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Defective killer cell activity in patients with chronic active Epstein-Barr virus infection.

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Abstract

Natural killer (NK) cell activity, lymphokine activated killer (LAK) activity and Epstein-Barr virus specific cytotoxic T lymphocyte (EBV-CTL) activity were examined in 10 children with chronic active EB-virus infection and an adult with persistently positive early antigen-antibody to EB-virus. NK cell activity against erythroleukemia cell line K-562 was significantly (p less than 0.005) lower in the patients (22.3 +/- 8.5%, mean +/- SD) than in normal controls (40.4 +/- 15.9%). Spontaneous cytotoxicity against an EB-virus transformed autologous lymphoblastoid cell line was 15.0 +/- 7.6% in the patients, and was comparable to spontaneous cytotoxicity activity in normal controls (11.7 +/- 4.3%). LAK activity against Raji cells was significantly (p less than 0.02) lower in the patients (14.6 +/- 11.4%) than in normal controls (29.2 +/- 15.9%). EBV-CTL activity against an EB-virus transformed autologous lymphoblastoid cell line was significantly (p less than 0.005) lower in the patients (11.8 +/- 5.5%) than in seropositive normal controls (33.7 +/- 14.7%). No regression of the lymphoblastoid cell line was observed when EBV-CTL activity of the patients was tested by regression assay. It is conceivable that defects in both EB-virus specific and nonspecific killer cell activities play important roles in the pathogenetic abnormalities which allow EB-virus infection to progress to a chronic active state.

KEYWORDS: chronic active EB-virus infection, EB-virus specific cytotoxic T lymphocyte, natural killer, lymphokine activated killer

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Defective Killer Cell Activity in Patients with Chronic Active Epstein-Barr Virus Infection

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Natural killer (NK) cell activity, lymphokine activated killer (LAK) activity and Epstein-Barr virus specific cytotoxic T lymphocyte (EBV-CTL) activity were examined in 10 children with chronic active EB-virus infection and an adult with persistently positive early antigen-antibody to EB-virus. NK cell activity against erythroleukemia cell line K-562 was significantly ($p < 0.005$) lower in the patients ($22.3 \pm 8.5\%$, mean $\pm$ SD) than in normal controls ($40.4 \pm 15.9\%$). Spontaneous cytotoxicity against an EB-virus transformed autologous lymphoblastoid cell line was $15.0 \pm 7.6\%$ in the patients, and was comparable to spontaneous cytotoxicity activity in normal controls ($11.7 \pm 4.3\%$). LAK activity against Raji cells was significantly ($p < 0.02$) lower in the patients ($14.6 \pm 11.4\%$) than in normal controls ($29.2 \pm 15.9\%$). EBV-CTL activity against an EB-virus transformed autologous lymphoblastoid cell line was significantly ($p < 0.005$) lower in the patients ($11.8 \pm 5.5\%$) than in seropositive normal controls ($33.7 \pm 14.7\%$). No regression of the lymphoblastoid cell line was observed when EBV-CTL activity of the patients was tested by regression assay. It is conceivable that defects in both EB-virus specific and nonspecific killer cell activities play important roles in the pathogenetic abnormalities which allow EB-virus infection to progress to a chronic active state.

Key words : chronic active EB-virus infection, EB-virus specific cytotoxic T lymphocyte, natural killer, lymphokine activated killer

X-linked lymphoproliferative syndrome (XLP) is well known as an immunodeficiency caused specifically by Epstein-Barr (EB) virus infection (1, 2). XLP is a disease with familial occurrence, and only males are affected. Natural killer (NK) cell and EB-virus specific cytotoxic T lymphocyte (EBV-CTL) activities as well as antibody response to EB-virus antigens are defective in XLP (3, 4). NK cell, lymphokine activated killer (LAK) and CTL play an important role in virus infections as well in malignancies.

Though there are no XLP families in Japan, chronic active EB-virus infection similar to XLP was reported (5—7) in a

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few children of both sexes. In the present study, we examined NK, spontaneous cytotoxicity, LAK and EBV-CTL activities in 10 children with chronic active EB-virus infection and a mother of one of the patients, in whom antibody against early antigen (EA) of EB-virus was persistently elevated. The activities were significantly lower in these patients than in normal controls.

Materials and Methods

Patients. Ten children (Table 1) from 2 to 12 years of age at the time of examination were diagnosed as having chronic active EB-virus infection according to the criteria of Rickinson (8) : 1) chronic or recurrent infectious mononucleosis-like symptoms lasting for a period of at least one year and often longer, 2) an unusual pattern of anti-EB-virus antibodies with raised anti-EA and/or absent anti-EB-virus nuclear antigen (EBNA) titers, and 3) no evidence of any prior immunological abnormality or of any other recent infection by which to explain the condition.

None of the patients had a past history of acute infectious mononucleosis. Age of onset varied from 1 month to 12 years. All but one patient, in whom recurrent exanthem and fever were problems, showed prominent hepatosplenomegaly and lymphadenopathy lasting more than one year. The titer of anti-EB-virus capsid antigen (VCA)-IgG antibody ranged from 1 : 640 to 1 : 5120, and anti-EA-IgG antibody ranged from 1 : 80 to 1 : 320 in anti-EA-antibody positive 8 cases and $<1:10$ in two cases. Anti-EBNA-antibody was also absent in the patinets whose anti-EA-IgG antibody was absent. The mother of case 6, who was asymptomatic but showed persistently elevated anti-VCA-IgG and anti-EA-IgG antibodies, and 120 normal controls, aged from 2 to 14 years, were also examined.

Effector cells. Venous blood was obtained by venipuncture with 1 unit/ml of preservative-free heparine, and centrifuged by Ficoll-Hypaque gradient methods. The mononuclear cells on the interface were washed 3 times with phosphate buffered saline and adjusted to 1×10^6 cells/ml in RPMI-1640 (Flow Labs) supplemented with penicillin G, streptomycin, sodium bicarbonate and heat-inactivated fetal calf serum, which was used as a complete culture medium.

Target cells. K-562 cells, the erythroleukemia cell line, were used as the target cells for testing NK cell activity. An autologous lymphoblastoid cell line (LCL), derived from B cells transformed by EB-virus (B95-8 strain; kindly supplied by Dr. Shoki Yano, Department of Third

Table 1 Clinical data of the patients

Case	Sex	Age[a] (y-m)	Size[b] Liver (cm)	Size[b] Spleen (cm)	EB-virus antibody VCA-IgG	EB-virus antibody EA-IgG	EB-virus antibody EBNA
1. M.M.	M	0-1	6.5	6.0	640	<10	<10
2. T.N.	M	0-4	9.0	5.0	640	<10	<10
3. Y.C.	M	0-3	5.0	9.0	640	80	<10
4. K.I.	M	12-0	4.0	15.0	2,560	80	80
5. K.K.	M	8-5	1.5	0.5	1,280	320	40
6. R.F.	M	2-4	5.0	9.0	5,120	640	160
7. F.A.	F	7-6	1.0	5.0	5,120	1,280	160
8. M.I.	F	5-11	0	0	1,280	80	160
9. M.W.	F	12-1	10.0	12.0	2,560	320	160
10. R.O.	M	10-0	3.0	1.0	640	80	20
11. S.F.	F	24-0	0	0	640	40	80

a: Age of onset.

b: Size means the length of liver or spleen below the costal margin.

Abbreviations: EB-virus, Epstein-Barr virus; VCA, virus capsid antigen; EA, early antigen; EBNA, EB-virus nuclear antigen.

Internal Medicine, Kochi Medical School), was used as the target cells for testing spontaneous cytotoxicity and EBV-CTL activities. Raji cells were used as target cells for testing LAK activity.

NK and spontaneous cytotoxicity test method. NK and spontaneous cytotoxicity activities were measured by a previously reported method (9). Briefly, two hundred μCi of ^{51}Cr (Na_2CrO_4 in 0.9% NaCl, 134 mCi/mg Cr, JRIA) was mixed with the target cell suspension (1×10^7 cells/ml) and incubated for 45 min at 37°C in a 5% CO_2 atmosphere. ^{51}Cr-labelled target cells were washed 3 times with 4°C-cold culture medium and adjusted to 1×10^5 cells/ml in culture medium. One hundred μl of effector cell and target cell suspensions were mixed in wells of round bottomed 96 well microtiter plates (Nunc) and settled at 37°C in a 5% CO_2 atmosphere for 4 h (NK) or 18 h (spontaneous cytotoxicity). The cell mixtures were centrifuged, and 100 μl aliquots of the supernatant were harvested and counted in a gamma scintilation counter. Killer activity was expressed as % lysis:

$$\%\ \text{Lysis} = \frac{\text{Experimental release (cpm)} - \text{Spontaneous release (cpm)}}{\text{Maximum release (cpm)} - \text{Spontaneous release (cpm)}} \times 100.$$

Maximum release was obtained from the supernatant of target cells frozen-and-thawed 3 times, and spontaneous release was obtained from the supernatant of target cells maintained for 4 h (NK) or 18 h (spontaneous cytotoxicity) without effector cells. All samples were tested in triplicate, and data were expressed as the mean value.

LAK test method. LAK activity was measured by a previously reported method (10). Briefly, 0.2 U/ml of recombinant interleukin-2 (IL-2; Takeda Co., Ltd.) was added to mononuclear cells, and the cells were cultured for 4 days at 37°C in a 5% CO_2 atmosphere to activate them for use as effector cells. ^{51}Cr-labelled Raji cells were adjusted to 2×10^5 cells/ml, and 100 μl each of the effector cell and target cell suspensions were mixed in round-bottomed 96-well microtiter plates and settled at 37°C in a 5% CO_2 atmosphere for 4 h. LAK activity was expressed as net % lysis (% lysis by LAK − % lysis by nonactivated effector cells).

EBV-CTL test method. EBV-CTL activity was measured by a previously reported method (9). Briefly, the effector cell suspension (100 μl) was inoculated with 10 μl EB-virus solution, B95-8 strain, and cultured for 14 days in round-bottomed 96 well microtiter plates at 37°C in a 5% CO_2 atmosphere. ^{51}Cr-labelled target cells were adjusted to 1×10^5 cells/ml, and 100 μl of the target cell suspension was added to each well containing effector cells after 14 days of culture. One hundred μl of supernatant was harvested 18 h later. EBV-CTL activity was expressed as net % lysis (% lysis by EB-virus primed killer cells − % lysis by non-primed killer cells).

EBV-CTL activity was also measured by regression assay as described by Moss *et al.* (11). A suspension of EB-virus infected mononuclear cells (2×10^6 cells/ml) was distributed into 5 replicates of 0.2 ml each in flat-bottomed 96 well microtiter plates (Nunc). Cultures were observed for 4 weeks until complete regression of transformed cells was observed. Cell counts required for 50% incidence of regression were estimated by the method of Reed and Muench (12).

Results

The results of various killer cell activities are summarized in Table 2.

The patients' NK cell activity varied from 12.5% to 39.7% (mean ± standard deviation, 22.3 ± 8.5%) which was significantly ($p < 0.005$) lower than the NK cell activity of normal controls (40.4 ± 15.9%).

The patients' spontaneous cytotoxicity activity varied from 6.5% to 26.0% (15.0 ± 7.0%) which was almost the same as the spontaneous cytotoxicity activity of normal controls (11.7 ± 4.3%).

The patients' LAK activity varied from 3.3% to 40.4% (14.6 ± 11.4%) which was significantly ($p < 0.02$) lower than that of normal controls (29.2 ± 15.9%).

The patients' EBV-CTL activity tested by ^{51}Cr-release assay varied from 2.1% to 19.3% (11.8 ± 5.5%) which was significantly ($p < 0.005$) lower than that

Table 2 Killer cell activities of the patients

Case	Natural killer[a]	Spontaneous cytotoxicity[a]	LAK[a]	EBV-CTL	
				REG50[b]	% Lysis[a]
1. M.M.	29.5	9.1	13.1	>40	14.6
2. T.N.	n.t.[c]	12.0	n.t.	>40	8.3
3. Y.C.	14.5	26.0	10.4	>40	7.5
4. K.I.	39.7	n.t.	n.t.	>40	17.4
5. K.K.	20.2	9.2	5.0	>40	8.8
6. R.F.	24.3	21.5	3.3	>40	13.6
7. F.A.	21.4	20.4	13.0	n.t.	18.4
8. M.I.	12.5	6.5	40.4	>40	13.2
9. M.W.	14.9	n.t.	n.t.	>40	2.1
10. R.O.	n.t.	n.t.	17.2	n.t.	19.3
11. S.F.	23.6	n.t.	14.6	n.t.	6.8
Mean $\pm$SD	22.3 ± 8.5[d]	15.0 ± 7.6	14.6 ± 11.4[e]	>40[d]	11.8 ± 5.5[d]
Control	40.4 ± 15.9 (n = 114)	11.7 ± 4.3 (n = 18)	29.2 ± 15.9 (n = 44)	13.36 ± 8.27 (n = 49)	33.7 ± 14.7 (n = 46)

a: Killer cell activities were expressed as % lysis. Target cells used were K-562 for natural killer assay, autologous lymphoblastoid cell line for spontaneous cytotoxicity and Epstein-Barr virus specific cytotoxic T lymphocyte (EBV-CTL) assay, and Raji cells for lymphokine activated killer (LAK) assay.

b: 50% incidence of regression measured by regression assay. Figures indicate lymphocyte counts ($\times10^4$/well) required for REG50.

c: Not tested. *d*: $p<0.005$. *e*: $p<0.02$.

of seropositive normal controls ($33.7\pm14.7\%$). No patient showed lymphoblastoid cell line regression when tested by regression assay (REG50 $>40\times10^4$/well). The value of REG50 was 13.36 ± 8.27 ($\times10^4$/well) in seropositive normal controls, and the difference was highly significant ($p<0.005$).

Discussion

Chronic active EB-virus infection is a disease of lymphoproliferative disorders such as giant hapatosplenomegaly, lymphadenopathy, and malignant lymphomas as a late complication (5, 6). Though a very high titer of anti-VCA-IgG antibody and persistently elevated anti-EA-antibody are the serological characterisitics of the disease (5, 6), clinical signs and symptoms of the patients with chronic active EB-virus infection very much mimic XLP (8). It is usually severe in pediatric patients and a high mortality rate is observed. Okano *et al.* (5) reported that 3 out of 6 patients died, and we (6) reported that 4 out of 13 patients died from malignant lymphoma, virus associated hemophagocytic syndrome or pneumonitis. Though no adult case has been reported in Japan, chronic active EB-virus infection in adults, whose symptoms are depression, myalgia, recurrent fever or severe allergic diseases, has been reported by Tobi *et al.* (13), Straus *et al.* (14) and Jones *et al.* (15). Miller *et al.* (16) called the disease "fatigue syndrome".

Concerning the immunological abnormalities of patients with chronic active EB-virus infection, Kibler *et al.* (17) reported defective NK cell activity and elevated T suppressor activity to NK cells. It is well known that NK cells are important in eliminating virus infected cells (18). Harada *et al.* (3) reported that NK cell activity was defective in XLP patients. We reported

that NK cell activity to K-562 cells was defective in 4 children with chronic active EB-virus infection (9). In this study, NK cell activity of 8 children with chronic active EB-virus infection and an asymptomatic adult with persistently elevated EA-antibody was tested, and the results were the same as in both our previous report (9) in which 4 patients were tested, and the report of Kibler *et al.* (17). Yanagisawa *et al.* (19) reported a patient with fatal EB-virus infection in whom low spontaneous cytotoxicity, LAK and CTL activities against autologous LCL were observed, though NK cell activity against K-562 cells was the same as in normal controls. They induced EBV-CTL activity by mixed culture with autologous LCL for 6 days. However, the spontaneous cytotoxicity activity to autologous LCL in the patients tested in our study was almost the same as that of normal controls. Spontaneous cytotoxicity activity to LCL was tested by an 18-h ^{51}Cr-release assay, because killer activity was hardly detected in a 4-h assay not only in the patients but also in normal controls. Yanagisawa *et al.* tested the killer activity in a 4-h assay, and the different results might be due to the difference in the reaction time.

LAK activity is known to be important in nonspecific killer activity to malignant tumors (20). LAK may be important in virus infections also, especially EB-virus infection, because EB-virus infected B cells are reported to be resistant to NK cells but sensitive to LAK cells (21). As in the case of Yanagisawa *et al.* (19), the LAK activity of the 8 patients we tested was significantly lower than that of normal controls.

Harada *et al.* (3) reported that EBV-CTL activity tested by regresseion assay was defective in XLP. We have reported previously that EBV-CTL activity was low in 5 children with chronic active EB-virus infection (9). In this study, we examined EBV-CTL activity in 8 patients by regression assay and in 11 patients by ^{51}Cr-release assay, and found the activity to be significantly lower than that of normal controls by either method.

It is conceivable that defective activity of both nonspecific and specific killer cells allow EB-virus infection to progress to a chronic active disease or fulminant fatal disease, and may be due to the high incidence of malignant diseases in patients with severe chronic active EB-virus infection.

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