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Expression of Cytokine mRNA in Leukemic Cells from Adult T Cell Leukemia Patients

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HTLV-I infection of peripheral mature T cells appears to induce the expression of cellular genes including those of some cytokines and their receptors. We examined the expression of interleukin-1α (IL-1α), IL-1β, IL-2, IL-3, IL-4 and granulocyte/macrophage colony-stimulating factor (GM-CSF) at the mRNA level in fresh leukemic cells from 20 adult T cell leukemia patients to see whether there is any association between cytokine expression and HTLV-I expression and between their expression and clinical manifestations such as hypercalcemia or neutrophilia. IL-1α, IL-1β and IL-3 expression was observed in 3, 7 and 1 of 20 cases examined, respectively. However, there seemed to be no association between IL-1 expression and clinical manifestations. IL-2, IL-4 and GM-CSF mRNA expression was not detected. HTLV-I viral RNA expression was detected only in one case in which IL-3 mRNA was expressed in both peripheral blood and lymph node cells and a relatively high proportion of leukemic cells expressed IL-2 receptor (p55, Tac). Thus, in the present study we could not find any correlation between cytokine expression and HTLV-I expression in peripheral blood fresh leukemic cells except in one unusual case.

Key words: Cytokine expression — Adult T cell leukemia — Human T cell leukemia virus type I

Adult T cell leukemia (ATL)[1] is a leukemia of human T cell leukemia virus type I (HTLV-I)-infected peripheral mature T cells.[2] Peripheral mature T cells, when activated with appropriate stimuli, not only express activation-associated cellular antigens such as interleukin-2 (IL-2) receptors and HLA-DR antigens, but also produce a variety of cytokines including IL-2, IL-3, IL-4, IL-5, IL-6, transforming growth factor-β (TGF-β) and granulocyte/macrophage colony-stimulating factor (GM-CSF).

It has been reported that HTLV-I-infected cell lines abnormally express IL-2 receptors[3] and produce some cytokines.[4, 5] Recent reports have shown IL-1[6] and TGF-β[7] production by fresh leukemic cells in the majority of ATL patients examined, suggesting that they play a role in the development of characteristic clinical manifestations such as neutrophilia, osteolytic lesions and hypercalcemia, which are often observed in ATL.

In the present studies we examined IL-1α, IL-1β, IL-2, IL-3, IL-4 and GM-CSF mRNA expression in freshly isolated leukemic cells and compared the expression of HTLV-I viral RNA and clinical characteristics in ATL patients.

MATERIALS AND METHODS

Patients and diagnosis Twenty patients with ATL were studied (Table I). The diagnosis of ATL was made on the basis of clinical features, morphological characteristics,[1] cell surface phenotypes of leukemic cells,[8] serum antibodies to HTLV-I associated antigens[2] and HTLV-I proviral DNA integration in leukemic cells.[9] Of 20 ATL patients examined, 14 cases were acute-type and 6 cases were chronic-type.

Cell preparation Mononuclear cells were separated from heparinized peripheral blood by Ficoll-Conray density gradient centrifugation. Lymph nodes of ATL patients and normal spleen of a splenectomized patient with gastric cancer were each minced in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) containing 10% fetal calf serum (FCS) (Whittaker M. A. Bioproducts, Inc., Walkersville, MD) to give a single cell suspension. In the studies of IL-1 mRNA expression, the percentage of contaminating monocytes was determined by flow-cytometric analysis or by morphological examination of cytocentrifuge preparations. It was less than 5% of all mononuclear cells in 4 cases examined of which the leukemic cells expressed IL-1α or IL-1β mRNA. As a negative control, HL 60 cells (human acute promyelocytic leukemia cell line)[10] were cultured in RPMI 1640 medium containing 10% FCS and 20 μg/ml tobramycin. As positive controls for IL-1α and IL-1β mRNA expression HL60 cells were cultured with 50 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma Chemical Co., St. Louis, MO) in RPMI 1640 medium containing 10% FCS at 37°C for 24 h or normal spleen cells were cultured with 10 ng/ml TPA and 0.1% phytohemagglutinin-P (PHA-P) (Difco Laboratories, Detroit, MI) in RPMI 1640 medium containing 10% FCS at 37°C for 12 h. As positive controls for IL-1α

Table I. Summary of Patients Examined

Patient	Age	Sex[a]	Type[b]	WBC[c] (/mm^3)	Granulocyte (%)	RBC[d] ($\times 10^4$/mm^3)	Plt[e] ($\times 10^4$/mm^3)	Serum Ca (mEq/liter)	Tac[f] (%)	Mo2[g] (%)	IL-1α[h]	IL-1β[i]
K.M.	63	F	C	24,800	29.0	494	23.6	4.5	12.1	0	N	P
Y.N.	70	F	A	69,700	9.0	406	11.4	5.9	15.7	2.4	N	N
S.Y.	38	M	C	156,900	12.0	279	22.2	4.1	17.7	5.9	N	N
Y.T.	68	F	C	16,400	20.5	356	37.4	4.6	12.2	9.4	P	P
S.F.	53	M	A	149,000	10.0	354	9.9	4.2	54.6	3.9	N	N
T.N.	60	M	C	66,300	60.0	500	14.8	5.6	ND[k]	ND	P	P
K.Y.	52	F	C	21,900	30.0	445	23.2	4.6	60.1	ND	P	P
S.M.	40	F	A	71,000	22.0	417	4.9	6.1	54.0	6.4	N	N
T.I.	62	F	A	119,500	5.0	477	6.9	5.6	71.4	7.5	N	N
M.A.[j]	44	M	A	15,500	41.0	431	17.0	4.4	63.4	3.4	N	N
Y.S.	38	M	C	137,600	15.0	300	20.0	4.0	15.8	0	N	P
M.S.	26	M	A	45,900	11.5	270	9.5	4.6	38.3	ND	N	P
H.K.	59	F	A	148,500	ND	205	2.3	5.4	9.0	ND	N	N
S.M.	48	M	A	121,000	<5.0	340	7.5	6.7	ND	ND	N	N
S.M.	51	F	A	49,700	62.0	326	3.9	7.1	54.4	14.0	N	N
Y.O.	44	F	A	14,600	23.0	459	17.5	5.2	70.0	ND	N	N
K.H.	57	M	A	65,000	<10.0	326	ND	7.3	18.0	ND	N	P
D.M.	61	M	A	127,500	25.0	489	16.7	8.1	23.0	<1.0	N	N
Y.T.	48	M	A	48,800	35.0	483	16.6	4.5	4.0	3.6	N	N
F.N.	78	M	A	21,900	60.5	447	19.8	8.4	17.0	3.3	N	N

a) M, male; F, female.
b) Clinical type of the patients. A, acute-type; C, chronic-type.
c) White blood cell count of peripheral blood.
d) Red blood cell count of peripheral blood.
e) Platelet count of peripheral blood.
f) Percentage of Tac-positive cells.
g) Percentage of Mo2-positive cells.
h) IL-1α mRNA expression (P, positive; N, negative).
i) IL-1β mRNA expression (P, positive; N, negative).
j) In this case, IL-3 mRNA and HTLV-I viral RNA expressions were observed.
k) ND, measurement was not done.

mRNA and HTLV-I viral RNA expression, ATL-2[11] cells (HTLV-I-infected cell line) were cultured in RPMI 1640 medium containing 10% FCS at 37°C. As positive controls for IL-2, IL-3, IL-4 and GM-CSF mRNA expression, normal spleen cells were cultured with 10 ng/ml TPA and 0.1% PHA-P in RPMI 1640 medium containing 10% FCS at 37°C for 12 h.

Cells were washed with phosphate-buffered saline (PBS) three times and frozen at −70°C.

Northern blot hybridization Total cellular RNA was isolated by ultracentrifugation on a guanidine isothiocyanate/CsCl gradient. Twenty micrograms of total RNA was electrophoresed in 1% agarose/formaldehyde denaturing gels[12] and was transferred to nitrocellulose filters. After prehybridization, the filters were hybridized with labeled probes and washed at 45°C. Exposure to X-ray film (RXO, Fuji Film, Co., Tokyo) was done at −70°C with an intensifying screen (Grenex G8, Fuji Film Co.).

Southern blot hybridization Southern blot analysis was performed as described previously.[13] Hybridization, washing and autoradiography were performed as described for the Northern blot hybridization except that washing was carried out at 65°C.

Probes *Hind* III-*Hinc* II fragment of human IL-1α cDNA provided by Dr. J. Yodoi (Kyoto University, Kyoto) and human IL-1β cDNA in pcDV$_1$ vector provided by Dainippon Pharmaceutical Co. (Osaka) were used as probes for IL-1α and IL-1β, respectively. *Hinf* I fragment of pIL-2-50A, provided by Dr. T. Taniguchi[14] (Osaka University, Osaka), was used as a probe for IL-2. *Bam* HI fragment of pcD-SRα-hIL-3, *Bam* HI fragment of pcD-hIL-4[15] and *Bam* HI fragment of pcD-hGM-CSF, provided by Dr. K. Arai (DNAX Research Insti-

tute, CA), were used as probes for IL-3, IL-4 and GM-CSF respectively. *Acc* I-*Sma* I fragment of HTLV-I pX region,[16] provided by Dr. T. Honjo (Kyoto University, Kyoto), was used as a probe for HTLV-I. These probes were ^{32}P-labeled using the Multiprime DNA labeling system (Amersham Japan, Tokyo) or nick translation kit (Amersham Japan).

Flow cytometric detection Cell surface antigens of leukemic cells were detected by flow cytometric analysis after indirect immunofluorescence staining with OKT3, OKT4, OKT8, OKT11, OKIal (Ortho Diagnostic Systems, Westwood, MA), anti-Tac[17] and Mo2 (Coulter Immunology Hialeah, FL) monoclonal antibodies as described previously.[18]

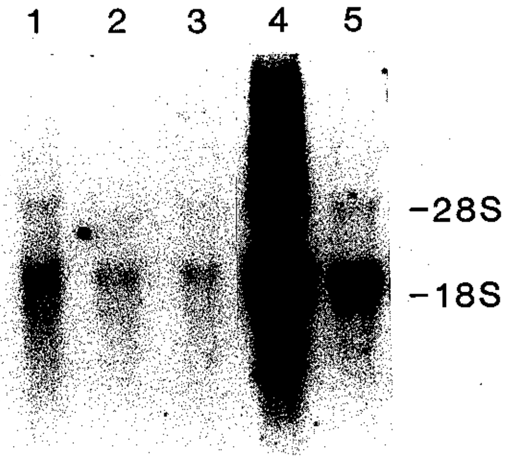

Fig. 1. Northern blot analysis of IL-1α mRNA expression. Lane 1, case K.Y.; lane 2, case T.N.; lane 3, case Y.T.; lane 4, ATL-2 cell line; lane 5, HL-60 cell line stimulated with 50 ng/ml TPA for 24 h. The size markers are 18S and 28S ribosomal RNAs.

RESULTS

IL-1α and IL-1β mRNA expression, and their relation to clinical features To examine the expression of IL-1 mRNA, Northern blot analysis was performed (Fig. 1 and Fig. 2). Freshly isolated normal spleen cells did not express any cytokine mRNA, but they expressed IL-1α, IL-1β, IL-2, IL-3, IL-4 and GM-CSF mRNA when stimulated with TPA and PHA-P. When stimulated with TPA, HL-60 cells began to express IL-1α and IL-1β mRNA. ATL-2 cells constitutively expressed IL-1α mRNA and its level was much higher than that of HL-60 cells stimulated with TPA. IL-1α mRNA was expressed in 3 of the 20 cases and IL-1β mRNA was expressed in 7 of the 20 cases examined. Peripheral blood leukemic cells expressed both IL-1α and IL-1β mRNA in 3 cases. The sizes of IL-1α and IL-1β mRNA were about 2.5 kb and 1.8 kb, respectively, which were the same sizes as those of normal IL-1α and IL-1β mRNA.[19] No gross rearrangement or obvious amplification of IL-1 genes was detected by Southern blot analysis (data not shown). Hypercalcemia (serum Ca $>$5 mEq/liter) was observed in 2 of the 7 cases in which IL-1α and/or IL-1β mRNA expression was detected. Of 10 patients with hypercalcemia, IL-1α or IL-1β mRNA was expressed in peripheral blood leukemic cells from two patients. Nine patients showed neutrophilia, with levels greater than 10,000/mm^3. Leukemic cells from two of those patients expressed IL-1α and/or IL-1β mRNA.

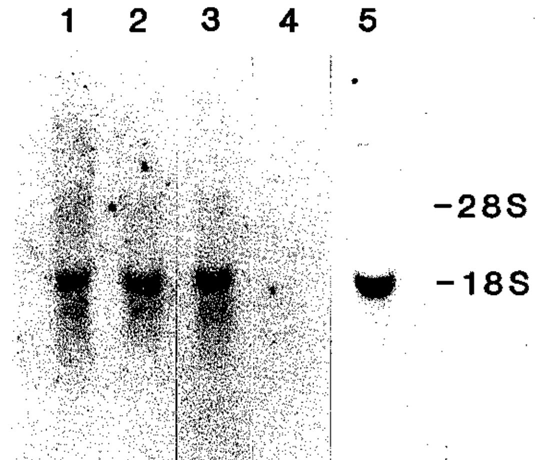

Fig. 2. Northern blot analysis of IL-1β mRNA expression. Lane 1, case K.Y.; lane 2, case T.N.; lane 3, case Y.T.; lane 4, ATL-2 cell line; lane 5, normal spleen cells stimulated with 10 ng/ml TPA and 0.1% PHA-P for 12 h. The size markers are 18S and 28S ribosomal RNAs.

IL-2, IL-3, IL-4 and GM-CSF mRNA expression IL-2, IL-4 and GM-CSF, which have T cell growth-promoting activity, were not expressed at the mRNA level in the 20 cases examined. IL-3 mRNA was expressed in one of the 20 cases examined (Fig. 3). The size of IL-3 mRNA was about 1.2 kb.[20] No gross rearrangement or amplification of IL-3 gene was observed by Southern blot analysis (data not shown). This case was a lymphoma type ATL in which both peripheral blood mononuclear cells and lymph node cells were mostly leukemic cells expressing IL-3 mRNA. However, in this case clinical manifestations which may be related to overproduction of IL-3, for example, elevation of cell numbers in any lineage of peripheral blood cells or hypercellular bone marrow, were not observed.

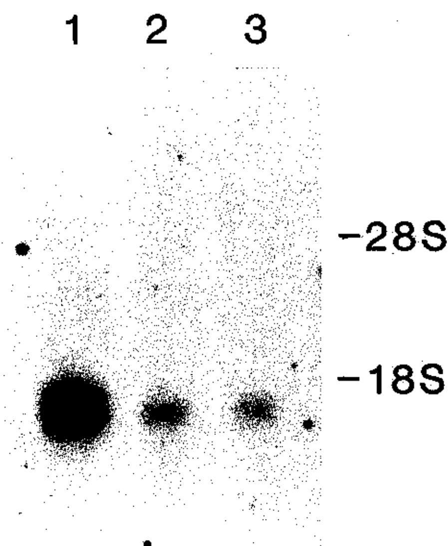

Fig. 3. Northern blot analysis of IL-3 mRNA expression. Lane 1, normal spleen cells stimulated with 10 ng/ml TPA and 0.1% PHA-P for 12 h; lane 2, case M.A. (lymph node cells); lane 3, case M.A. (peripheral blood mononuclear cells). The size markers are 18S and 28S ribosomal RNAs.

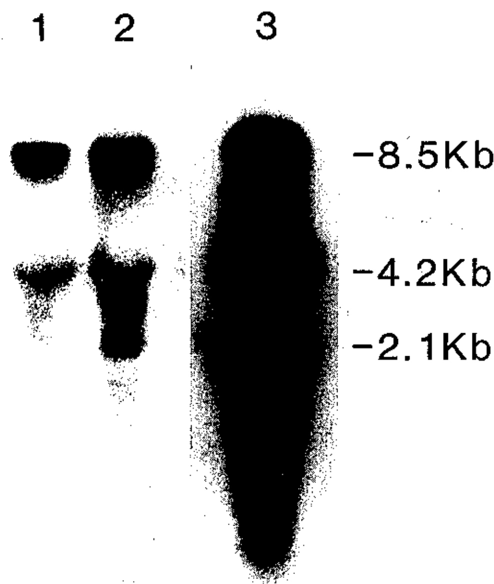

Fig. 4. Northern blot analysis of HTLV-I viral RNA expression. Lane 1, case M.A. (lymph node cells); lane 2, case M.A. (peripheral blood mononuclear cells); lane 3, ATL-2 cell line. 8.5 kb RNA is full length viral RNA. 4.2 kb RNA is mRNA for *env*. 2.1 kb RNA is mRNA for pX of HTLV-I.

HTLV-I viral RNA expression Although ATL cell lines and short-term-cultured ATL cells frequently express HTLV-I viral RNA, fresh ATL cells rarely express it.[21] In our study, leukemic cells from only one out of the 20 patients expressed HTLV-I viral RNA (Fig. 4). Interestingly, IL-3 mRNA expression was also detected in this case. Northern blot analysis of ATL-2 cells using HTLV-I pX cDNA as a probe revealed three bands corresponding to 8.5 kb RNA (full-length viral RNA), 4.2 kb RNA (*env*) and 2.1 kb RNA (pX).[22] These three bands were also detected in both the peripheral blood and lymph node leukemic cells from this patient, although 2.1 kb RNA expression of lymph node leukemic cells was very weak.

DISCUSSION

As described by Wano *et al.*,[6] IL-1α and/or IL-1β mRNA expression in peripheral blood leukemic cells was also observed in 7 of 20 cases we studied. IL-1β mRNA expression was more frequently observed. IL-1 has been demonstrated to have various biological activities including osteoclast-activating factor activity and granulopoiesis-promoting activity.[23] It is possible that IL-1 produced by leukemic cells is responsible for the development of hypercalcemia, osteolytic lesions and neutrophilia seen in ATL. However, our results show little association between IL-1 production and such symptoms and signs, suggesting the involvement of other factors.

Arima *et al.* classified the leukemic cells of ATL patients into four groups according to their IL-2 responsiveness and IL-2 production.[24] They discussed the correlation between them and clinical types of ATL (i.e., acute or chronic). In our results, 3 cases in which IL-1α mRNA was expressed were all chronic-type. Of 7 cases in which IL-1β mRNA was expressed, 2 cases were acute-type and the other 5 cases were chronic-type. In 5 cases out of 6 chronic-type cases we examined, IL-1α and/or IL-1β mRNA expression was observed, although it was observed only in 2 out of 14 acute-type cases examined. IL-1 mRNA may be expressed more frequently in chronic-type cases.

IL-2, IL-4 and GM-CSF have T cell growth-promoting activity and peripheral blood leukemic cells from some ATL patients proliferate in response to exogenous IL-2 or IL-4,[25] which suggests cell growth of ATL cells by autocrine mechanisms.[26] Arima *et al.* reported that leukemic cells from some ATL patients secreted IL-2 and proliferated in mitogen-free medium, and the proliferation was inhibited by anti-Tac or anti-IL-2 monoclonal antibody.[24] They also reported that these cases were all acute-type. However, IL-2 mRNA expression was not detected by Northern blot analysis in any of the

14 acute-type ATL cases we examined. No expression of IL-2 or IL-4 mRNA indicates that IL-2 or IL-4 autocrine mechanisms do not work, at least, in most of the peripheral blood leukemic cells.[27]

It has been reported that HTLV-I-infected cell lines produce various cytokines such as IL-1, IL-5, IL-6, GM-CSF and IFN-γ and constitutively express IL-2 receptor (Tac). In addition some HTLV-I-infected cell lines are known to express HTLV-I viral RNA and proteins which are rarely expressed in freshly isolated leukemic cells. Umadome *et al.* reported the close association between HTLV-I viral RNA expression and IL-2 receptor (Tac) mRNA expression in short-term-cultured leukemic cells from ATL patients.[28] These observations strongly suggest that HTLV-I viral products might induce the gene expression of some cytokines and IL-2 receptor (Tac). In fact, one of the HTLV-I viral products, $p40^{tax}$, was revealed to have a transacting transcriptional activity and to induce the expression of IL-2 and IL-2 receptor (Tac) genes in transient transfection studies.[29, 30] Furthermore, Arai *et al.* recently reported that $p40^{tax}$ activated the cotransfected human GM-CSF and IL-3 promoter genes in T cell lines in a transient transfection assay.[31]

In the case of M.A., both IL-3 mRNA and HTLV-I viral RNA expression in leukemic cells were clearly demonstrated by Northern blot analysis. In addition, Tac peptide of IL-2 receptor was also expressed in 54.2% and 63.4% of lymph node cells and peripheral blood mononuclear cells, respectively, which are higher than the values usually detected in ATL patients. This unusual case appears very interesting, because freshly isolated leukemic cells expressed both HTLV-1 viral RNA and IL-3 mRNA. There may be a close association between HTLV-I viral product(s) and the activation of IL-3 gene *in vivo.*

The failure to detect HTLV-I viral RNA expression in the remaining cases in which leukemic cells expressed IL-1α and/or IL-1β mRNA may suggest that the expression of some cytokines is not associated with HTLV-I expression. Alternatively, the expression of an undetectable amount of HTLV-I RNA is enough to induce gene expression of some cytokines.

Finally, IL-3 produced by leukemic cells may modulate the clinical features or affect the host defense mechanisms in this case, although characteristic abnormalities which may be ascribed to the overproduction of IL-3 have not yet been observed.

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