%)	368* (11.6%)	120* (3.8%)	2356* (74.3%)	2379* (75.0%)	60
Patient 2	4000	2040 (51%)	1954 (95.8%)	337 (16.5%)	204 (10.0%)	700 (34.3%)	57 (2.8%)	1564 (76.7%)	1552 (76.1%)	15
Patient 3	5300	2703 (51%)	2273 (84.1%)	711 (26.3%)	489 (18.1%)	397 (14.7%)	289 (10.7%)	1494 (55.3%)	1241 (45.9%)	25

* Absolute count (/μl).

Immunophenotypic evaluation

Surface analysis of the PBMC and activated NK cells was conducted by direct immunofluorescence using FITC- or PE-conjugated anti-CD2, anti-CD3, anti-CD16, anti-CD21, anti-CD56, anti-CD57, anti-IL-2R α (Coulter), anti-IL-2R β , anti-IL-2R γ (Pharmingen, San Diego, CA), anti-HLA class I (W6/32; Dako, Glostrup, Denmark), anti-HLA class II (I2; Coulter) and anti-Fas (MBL, Nagoya, Japan) antibodies. PBMC were stained as previously described [15] and analysed using a fluorescence-activated cell sorter (Epics XL; Coulter).

Analysis of EBV DNA and mRNA

For Southern blotting analysis, genomic DNA was extracted and digested with BamHI. Five micrograms of digested DNA were electrophoresed on 0.7% agarose gels, transferred to nylon filters, and hybridized to ^{32} P-labelled 1.9 kb XhoI fragment, a probe of the terminal region of EBV [16].

In situ hybridization was performed according to Hironaka *et al.* [17] using an alkaline phosphatase-linked antisense oligonucleotide probe (AGCAGAGTCTGGGAAGACAACACAGACACCGTCTCACC) or sense probe to EBER1.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed essentially according to Imai *et al.* [18–20]. Total cellular RNA was isolated using TRIzol (GIBCO BRL, Rockville, MD), and cDNA synthesis was performed with Moloney murine leukaemia virus RTase (GIBCO BRL). The cDNA samples were then subjected to 35 cycles of PCR. The PCR products were electrophoresed, Southern transferred onto nylon membranes, and hybridized with ^{32} P-end-labelled probes. Primers and probes used were as follows; LMP2A 5' primer ATGACTCATCTCAACACATA; LMP2B 5' primer CAGTGTAATCTGCACAAAGA; 3' primer CATGTTAGGCAAATTGCAAA; LMP2 probe ATCCAGTATGCCTGCCTGTA; β -actin 5' primer CCTTCCTGGG CATGGAGTCTT; 3' primer CCTCGTTACTAGAACTAGAAG; β -actin probe ACATCCGCAAAGACCTGTACGCCAACACAG.

Immunoblotting

The equivalent of 2×10^5 cells were boiled and loaded into 10% Tris-glycine gels. The electrophoresed proteins were transferred onto nitrocellulose membranes. Blots were incubated with a human serum with high-titre antibodies against EBNA, a MoAb directed against LMP1 (CS1-4; Dako), or a MoAb directed against EBNA 2 (PE2; Dako). Immunoblots were developed using the ECL Western blotting detection systems (Amersham Life Science, Arlington Heights, IL).

Detection of apoptosis

Anti-Fas induced apoptosis was evaluated by flow cytometric assay according to Nicoletti *et al.* [21]. Activated NK cells were cultured with anti-Fas antibody (CH-11; MBL) for 18 h. Cells were then fixed with 70% ethanol, resuspended in a PBS buffer containing RNase A and propidium iodide (PI; Sigma, St Louis, MO), incubated at room temperature for 15 min, and analysed by Epics EL.

Target-induced death of activated NK cells was evaluated by growth inhibition by K562 cells. NK cells (1×10^5) were seeded in a U-bottomed 96-well microplate with or without 5×10^4 irradiated K562 cells or LCL in RPMI 1640 supplemented with 10% FCS. After 48 h of culture, cells were pulsed with $2 \mu\text{Ci/ml}$ of ^3H -TdR for 2 h, and harvested onto a glassfibre filter for determination of ^3H -TdR retention. Growth inhibition was calculated as (^3H -TdR without inhibitor – (^3H -TdR with inhibitor – ^3H -TdR inhibitor alone))/ ^3H -TdR without inhibitor.

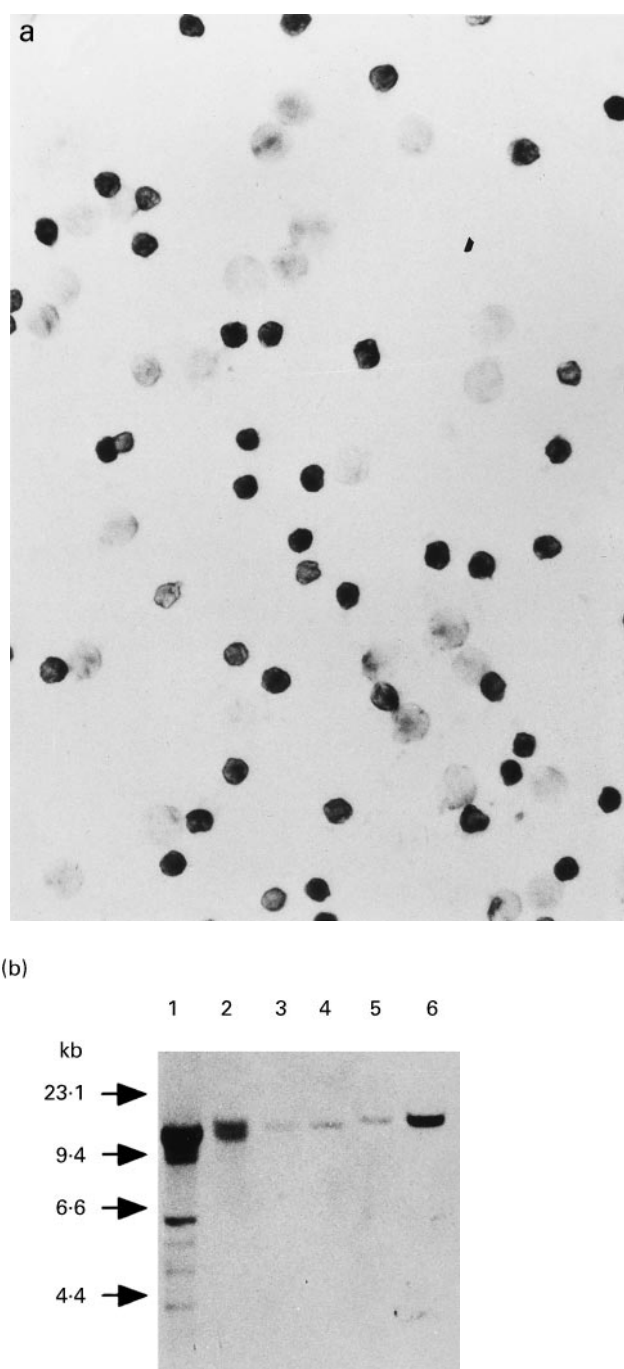


Fig. 1. (a) Peripheral blood mononuclear cells (PBMC) from patient 1 were sorted into CD16 $^{+}$, CD56 $^{+}$ by EPICS Elite, and then *in situ* hybridized with an EBER1 probe. (b) Southern blot analysis of terminal fragments of the EBV genome. Genomic DNA from B95-8 cells (lane 1), lymphoblastoid cell lines (LCL) from patient 2 (lane 2), PBMC from patient 1 (lane 3), patient 2 (lane 4), patient 3 (lane 5) and KAI3 cells (lane 6) were digested with BamHI and Southern hybridized with a ^{32} P-labelled probe.

RESULTS

Increased CD56 $^{+}$ CD16 $^{+}$ NK cell population in patients with severe mosquito allergy

The phenotype of PBMC of three patients is shown in Table 2. Both percentage and absolute counts of CD16 $^{+}$ CD56 $^{+}$ NK cells

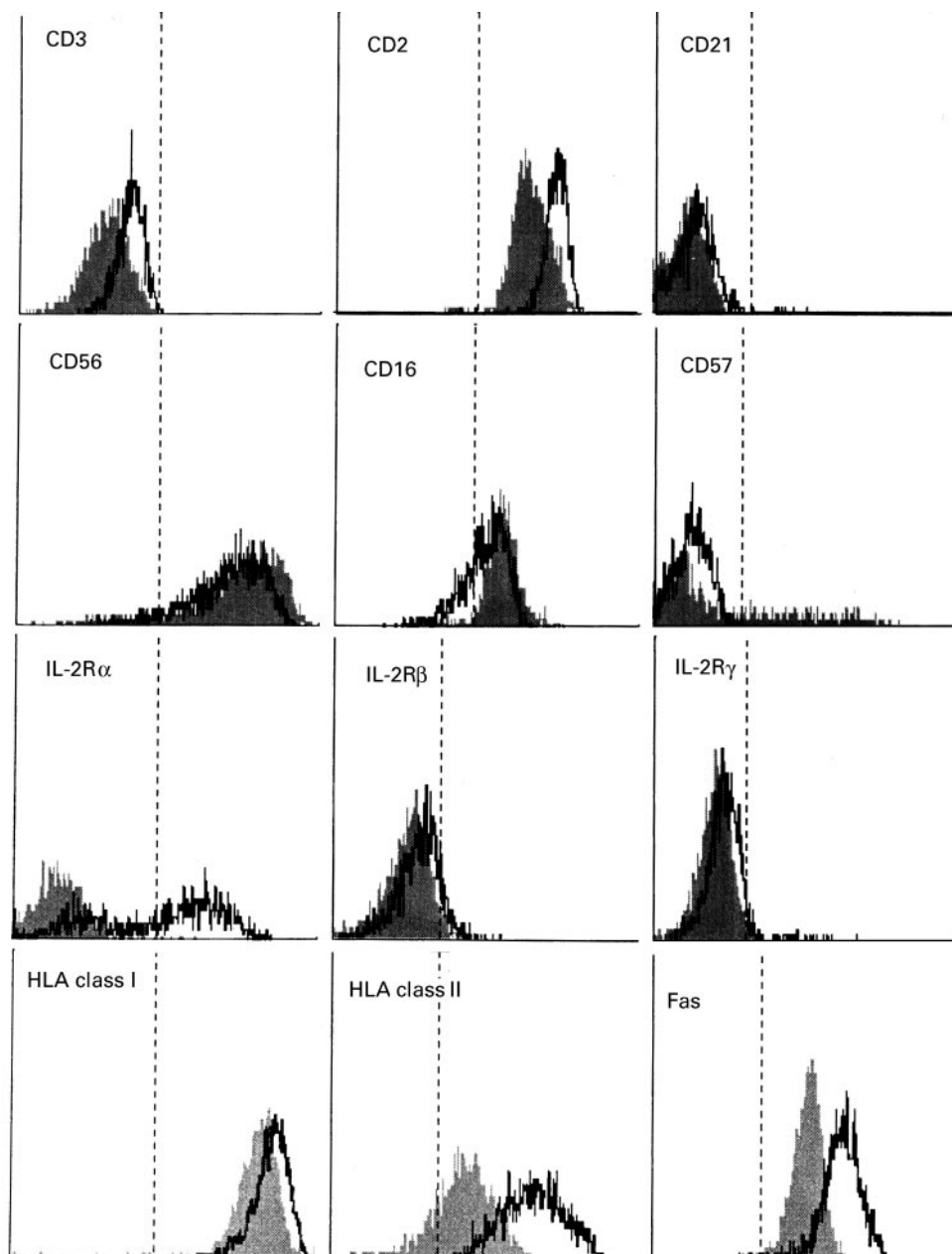


Fig. 2. Flow cytometric analysis of KAI3 cells (solid line) compared with IL-2-activated normal NK cells (grey shadow). The dotted lines represent background levels determined by isotype controls.

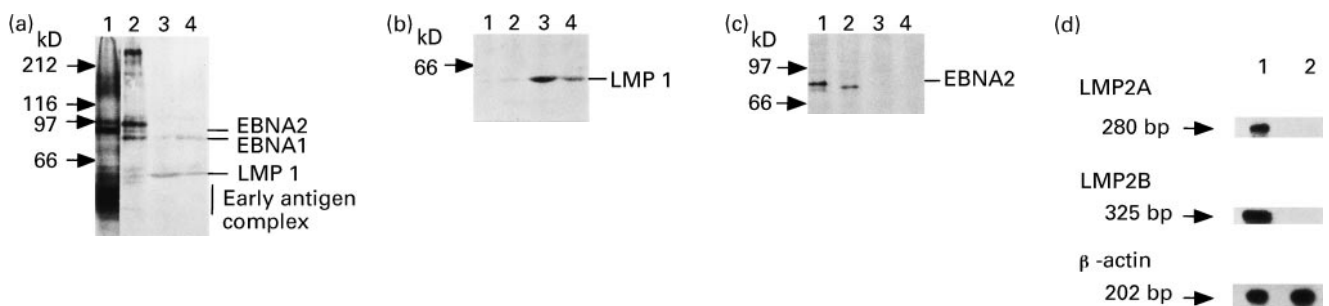


Fig. 3. Analysis of EBV gene expression. (a–c) Cell extracts from B95-8 cells (lane 1), lymphoblastoid cell lines (LCL) from patient 2 (lane 2), KAI3 cells (lane 3) and enriched NK cells from patient 1 (lane 4) were electrophoresed through 10% Tris-glycine gels, transferred onto nitrocellulose membranes, probed with a human serum containing high titre antibodies to EBV (a), a murine MoAb to LMP-1 (b) and a murine MoAb to EBNA 2 (c). (d) EBV gene expression in LCL from patient 3 (lane 1) and KAI3 cells (lane 2) as assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. PCR products were hybridized with ^{32} P-labelled probes.

were increased in all three patients. During the 3–4 years of follow up, the NK cell counts fluctuated, and did not exhibit progressive proliferation. Morphologically, large granular lymphocytes (LGL) were increased in the peripheral blood of all three patients.

EBV-infected cells were found exclusively in the NK cell population and clonally expanded

CD16⁺CD56⁺ cells and CD16⁺CD56[−] cells highly purified by EPICS XL were prepared from the three patients, and *in situ* hybridized with antisense oligonucleotide probe to EBER1. Of NK cells from patients 1, 2 and 3, 60%, 15% and 25%, respectively, were EBER1⁺ (Table 2, Fig. 1A). In contrast, EBER1⁺ cells were <0.01% in CD16⁺CD56[−] cell populations.

The clonality of EBV DNA-positive cells was assessed by Southern blot hybridization with 1.9-kb XhoI fragment, a probe of the terminal region of EBV [16]. PBMC from the three patients showed single bands, showing that EBV DNA-positive cells of the patients expanded clonally (Fig. 1B, lanes 3–5). To analyse the karyotype of expanded NK cells, mitosis of enriched NK cells was induced by stimulation with IL-2 for 3 days. All of the patients showed normal karyotypes (data not shown).

Establishment and characterization of an EBV-infected NK-like cell line

To analyse further EBV-infected NK cell proliferation in the patients, an IL-2-dependent cell line was established from patient 3. An established NK-like cell line designated KAI3 has been growing stably for >16 months in the presence of IL-2. Cultures were maintained by changing a half volume of the IL-2-containing medium twice a week. Cells have so far been unable to grow without IL-2. In contrast, NK cells from normal donors could not be maintained even in IL-2-containing medium for longer than 2–3 weeks.

Immunophenotypic analysis demonstrated that the established line, designated KAI3, was positive for CD2, CD16, CD56, IL-2R α , HLA class I, HLA class II and Fas antigens but negative for CD3, CD4, CD8, CD19, CD20, CD21, and CD57 antigens (Fig. 2). The fluorescence intensity of CD2, IL-2R α , HLA class II and Fas antigens in KAI3 cells was stronger than that observed in IL-2-activated NK cells from three EBV⁺ normal donors. The expression level of IL-2R α in KAI3 cells was comparable to that of the short-term (5–15 days) cultured infected NK cells (data not shown), and the greater level of IL-2R α expression in KAI3 was not due to the greater length of culture with IL-2.

Morphologically, KAI3 cells appeared as large mononuclear cells. Azurophilic granules characteristic of LGL were not found, and NK cell activity against K562 target cells was not detected (data not shown). *In situ* hybridization using probes for EBER1 showed that >95% of KAI3 cells were infected with EBV. Southern blot analysis with the EBV TR probe showed the same single band found in PBMC from patient 3 (Fig. 1B, lane 6). This fact strongly suggests KAI3 cells were derived from EBV-infected circulating NK cells, although it might be possible that KAI3 were transformed *in vitro* by EBV derived from LCL used as feeders. Chromosomal analysis demonstrated that KAI3 cells possessed an abnormal karyotype, 46, XY, add(6)(p23), der(6)add(6)(p23)add(6)(q21).

Immunoblot assays showed that KAI3 cells and purified NK cells from patient 1 expressed EBNA 1 and LMP1, but other EBNA s were not detected. In contrast, LCL derived from patient 2 were found to express a full set of EBNA s and LMP1 (Fig. 3,a–c).

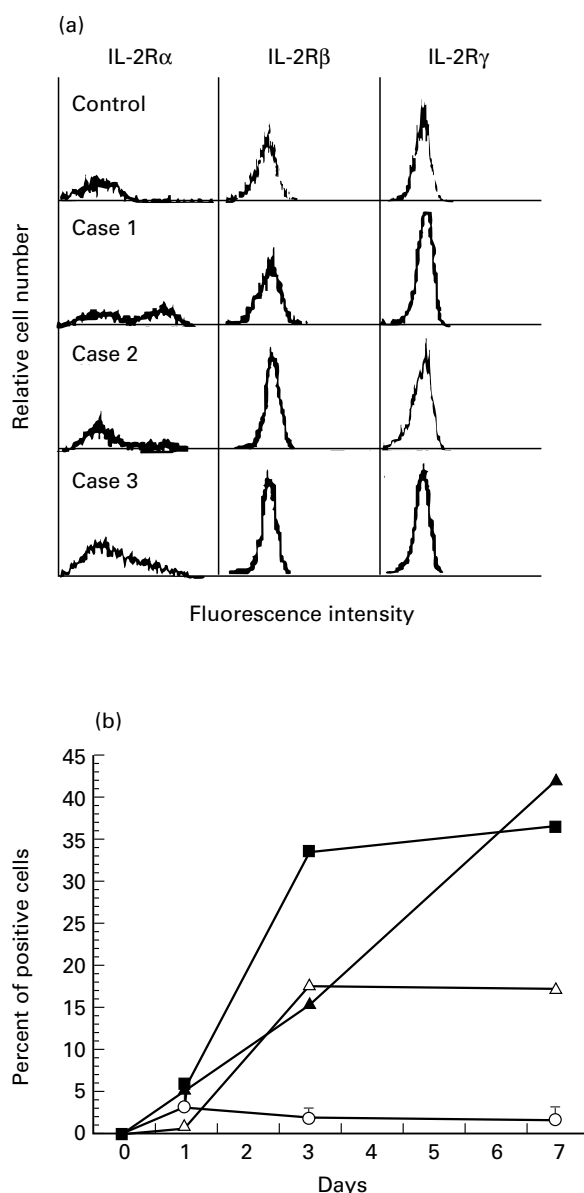


Fig. 4. (a) IL-2R expressions of NK cells from patients and a control after 7 days cultures with IL-2 were compared. (b) IL-2-induced IL-2R α expression. NK cells were enriched and cultured with IL-2 for days indicated. Activated NK cells were analysed with flow cytometry using anti-CD25 MoAb. $\blacktriangle$, Patient 1; $\blacksquare$, patient 2; $\triangle$, patient 3; $\circ$, normal donor ($n = 5$). Bars indicate s.d.

It is noteworthy that the LCL might be derived from EBV infection *in vivo* because the LCL showed a different EBNA 1 size to B95-8, although we established the LCL by culturing PBMC with B95-8 supernatant.

In RT-PCR analysis, LMP2A and -2B primers detected specific transcripts in LCL established from patient 3 but not in KAI3 cells (Fig. 3d). Although it is possible that our RT-PCR system could not detect low level transcription of LMP2 mRNA, the difference of expression levels between the LCL and KAI3 were apparent. The results of EBNA 1, 2 and LMP1 mRNA expression were consistent with results from immunoblotting analysis (data not shown).

IL-2-induced IL-2R α expression

As IL-2R α was up-regulated in the established KAI3 line in contrast to its low expression of NK cells derived from the peripheral blood of the patients, we examined the possibility that IL-2R α expression induced by IL-2 stimulation was augmented in EBV-infected NK cells. Enriched NK cells from the patients and normal subjects were cultured with IL-2, and IL-2R expression was assessed by flow cytometry. In Fig. 4a, IL-2R expressions on NK cells from patients were compared with a normal NK control after 7 days of culture with IL-2. While NK cells from patients expressed greater levels of IL-2R α than normal NK cells, the expression levels of IL-2R β and IL-2R γ were not different.

Kinetics of IL-2R α expression is demonstrated in Fig. 4b. While <5% of NK cells from normal donors expressed IL-2R α , and positive cells tended to decrease after longer culture periods, a high percentage of NK cells from the patients maintained IL-2R α expression.

Fas-induced and target cell-induced apoptosis were comparable in control and EBV-infected NK cells

The sensitivity of KAI3 and activated NK cells from the patients to the Fas apoptotic pathway was assessed by flow cytometry. Anti-Fas antibody induced apoptosis of EBV-infected NK cells in a dose-dependent manner (Fig. 5a), and the percentage of apoptotic cells was comparable to that observed in activated NK cells from a normal individual (Fig. 5b). Target cell-induced apoptosis was assessed by growth inhibition of KAI3 cells by K562 NK-sensitive cells. While K562 cells inhibited the growth of KAI3 cells by >80%, autologous and allogeneic LCL inhibit 23.6% and 25.7%, respectively, demonstrating KAI3 is sensitive to target cell-induced apoptosis (Fig. 5c).

DISCUSSION

In order to clarify the pathogenesis of persistent EBV-infected NK cell expansion, an IL-2-dependent NK-like cell line was established from one of the patients. To our knowledge, the established KAI3 is the first reported NK-like cell line established from patients with

SCAEBV before the onset of leukaemias or lymphomas. Although the peripheral blood NK cells from three patients possessed normal karyotypes, KAI3 cells exhibited an abnormal karyotype. EBV infection is not likely to be the sole reason for the immortalized state of KAI3 cells [22]. *In vitro* expansion by the culture with IL-2 might induce additional genetic damage that contributes to the immortalization. Alternatively, *in vitro* culture might select a pre-existent minor population with the abnormal karyotype.

Immunoblot and RT-PCR analysis revealed that the NK-like cell line expressed EBNA 1 and LMP1, but not EBNA 2 or other EBNA. This form of EBV latency (categorized as latency II [23–25]) is similar to that observed in a subset of nasopharyngeal carcinoma [19,20], Hodgkin's disease [26] and peripheral T cell lymphomas [27]. Because many of the EBV latent proteins can serve as target antigens for EBV-specific T cells [28], the down-regulation of EBNA may provide advantages for EBV-infected NK cells in evading immunological surveillance. However, it remains unclear why the LMP1 protein does not induce specific cytotoxic T lymphocytes (CTL). Further study is necessary to address these issues.

Even though EBV-infected NK cells might escape from immunological surveillance, this would not necessarily lead to the clonal expansion which we observed. Infected EBV might promote cell proliferation through EBV latent proteins. Expression of LMP1 in B cells provokes many of the phenotypic changes characteristic of B cell immortalization [29]. LMP1 can also induce cellular changes in cell types other than B cells [30,31]. Although most of the changes induced by EBV infection in the NK cell environment remain to be determined, we demonstrated that induction of IL-2R α expression by IL-2 is augmented in EBV-infected NK cells. As the percentage of IL-2R α -positive cells increased by >30-fold after 3 days exposure to IL-2, this can not represent a proliferation of the pre-existing low number of IL-2R α -positive cells. This augmented IL-2R α expression may contribute to promote expansion of infected NK cells.

Although the infected NK cells seemed to possess a growth advantage, as mentioned above, NK cell numbers in the peripheral blood of the patients were not progressively increased for several years of observation. As activation-induced cell death is regarded

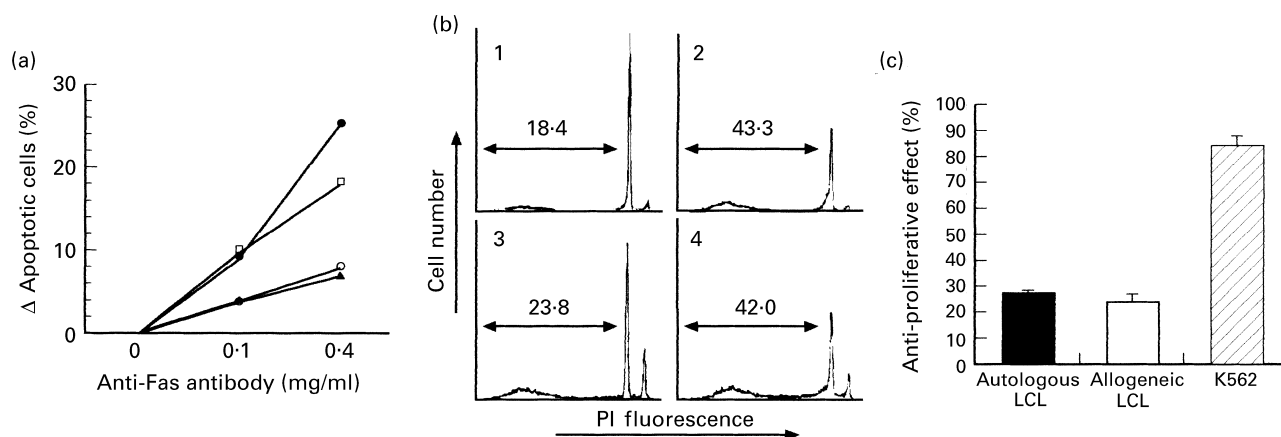


Fig. 5. Sensitivity to apoptosis of KAI3 cells and activated NK cells from patients. (a) Apoptosis induced by Fas antibody (Δ apoptosis = (Fas antibody-induced apoptosis) – (spontaneous apoptosis)). $\square$, KAI3; $\blacktriangle$, activated NK cell from patient 1; $\circ$, activated NK cell from patient 3; $\bullet$, activated NK cell from a normal donor. (b) Sensitivity of KAI3 cells (1,2) and activated NK cells from a normal donor (3,4) to Fas antibody (1, 3, 0 mg/ml; 2, 4, 0.4 mg/ml) were compared. Percentage of apoptotic cells was indicated. (c) Proliferation inhibition of KAI3 cells by autologous lymphoblastoid cell lines (LCL), allogeneic LCL and K562 cells.

as one of the mechanisms of NK cell regulation, we analysed sensitivity to apoptosis of infected cells. An anti-Fas antibody induced the same level of apoptosis in EBV-infected and control NK cells, although LMP1 was reported to induce expression of several molecules in B cells that function as inhibitors of the cell death pathway [32]. KAI3 cells were also sensitive to target cell-induced apoptosis [33].

Usually, reactions to mosquito bites are localized to skin, possibly mediated by IgE and cell-mediated hypersensitivity. However, Tokura *et al.* have reported NK cell-dominant mononuclear cell infiltration in mosquito bite sites of a severe HMB patient [34]. The relationship of mosquito antigen to NK cells is not understood at this time, and EBV-infected NK cell proliferation does not always exhibit severe HMB. Nonetheless, it could be speculated that accumulation of NK cells augments reactions on the skin, and that these NK cells may directly or indirectly mediate the systemic symptoms. On the other hand, the activated state of accumulated NK cells might persist for a longer time due to up-regulated IL-2-induced IL-2R α expression. Recurrent and prolonged activation may induce additional genetic damage including chromosomal changes that lead to genesis of leukaemias or lymphomas. The established NK-like cell line will contribute to further investigation of HMB pathogenesis and the relationship between EBV-infected NK cells and high incidence of leukaemias or lymphomas.

ACKNOWLEDGMENTS

The authors are most grateful to Professor Kazuyoshi Watanabe for his encouragement throughout this study. This work was supported in part by The Project of Human Science, The Ministry of Health and Welfare of Japan and The Japan Society for the Promotion of Science.

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