and 15 chronic-type ATL patients to determine whether ATL cells themselves can be immunogenic and evoke HTLV-I-specific T cell response. We demonstrated that in nearly 50% of the ATL patients tested HTLV-I Tax was inducible after short-term culture. Nucleotide sequences of HTLV-I *tax* at representative CTL epitopes in these ATL cells were mostly conserved. Interestingly, rats inoculated with formalin-treated uncultured ATL cells successfully developed helper T cell responses specific for Tax-expressing cells *in vivo*, indicating that ATL cells may express a small but sufficient amount of HTLV-I antigens for T cell response *in vivo*. Our findings suggest that ATL cases may be divided into 2 groups depending on the ability to express HTLV-I antigens and in nearly 50% the cases of ATL patients, ATL cells may potentially be recognized by HTLV-I-specific T cells *in vivo*.

Material and methods

Patients and PBMC preparation

Heparinized peripheral blood samples were donated under informed consent from 19 patients diagnosed as acute-type or chronic-type ATL at Ryukyu University Hospital, Imamura Bun-in Hospital in Kagoshima, and Nagasaki University Hospital and from uninfected healthy volunteers. The clinical status of these patients is summarized in Table I. The diagnosis and clinical subtype of ATL were made according to Shimoyama's criteria.[8] The ATL patients did not receive any chemotherapy when tested. Two samples from Patient 1 were used in our study. The first sample (1-i) was taken at a chronic phase and the other (1-vi) was taken at the acute crisis after an 18-month interval. Otherwise, one sample per patient was used. PBMC were isolated by using Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) density centrifugation and cryopreserved in liquid nitrogen until use.

Animals

Inbred female F344/N Jcl-rnu/+ rats (F344 n/+; 4-week-old) were purchased from Clea Japan, Inc. (Tokyo, Japan). Rats were treated under the experimental protocol of the Animal Care Committee of our university.

Cell lines

HTLV-I-negative human T cell line Molt-4[31] and HTLV-I-producing human T cell line MT-2[32] were maintained in 10% heat-inactivated FBS (Sigma, St. Louis, MO), 100 U/mL of penicillin, 100 μg/mL of streptomycin in RPMI 1640 medium (Sigma) (10% FBS-RPMI).

ILT-Hod,[33] an IL-2-dependent HTLV-I-infected human T cell line was maintained in the presence of 10 U/mL of recombinant human IL-2 (rhIL-2; Shionogi Co., Osaka, Japan) in 10% FBS-RPMI. In addition, 2 other IL-15-dependent HTLV-I-infected human T cell lines, ILT-79 and ILT-85, were established from ATL Patients 79 and 85, respectively. To establish these lines, a CD4-positive cell-enriched fraction negatively separated from PBMC by using Dynabeads M-450 CD8 (Dynal, Oslo, Norway) and Dynabeads M-450 CD19 (Dynal) was stimulated with 1 μg/mL of phytohemagglutinin (PHA-p; Difco Laboratories, Detroit, MI) for 24 hr, washed and cultured in 10% FBS-RPMI containing 10 ng/mL of rhIL-15 (Sigma) for 1–3 months.

HLA-A24-restricted HTLV-I Tax-specific $CD8^+$ CTL line was induced from PHA-p-stimulated PBMC of a post-HSCT ATL patient by repeated stimulation with formalin-fixed autologous HTLV-I-infected cells established before the HSCT.[29] The CTL line was maintained in the presence of 100 U/mL of rhIL-2 with periodical stimulation with formalin-fixed autologous HTLV-I-infected cells at 10–14 day intervals.

HTLV-I-infected rat T cell line, FPM1,[12] derived from an F344 n/+ rat, were cultured in 10% FBS-RPMI. G14[14] is IL-2-dependent HTLV-I negative $CD8^+$ T cell line established from a F344 n/+ rat. G14-Tax[14] is a stable transfectant of G14 with HTLV-I Tax-expressing plasmids. G14 and G14-Tax were maintained in 10% FBS-RPMI containing 5.5×10^{-5} M of 2-mercaptoethanol and 10 U/mL of rhIL-2.

Monoclonal antibodies

To detect intracellular HTLV-I antigens, mouse monoclonal antibodies (mAbs), Lt-4 (anti-p40 Tax, mouse IgG3),[34] NOR-1 (anti-p24 and p53 Gag; mouse IgG1),[35] GIN-7 (anti-p19, p28 and p53 Gag; mouse IgG2b)[35] and biotinylated GIN-7 were used.

For cell surface characterization, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse anti-human CD4, CD8, CD25, CD40, CD40L, CD86, OX40, HLA-A, B, C, HLA-DR (IgG1; BD Pharmingen Co., San Diego, CA), CD80 (IgG1; Immunotech, Marseille, France) and OX40L (TAG-34, IgG1)[36] mAbs were used. In addition, FITC-conjugated mouse anti-rat CD4 and PE-conjugated mouse anti-rat CD8 mAbs (IgG1; BD Pharmingen Co.) were used.

TABLE I – CLINICAL STATUS OF ATL PATIENTS TESTED

Patient ID	Age	Gender	Type of ATL	WBC number/ μL	Mononuclear cells/ WBC (%)	Abnormal lymphocytes/ WBC (%)
#1-vi[1]	60	F	Acute	42,000	63	49
#22	38	M	Acute	16,100	50	35
#80	39	M	Acute	141,000	>95	91
#85	77	M	Acute	67,400	74	67
#91	66	F	Acute	27,200	89	61
#1-i[1]	58	F	Chronic	21,500	87	55
#5	72	M	Chronic	12,200	64	25
#6	70	F	Chronic	14,400	71	33
#7	62	F	Chronic	8,300	69	34
#8	60	F	Chronic	17,400	70	47
#20	64	M	Chronic	14,700	48	28
#23	68	M	Chronic	14,200	74	58
#29	58	M	Chronic	6,000	59	21
#42	54	F	Chronic	8,800	48	15
#54	63	F	Chronic	10,700	73	43
#69	54	F	Chronic	13,300	68	36
#79	63	F	Chronic	19,500	64	6
#89	50	F	Chronic	28,000	41	27
#90	53	M	Chronic	7,700	30	8
#92	66	F	Chronic	16,900	83	76

[1]Pt. #1-i and Pt. #1-vi are the identical individual with an initial diagnosis of chronic ATL, whose diagnosis changed to acute ATL associated with elevated levels of serum LDH after 18 months.

Intracellular and surface staining and flow cytometric analysis

For intracellular HTLV-I-staining, cells were fixed with 1% paraformaldehyde in PBS containing 20 μg/mL of lysolecithin (Sigma) for 2 min at room temperature. The cells were then centrifuged and resuspended in cold methanol. After incubation for 15 min at 4°C, the cells were centrifuged and incubated in 0.1% Triton-X in PBS for 5 min at 4°C. The cells were then washed with PBS containing 1% FBS and 0.1% NaN_3 (staining buffer), and incubated with mouse mAbs to HTLV-I antigens or BALB/c control ascites, and subsequently with FITC-conjugated goat anti-mouse IgG + IgM mAbs (Immunotech) for 30 min at room temperature. The optimal concentrations of these mAbs were determined before use. Cells were washed twice, fixed with 1% formalin in PBS and analyzed using a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA). Live cells were gated based on a pattern of SSC and FSC for approximately 1×10^4 cells.

An alternative permeabilizing method using saponin was also employed for intracellular staining. Briefly, cells were fixed with 4% formalin in PBS, then permeabilized with 0.5% saponin (Sigma) in staining buffer for 10 min at room temperature. Permeabilized cells were further incubated with mAbs to HTLV-I antigens as described above.

For surface staining, cells washed and stained with FITC- or PE-conjugated mAbs and appropriate isotype control mAbs. Cells were further stained with 7-ADD (BD Pharmingen Co.) and stained cells were gated out on FACS analysis to eliminate dead cells.

For two-color analysis of intracellular and cell surface antigens, cells were stained with FITC-conjugated mouse anti-human mAbs (CD80, CD86, OX40), fixed and permeabilized by saponin treatment. Permeabilized cells were further stained with biotinylated GIN-7, and subsequently with Cy-chrome streptavidin (BD Pharmingen Co.). After extensive washing, the cells were subjected to two-color flow cytometry.

Long PCR and nucleotide sequences

Genomic DNA was prepared from PBMC by sodium dodecyl sulfate-proteinase K digestion, followed by phenol-chloroform extraction and subjected to long PCR (Expand Long Template PCR system, Boehringer Mannheim, Mannheim, Germany) to detect deletion of HTLV-I provirus. The primers of HTLV-I long terminal repeat (LTR) were 5′-LTR (5′-GTTCCACCCCTT-TCCCTTTCATTCACGACTGACTGC-3′) and 3′-LTR (5′-GGC-TCTAAGCCCCCGGGGGAT-3′) as described before.[37] Each 500 ng of genomic DNA was subjected to 10 cycles of denaturation (94°C, 10 sec), annealing (65°C, 30 sec) and extension (68°C, 8 min), and additional 20 cycles of denaturation (94°C, 10 sec), annealing (65°C, 30 sec) and extension (68°C, 8 min + 20 sec/cycle), then finalized by elongation of the product (68°C, 7 min). The PCR products were visualized by ethidium bromide staining after 0.6% agarose gel electrophoresis. The expected size of the amplified fragments with these LTR primers from a full-length HTLV-I provirus was 7.7 kbp. Long PCR products were partially sequenced on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.) by using the Big Dye terminator and the primers pX4 (5′-GGGGAAGGAGGGGAGTCG-AGGGATAAGGAA-3′) or pX12 (5′-TTGCCCACCACCCTT-TTCCAGC-3′) in accordance with the manufacturer's instructions. Amino acid sequences at CTL epitopes, Tax 11-19 and Tax 301-309 restricted by HLA-A2 and A24, respectively, were then determined according to the nucleotide sequences.

Lymphocyte proliferation assay

For the mixed lymphocyte reaction (MLR), cryopreserved PBMC (2×10^5/well) from a healthy volunteer were co-cultured with formalin-fixed ATL cells with or without preculture *in vitro* (5×10^4/well) in 96-well U-bottom plates in triplicate at 37°C for 4 days. Cultures were pulsed with 37 kBq/well of [^{3}H]-thymidine ([^{3}H]-TdR) for an additional 16 hr to assess cell proliferation. Cells were harvested with a Micro 96 Harvester (Skatron, Lier, Norway) and [^{3}H]-TdR uptake into cells was measured in a microplate β counter (Micro Beta Plus; Wallac, Turku, Finland). Proliferation of HTLV-I-specific CTL (1×10^5/well) co-cultured with formalin-fixed ATL cells (5×10^4/well) for 2 days, and proliferation of rat spleen T cells (1×10^5/well) co-cultured with formalin-fixed various syngeneic rat cells (1×10^5/well) for 3 days were also similarly measured.

ELISA

Human and rat interferon-gamma (IFN-γ) production in 100 μL of culture supernatants was measured by Human IFN-γ ELISA kit (Endogen, Woburn, MA) and Rat IFN-γ ELISA kit (BioSource Inc., Camarillo, CA), respectively. Absorbances were measured at 450 nm using microplate reader (BioRad, Hercules, CA) and analyzed with Microplate Manager III software.

Inoculation of ATL cells in rats

Ten million formalin-fixed PBMC from ATL patients or uninfected healthy volunteers, with or without pre-culture *in vitro*, were subcutaneously administered to 4-week-old female F344n/+ rats twice with a 2-week interval. The rats were sacrificed at 1 month after second immunization. Spleen T cells from these rats were enriched through a nylon-wool column, and their IFN-γ production and proliferation against formalin-fixed syngeneic G14, G14-Tax or FPM1 cells were examined by IFN-γ ELISA and a [^{3}H]-TdR uptake assay, respectively, as described elsewhere.[38]

DNA-vaccination to rats

Plasmids containing wild-type *tax* cDNA controlled under the human β-actin promoter (pβMT-2 Tax) and its control plasmid pHβAPr.1-neo vector[39] were coated on Au particles and inoculated into rats by using Gene Gun as described previously.[14] Immunization was carried out 3 times with a 1-week interval. One week after final immunization, rats were sacrificed and spleen cells were collected.

Cytotoxicity assay

Spleen cells (5×10^6 cells) from immunized rats were used as effector cells after 7 days of co-culture with formalin-fixed G14-Tax (2×10^6 cells) in 24-well plate. Target cells (G14 or G14-Tax) were incubated with 370 kBq of [^{3}H]-TdR per 10^6 cells for 12 hours at 37°C, followed by extensive washing. These target cells (1×10^4/well) and effector cells (1×10^5/well) were plated in 96-well U-bottom plates at the effector/target ratio of 10. After 6 hr of incubation at 37°C, cells were harvested to glass filters and radioactivities remaining in the target cells were measured in a microplate β counter. The percentage of specific cell lysis was calculated as ([cpm without effector − cpm with effector]/cpm without effector) × 100.

Statistical analysis

Results are expressed as the mean ± SD. Differences between the 2 groups were analyzed for significance by Student's *t*-test. Differences among 3 groups were evaluated by Dunnett's *t*-test using SPSS Base 11.0J (SPSS Inc., Chicago, IL); p-values <0.05 were considered to be statistically significant.

Results

Detection of intracellular HTLV-I antigens in cell lines

Initially, to detect intracellular HTLV-I antigens by flow cytometry, the conditions of cell permeabilization and staining methods were determined using established cell lines. We used lysolecithin-paraformaldehyde, methanol and Triton-X to fix and permeabilize the cell membranes, and stained the cells with mAbs to HTLV-I p40 Tax (Lt-4), p24 Gag (NOR-1), and p19 and 28 Gag (GIN-7). The staining patterns under the optimal conditions are shown in Figure 1. HTLV-I-producing human T cell line MT-2, but not HTLV-I-negative Molt-4 cell line, was strongly stained with all of

these mAbs (Fig. 1*a,b*). Under the same conditions, an IL-2-dependent ILT-Hod cell line established previously from an ATL patient, exhibited 2 peaks, consisting of a large population weakly expressing HTLV-I antigens and a small population expressing substantial levels of the HTLV-I antigens (Fig. 1*c*). The levels of intracellular HTLV-I antigens, especially p40 Tax, fluctuated and were influenced by the culture conditions (data not shown).We

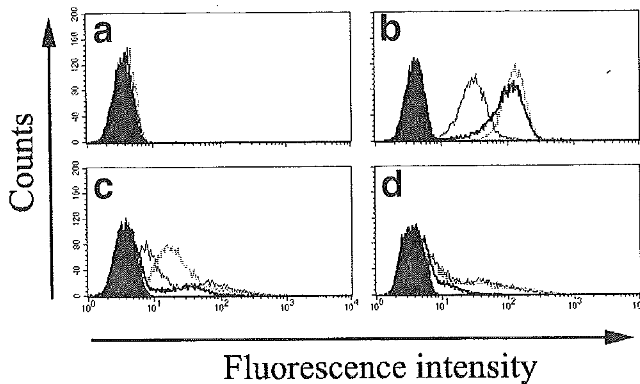


FIGURE 1 – Detection of intracellular HTLV-I antigens in cell lines. Molt-4 (*a*), MT-2 (*b*), and ILT-Hod (*c,d*) cells were permeabilized with Triton-X (*a–c*) or saponin (*d*) treatment (see Material and Methods), stained with control ascites (closed histogram), anti-p40 Tax mAb (Lt-4, solid lines), anti-p24 Gag mAb (NOR-1, thin lines) or anti-p19 and p28 Gag mAb (GIN-7, dashed lines) and subsequently with FITC-conjugated anti-mouse IgG + IgM. The cells were fixed with 1% formalin-PBS and analyzed using a flow cytometer.

also used saponin to permeabilize the cell membranes and compared the staining efficiency for detecting intracellular antigens with the methods using Triton-X. As shown in Figure 1*d*, saponin-treated ILT-Hod cells could also be stained with mAbs to HTLV-I but the detection levels were significantly lower than those in Triton-X-treated ILT-Hod cells. Thereafter, the permeabilization method using Triton-X was used primarily.

Induction of HTLV-I antigens in PBMC from ATL patients

The clinical status of the ATL patients tested is summarized in Table I. Expression of HTLV-I antigens in ATL cells from 5 acute and 15 chronic ATL patients who had not received chemotherapy were investigated. Cryopreserved PBMC from ATL patients were permeabilized, and stained with mAbs against HTLV-I antigens, Lt-4, NOR-1 and GIN-7 immediately (Day 0) or after *in vitro* 1-day cultivation. When viral expression was detected in 1 day, cells were kept in culture for 3–9 days if available. Representative data of intracellular HTLV-I-expression in the PBMC from an acute ATL patient (Patient 85) is shown in Figure 2. Although HTLV-I antigens were not detectable in the PBMC of the ATL patients before culture, a large number of live cells strongly expressed HTLV-I antigens in a 1-day incubation. The HTLV-I-positive cell number increased with further incubation, whereas the live cell number decreased (data not shown). The intensity of p40 Tax in the positive population nearly reached the maximal level in 1 day, whereas the intensity of p24 or p19 and p28 Gag antigens was further enhanced in 3 days of incubation. Similar induction was observed in the PBMC of 3 (Patients 22, 85, 91) of 5 acute-type ATL patients tested, although the proportion of HTLV-I-

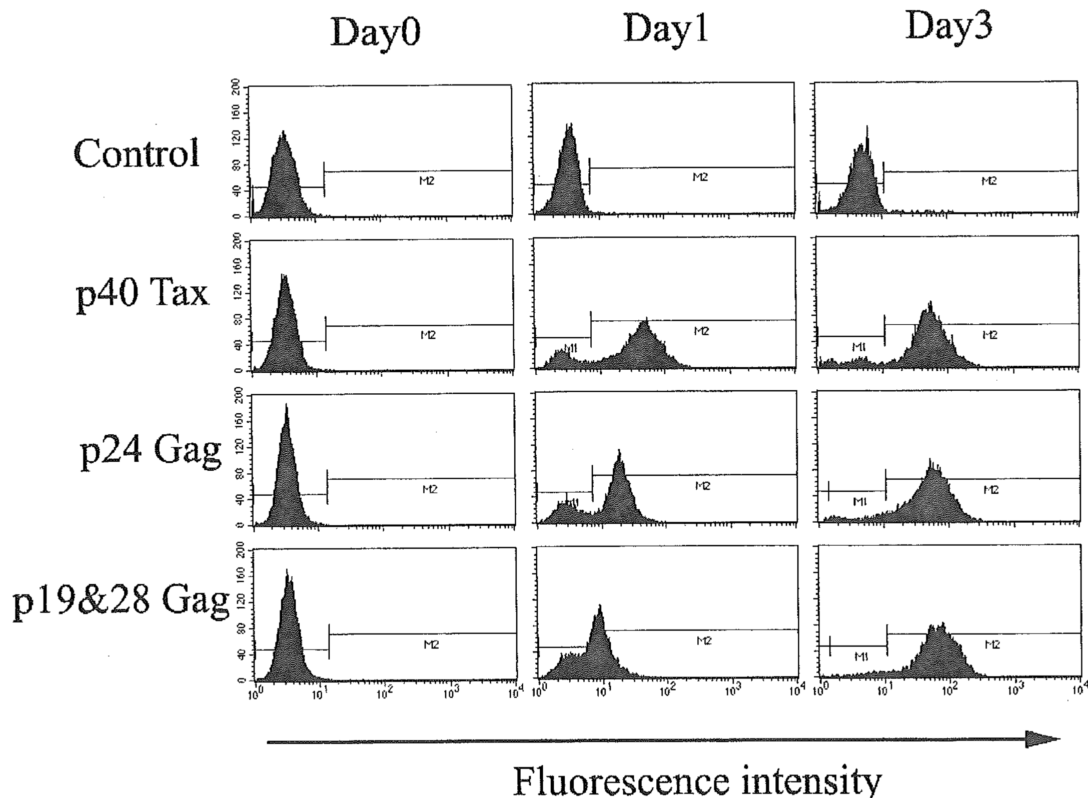


FIGURE 2 – Induction of HTLV-I antigens in PBMC of ATL patients after *in vitro* cultivation. Cryopreserved PBMC from Patient 85 (acute ATL) were incubated for the indicated periods in 10% FBS-RPMI, and permeabilized with Triton-X for intracellular staining with control ascites, Lt-4 (p40 Tax), NOR-1 (p24 Gag) or GIN-7 (p19 & p28 Gag) and subsequently with FITC-conjugated anti-mouse IgG + IgM. The M2 region shown in each histogram is regarded as positive.

expressing cells differed among individuals (Table II). In Patient 80 (acute ATL), only a small percentage of the cells expressed HTLV-I antigens after 9 days of incubation that probably arose from a minor population of the PBMC. The PBMC of Patient 1-vi who converted to acute-type ATL from chronic-type ATL (Patient 1-i) did not show any detectable levels of HTLV-I expression during 1 day of incubation.

In the chronic ATL patients, the results were more variable. In the PBMC of 3 (Patients 7, 79, 90) of 15 chronic ATL patients tested, more than 30% (range = 35.4–66.5%) of live cells expressed detectable levels of HTLV-I antigens in 1 day of incubation. In 3 chronic ATL patients (Patients 42, 54, 69), HTLV-I antigens were also induced but at a lower proportion (range = 6.8–11.3%) of the PBMC. No detectable levels of HTLV-I-expression were observed in the PBMC from the rest of the chronic ATL patients tested.

HTLV-I-induction was observed 3 of 5 acute-type and 6 of 15 chronic-type ATL patients, when HTLV-I-expression in >5% of the 1-day cultured PBMC was regarded as positive.

Conservation of representative CTL epitopes in ATL cells

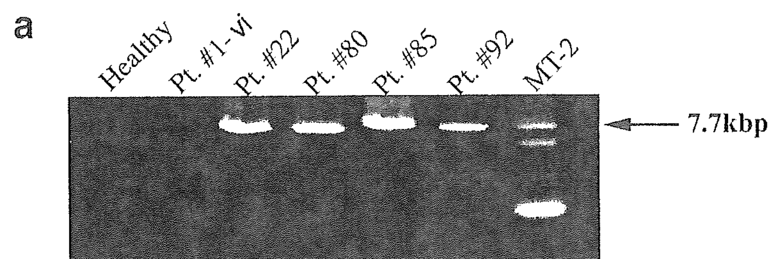
Because HTLV-I Tax is a major target antigen of HTLV-I-specific CTL,[16,17] we investigated whether ATL cells possessed mutations at the CTL epitopes in Tax. At first, the HTLV-I proviral genome integrated in the PBMC from 5 acute-type ATL patients was amplified by a long PCR method using LTR primers. As shown in Figure 3*a*, in 4 of 5 samples tested, comparable sizes of DNA fragments with a full-length provirus were amplified. No PCR product was obtained from the remaining case (Patient 1-vi). The DNA fragments amplified from the 3 patients were then examined for their nucleotide sequences at the regions corresponding to Tax 11–19 and Tax 301–309, representative CTL epitopes restricted by HLA-A2 and A24, respectively (Fig. 3*b*). Of the 4 acute-type ATL patients tested, 2 had HLA-A2, and all 4 had HLA-A24. The nucleotide sequences at Tax 11–19 were conserved in all patients regardless of the presence of HLA-A2. The sample from Patient 91 had a single mutation resulting in the substitution of serine (S) to asparagine (N) at the position 304. In the other 3 patients, nucleotide sequences at the Tax 301–309 region were identical to the prototype HTLV-I.[40]

TABLE II – INDUCTION OF HTLV-I ANTIGENS IN PBMC OF ATL PATIENTS FOLLOWING *IN VITRO* CULTIVATION

Patient	Type of ATL	Culture period (days)[1]	Percentage of positive cells for			
			Control antibody	Tax (Lt-4)	p24 (NOR-1)	p19&p28 (GIN-7)
#1-vi	Acute	0	0.1	0.0	0.0	0.1
		1	0.0	0.1	0.1	0.0
#22	Acute	0	0.0	0.0	0.0	0.0
		1	0.0	15.0	17.8	10.2
		3	0.0	26.4	56.4	38.1
#80	Acute	0	0.0	0.1	0.1	0.1
		1	0.0	0.5	0.3	0.2
		9	0.0	0.1	3.2	0.3
#85	Acute	0	0.1	0.0	0.0	0.0
		1	0.3	80.9	74.9	45.6
		3	0.6	91.4	93.9	97.3
#91	Acute	0	0.0	0.0	0.0	0.0
		1	0.2	31.4	25.8	22.5
#1-i	Chronic	0	0.0	0.0	0.0	ND[2]
		1	0.0	0.1	0.1	ND
#5	Chronic	0	0.0	0.0	0.0	0.0
		1	0.2	1.2	1.5	0.8
		9	0.1	0.9	7.0	6.7
#6	Chronic	0	0.2	0.1	0.1	ND
		2	0.1	0.2	0.1	ND
#7	Chronic	0	0.1	0.0	0.1	0.1
		1	0.2	52.3	66.5	62.1
#8	Chronic	0	0.1	0.1	0.1	0.0
		1	0.1	0.2	0.3	0.1
#20	Chronic	0	0.0	0.1	0.0	0.0
		1	0.3	0.5	0.4	0.4
#23	Chronic	0	0.0	0.0	0.1	0.0
		1	0.0	0.5	0.4	0.2
#29	Chronic	0	0.0	0.0	0.0	0.2
		1	0.1	0.3	0.4	0.1
#42	Chronic	0	0.0	0.0	0.0	0.0
		1	0.0	11.3	0.2	0.1
#54	Chronic	0	0.0	0.2	0.1	0.0
		1	0.1	9.7	10.5	5.4
#69	Chronic	0	0.0	0.3	0.2	0.1
		1	0.4	6.0	6.8	3.3
#79	Chronic	0	0.0	0.1	0.0	0.0
		1	0.0	63.4	59.2	33.2
#89	Chronic	0	0.0	0.0	0.0	0.1
		1	0.1	1.3	1.1	0.6
#90	Chronic	0	0.1	0.1	0.1	0.0
		1	0.1	35.4	32.0	15.9
#92	Chronic	0	0.0	0.0	0.0	0.0
		1	0.3	0.9	1.1	0.8

[1]Cryopreserved PBMC were thawed and expression of intracellular HTLV-I antigens were analyzed immediately (Day 0) or following incubation for the indicated days in 10% FBS RPMI. When the cells were cultured for longer than 3 days, 100 U/mL of IL- 2 was added to the culture medium to maintain cell viability.–[2]ND, not done.

a



b

Patients	HLA		Amino acid sequences	
	A2	A24	Tax11-19	Tax301-309
Pt. #1-vi	+	+	(not amplified)	(not amplified)
Pt. #22	+	+	LLFGYPVYV	SFHSLHLLF
Pt. #80	-	+	LLFGYPVYV	SFHSLHLLF
Pt. #85	-	+	LLFGYPVYV	SFHSLHLLF
Pt. #91	+	+	LLFGYPVYV	SFHN*LHLLF
Prototype HTLV-I			LLFGYPVYV	SFHSLHLLF

FIGURE 3 – Conservation of representative CTL epitopes in the proviruses from acute ATL cells. (*a*). Detection of full-length HTLV-I provirus in ATL cells. Genomic DNA (500 ng) extracted from PBMC of a healthy volunteer (*lane 1*), five acute ATL patients (*lane 2–6*), or MT-2 cells (*lane 7*) were amplified by a long PCR system with specific primers for 5′ and 3′ HTLV-I LTR. PCR products were visualized by ethidium bromide. The expected size of the fragments amplified from a full-length HTLV-I provirus was 7.7kbp in this system. (*b*) Nucleotide sequences of the long PCR products from acute ATL cells prepared in (*a*) were determined and the sequences at the regions corresponding to representative CTL epitopes, Tax 11-19 and Tax 301-309 restricted by HLA-A2 and A24, respectively, are shown as amino acid sequences. *Indicates the site of a single mutation (agt → aat) found.

Induction of co-stimulatory molecules in PBMC from ATL patients

It has been reported that HTLV-I-infected cell lines express a variety of surface molecules of activated T cells.[28] We investigated the expression of co-stimulatory molecules in ATL cells. It is known that typical ATL cells usually express CD4, CD25 and HLA-DR.[28] Besides these antigens, we assessed the expression of co-stimulatory molecules such as CD40, CD40L, CD80, CD86, OX40 and OX40L that participate in the interaction between antigen-presenting cells and T cells for efficient T cell-mediated immunity.[41] As shown in Figure 4, positive control ILT-Hod cells, an HTLV-I-infected T cell line established previously from an ATL patient, clearly expressed CD4, CD25, CD80, CD86, HLA-A, B, C and HLA-DR, and partially expressed OX40 and OX40L. The PBMC from an acute (Patient 85) and a chronic (Patient 79) ATL patient, that significantly expressed HTLV-I antigens after *in vitro* cultivation, were then analyzed before and after culture (Fig. 4). CD4, CD25 and HLA-A, B, C were detectable in uncultured PBMC from both patients. ATL cells from Patient 85 were double positive for CD4 and CD8. HLA-DR was detected in Patient 85 but not in Patient 79. In addition, small but detectable levels CD86 (14%) and OX40 (11%) were expressed in uncultured PBMC of Patient 85 and Patient 79, respectively (Day 0, closed histogram). After 1–3 days cultivation, expression of CD25, CD80, CD86 and OX40 was increased significantly. Similar induction of co-stimulatory molecules was also observed in other ATL cells with HTLV-I induction by culture (data not shown).

The results of two-color staining for HTLV-I Gag antigens and co-stimulatory molecules are shown in Figure 5. In 1-day cultured PBMC of Patient 85, the cells expressing intracellular HTLV-I Gag antigens partially expressed CD80 (23%) and CD86 (26%), and exclusively expressed OX40 (93%). This clearly indicated that HTLV-I and co-stimulatory molecules were co-expressed in ATL cells at the single cell level.

Among ATL cases without viral induction, the samples from Patient 1 expressed CD86 and OX40, and the sample from Patient 6 spontaneously expressed OX40 before culture (data not shown). The other samples tested did not express detectable levels of these co-stimulatory molecules. CD40 and CD40 ligand were not detectable in the PBMC of any ATL patients tested.

Augmentation of immunogenicity of ATL cells in vitro

We assessed the immunogenicity of short-term cultured ATL cells by *in vitro* MLR and HTLV-I-specific CTL assays. The results of MLR using allogeneic responder T cell and formalin-fixed PBMC from an acute (Patient 85) and a chronic (Patient 79) ATL patients were shown in Figure 6*a*. The levels of responder T cell proliferation were significantly enhanced upon mixing with 1-day or 3-day cultured ATL cells. Long-term cultured HTLV-I-infected T cell lines (ILT-85 and ILT-79) derived from these patients also induced significant levels of allogeneic MLR. It is of note that the ATL cells of Patient 79 expressed CD80 and OX40 but not HLA-DR (Fig. 4), suggesting that enhancement of MLR with ATL cells from this patient was not due merely to augmented HLA-DR. The levels of MLR against T cell-enriched fractions from 2 healthy volunteers were not markedly enhanced by preculture (Fig. 6*b*).

We assessed whether ATL cells could activate HTLV-I-specific CTL *in vitro* by mixing HLA-A24-restricted HTLV-I-specific $CD8^+$ CTL with formalin-fixed PBMC from HLA-A24-positive ATL patients (Patient 85 and Patient 1-vi) or a healthy volunteer. The results are shown in Table III. One- to three-day precultured, but not uncultured, PBMC of Patient 85 accelerated $[^3H]$-TdR-incorporation into the CTL, and induced marked levels of IFN-γ production in the CTL. In contrast, PBMC from a healthy volunteer or Patient 1-iv, in which HTLV-I antigens were not inducible, failed to enhance DNA synthesis or IFN-γ production in the CTL. Thus, short-term cultured ATL cells could be a specific stimulator as well as a target for HTLV-I-specific CTL in these *in vitro* assays.

In vivo *induction of HTLV-I-specific T cell responses by inoculating ATL cells in rats*

We investigated whether ATL cells could evoke HTLV-I-specific T cell immune responses *in vivo*. Twice with a 2-week interval, immunocompetent adult rats were subcutaneously inoculated with 10^7 cells/head of uncultured or 3 days-cultured PBMC from an acute ATL Patient 91 after formalin-treatment. Figure 7*a* shows the results of IFN-γ-production of spleen T cells from these rats 1 month after the last immunization. Surprisingly, in 2 of 2 rats inoculated with uncultured ATL cells, spleen T cells produced high levels of IFN-γ against stimulation with Tax-expressing syngeneic rat G14-Tax and HTLV-I-infected FPM1 cells but not with HTLV-I-negative G14

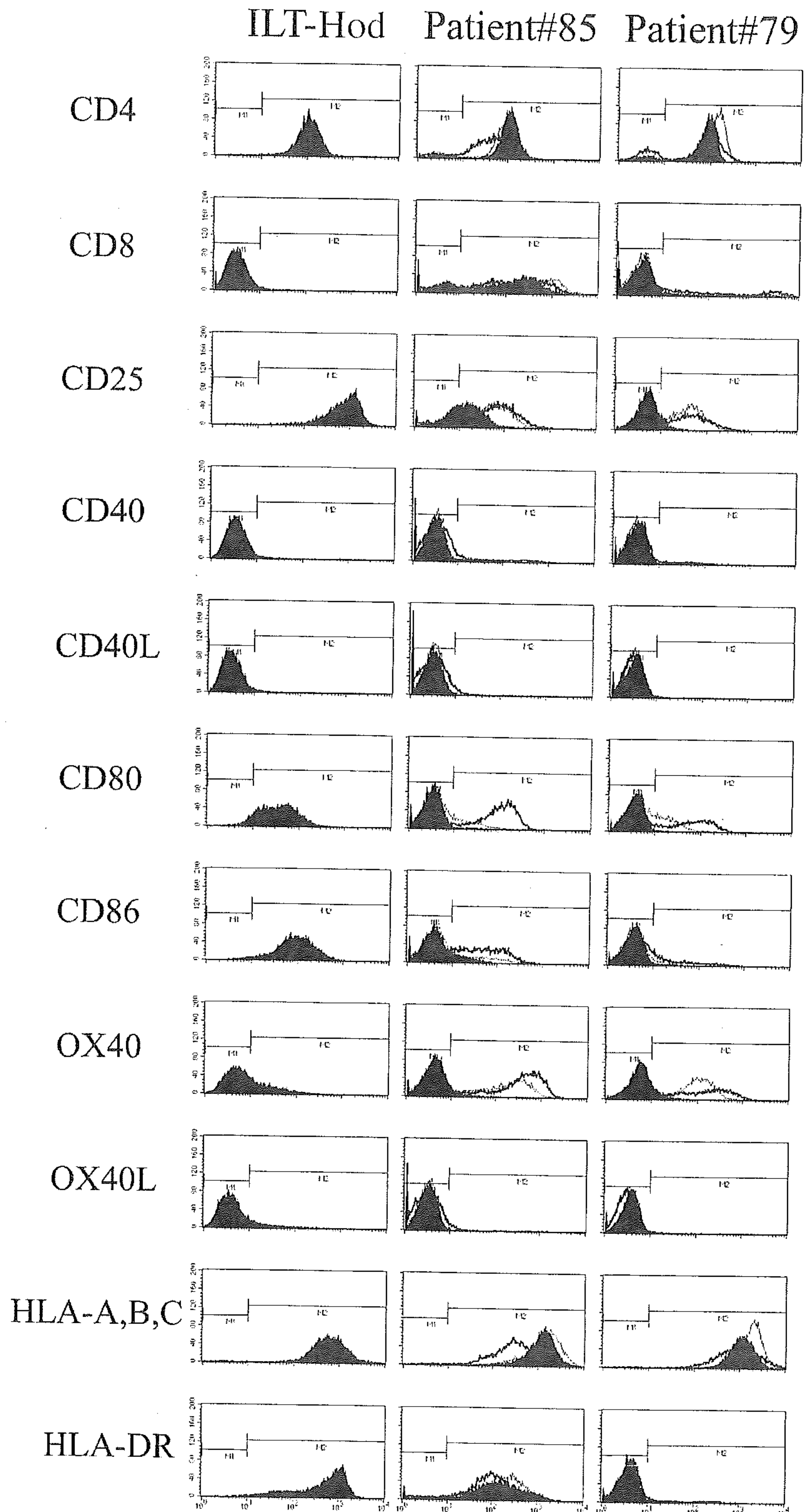


FIGURE 4 – Induction of co-stimulatory molecules in the PBMC of ATL patients after *in vitro* cultivation. Cryopreserved PBMC from an acute ATL Patient 85 and a chronic ATL Patient 79 were thawed and stained immediately (closed lines), or after incubation for 1 day (thin lines) or 3 days (solid lines) in 10% FBS-RPMI. FITC- or PE-conjugated anti-human mAbs (CD4, CD8, CD25, CD40, CD40L, CD80, CD86, OX40, OX40L, HLA-A, B, C, HLA-DR) as indicated. Cell surface antigen expression on ILT-Hod, an IL-2-dependent HTLV-I-infected cell line was analyzed by flow cytometry as a positive control. Live cells were gated and are shown as histograms. Each M1 region indicates where the cells stained with the isotype control mAb distributed (not shown). Accordingly, each remaining M2 region is regarded as positive.

cells (Fig. 7*a*; Rats 1 and 2). Similar or higher levels of HTLV-I-specific T cell responses were observed in the rats inoculated with 3-days cultured ATL cells from the same patient (Rats 3 and 4). T cells from control rats inoculated with PBMC from uninfected healthy human volunteers produced minimal levels of IFN-γ.

T cells from the rats inoculated with ATL cells also showed strong proliferative response against stimulation with G14-Tax and FPM1 cells but not with G14 cells (Fig. 7*b*). There was no significant difference between T cell response of the rats inoculated with uncultured and cultured ATL cells. CD4 positive cells became a dominant population in the spleen T cells from immunized rats after co-culture with formalin-fixed G14-Tax cells, whereas initially CD8 positive cells dominated before co-culture (Fig. 7*c*).

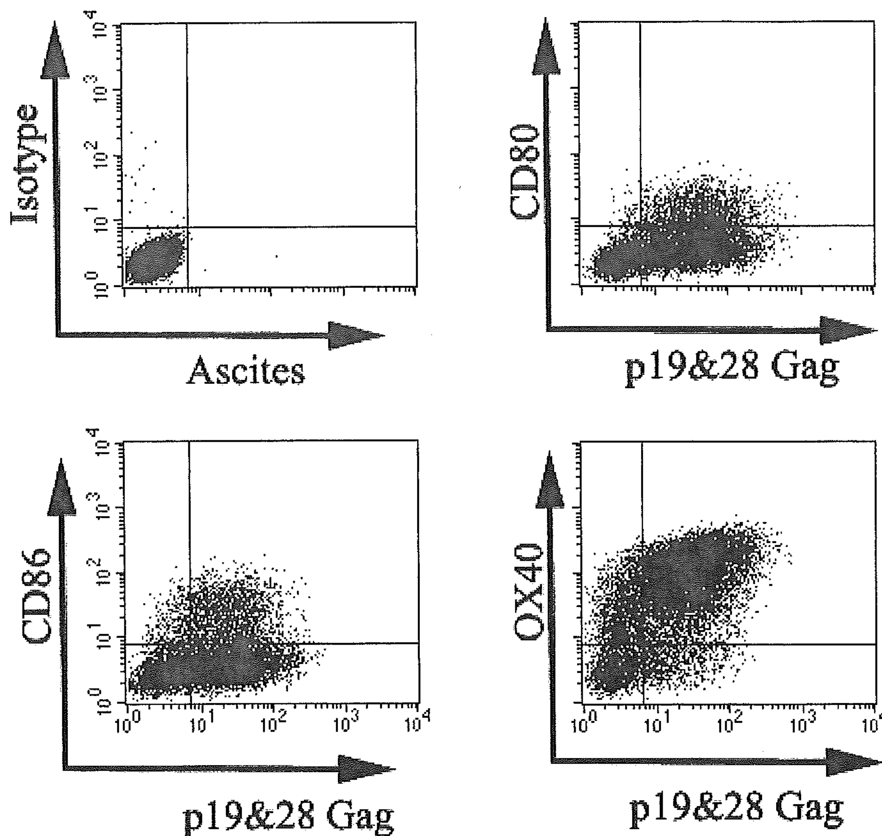


FIGURE 5 – Double induction of HTLV-1 and co-stimulatory molecules in the PBMC of ATL patients after *in vitro* cultivation. Cryopreserved PBMC from Patient 85 (acute ATL) were thawed and incubated for 1 day in 10% FBS-RPMI, and then subjected to a two-color analysis of cell surface antigens (CD80, CD86 or OX40) detected by FITC-conjugated mAbs and intracellular HTLV-I p19 and 28 Gag antigens detected by biotinylated GIN-7 mAb with Cy-Chrome-streptavidin after permeabilization with saponin (see Material and Methods). Live cells were gated and are shown as dot plots (FL-1/FL-3).

We tested cytotoxicity of the spleen cells from these rats inoculated with ATL cells after 7 days of *in vitro* co-culture with formalin-fixed G14 Tax cells. The results were shown in Figure 7*d*. The spleen cells from rats inoculated with ATL cells did not significantly kill Tax-expressing cells. In contrast, the positive control spleen cells from Tax-coding DNA-vaccinated rats showed strong cytotoxicity for G14-Tax but not for G14 cells.

These results suggest that not only precultured but also uncultured PBMC of ATL patients possessed sufficient amounts of antigens to evoke Tax-specific helper T cell response *in vivo*.

Discussion

We demonstrated that ATL cells retained the ability to express HTLV-I antigens including Tax in nearly 50% of the cases of ATL patients. Although the viral antigens became detectable in ATL cells by flow cytometric analysis only after short-term culture, the experiments with direct inoculation of the ATL cells to rats showed that uncultured ATL cells were also able to induce HTLV-I-specific T cell response *in vivo*.

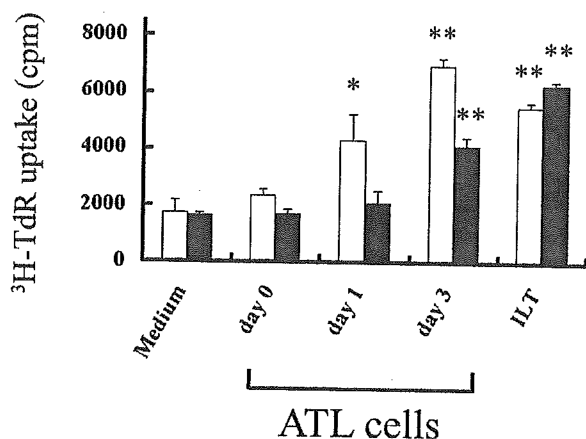
It has been noted that HTLV-I expression is inducible in ATL cells from some, but not all, ATL patients.[26,28] In our present study, induction of HTLV-I Tax and Gag in ATL cells was observed in 3 of 5 acute ATL, 6 of 15 chronic ATL, and 9 of 19 ATL cases tested (Table II). In one case with chronic ATL (Patient 79), HTLV-I expression was induced in many more cells than the number of morphologically identified abnormal lymphocytes, indicating that many peripheral HTLV-I-infected cells could appear as normal lymphocytes. It is intriguing that HTLV-I-expression was induced more frequently in acute-type ATL cells than in chronic-type ATL cells, despite the fact that acute-type ATL is supposed to be at a more advanced stage than chronic-type ATL. During *in vivo* evolution of HTLV-I-infected cells toward ATL, modification of viral expression may not be an absolute requirement.

Nucleotide sequences at 2 representative CTL epitopes, Tax 11–19 and Tax 301–309 restricted by HLA-A2 and A24, respectively, were highly conserved in proviruses from 4 acute ATL patients tested (Fig. 3). We chose these epitopes because they were predominantly recognized by CTL in 2 ATL patients after hematopoietic stem cell transplantation,[29] and also because genomic frequencies of HLA-A2 and A24 in Japanese are 24.7% and 35.6%, respectively.[42] ATL cells retained their ability to express viral antigens in 3 of 4 patients with conserved epitopes. These observations suggest that CTL escape mutants may not be the main reason for ATL-development in these patients.

Direct inoculation of fresh ATL cells into naive rats efficiently induced HTLV-I-specific helper T cell response *in vivo* (Fig. 7), despite the apparent absence of HTLV-I antigens in these cells by flow cytometry. Because ATL cells were derived from human, the inoculated rats might potentially respond to xenogenic antigens. Because we used syngeneic rat target cells for the cytokine production assay to evaluate immune response in the rats, however, reactions against xenogenic antigens should not be picked up by this assay. In addition, because we treated uncultured ATL cells with formalin before inoculation to rats, immune response of the rats was not due to further induction of viral antigens in the ATL cells or secondary HTLV-I-infection *in vivo* either. Spleen T cells from the rats inoculated with ATL cells reacted with syngeneic HTLV-I-infected or Tax-expressing rat cells but not uninfected cells, indicating that these T cells recognized HTLV-I antigens including Tax or Tax-induced antigens.

The *in vivo* HTLV-I-antigenicity of fresh ATL cells described above contrasted with the observation that only precultured but not uncultured ATL cells activated HTLV-I-Tax-specific CTL line *in vitro* (Table 3). This may be partly explained by the different sensitivity of the responding T cells between *in vivo* and *in vitro* detection systems. The Tax-specific CTL line used in the *in vitro* assay has been established by a long-term culture, whereas the spleen T cell population of immunized rats would be more heter-

a



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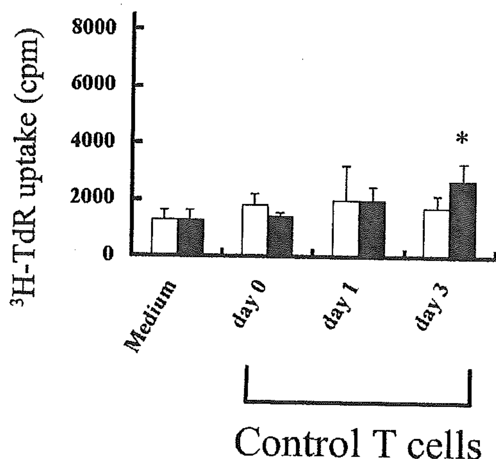

FIGURE 6 – Augmentation of antigenicity for allogeneic mixed lymphocyte reactions (MLR) in precultured PBMC from ATL patients. (*a*) PBMC from a healthy donor as responder cells were mixed with formalin-fixed PBMC from Patient 85 (acute ATL, open bar) and Patient 79 (chronic ATL, closed bar) that had been uncultured (Day 0) or precultured for the indicated periods (Day 1 or 3), or with formalin-fixed ILT cells derived from Patient 85 (open bar) and Patient 79 (closed bar). After 4 days of culture, [^{3}H]-TdR incorporated into the cells was measured. (*b*) Similar allogeneic MLR were carried out with responder PBMC from a healthy donor and formalin-fixed T cell-enriched PBMC fractions from two other healthy volunteers (open and closed bar) that had been precultured for the indicated periods. The results were expressed as the mean ± SD in counts per minutes (cpm) obtained from triplicate cultures. Differences between the 2 groups were analyzed for significance (*$p < 0.05$, **$p < 0.001$, compared to Day 0) by Student's t-test. Similar results were obtained in another set of independent experiments.

ogeneous. Antigens of formalin-fixed ATL cells inoculated to rats were presumably processed and presented mainly on MHC-II by professional antigen-presenting cells, which would favor CD4 positive helper T cell response *in vivo*. The amounts of antigen required for priming T cells *in vivo* might be smaller than those for activating the CTL line *in vitro*. In addition, the presence of co-stimulatory molecules on ATL cells might have been advantageous to induce T cell response *in vivo*.[41,43]

Short-term cultured ATL cells significantly expressed co-stimulatory molecules including CD80, CD86, and OX40 as well as HTLV-I antigens such as Tax and Gag at the single cell level. The levels of

TABLE III – PROLIFERATIVE RESPONSE AND IFN-γ PRODUCTION OF HTLV-I-SPECIFIC CTL IN RESPONSE TO ATL CELLS WITH OR WITHOUT *IN VITRO* PRECULTIVATION[1]

Stimulator PBMC		Responses of HTLV-I-specific CTL	
Subject	Preculture period (Day)	[^{3}H]-TdR uptake (cpm)	IFN-γ production (pg/mL)
Pt. #85	0	2,469 ± 246	Undetectable
	1	3,350 ± 423[2]	673 ± 45[3]
	3	4,498 ± 296[3]	534 ± 79[3]
Pt. #1-vi	0	2,493 ± 84	Undetectable
	1	2,470 ± 366	Undetectable
Healthy	0	2,737 ± 215	Undetectable
	1	2,554 ± 78	Undetectable
	3	2,748 ± 195	Undetectable
None	—	2,611 ± 115	Undetectable

[1]HLA-A24-restricted HTLV-I-specific CTL (1 × 10^5/well) were cocultured for 24 hr with formalin-fixed cryopreserved PBMC (5 × 10^4/well) from HLA-A24-positive ATL patients or healthy volunteers pre-incubated for the indicated periods (0, 1 or 3 days), and IFN-γ production in the culture supernatant and [^{3}H]TdR uptake for an additional 16 hr were measured. The results are expressed as the mean ± SD. Differences between the 2 groups were analyzed for significance.–[2]$p < 0.05$.–[3]$p < 0.001$, compared with Day 0 by Student's t-test. Similar results were obtained in another set of independent experiments.

Tax-expression in ATL cells reached the maximum in 1 day, whereas expression of Gag and co-stimulatory molecules increased with further incubation (3 days) (Fig. 4), suggesting that Tax was involved in the activation of the other molecules. A number of previous studies pointed to the potential transactivation of CD25,[44] OX40[45] and OX40L[46] by HTLV-I Tax. Induction of CD80 and CD86 in HTLV-I/II-infected cells has also been reported.[47] In our present study, spontaneous expression of OX40 and CD86 was sporadically observed in ATL cells before or without HTLV-I-induction. This indicates that Tax expression under detectable levels or some other mechanisms may be involved in activating co-stimulatory molecules in these ATL cells.

ATL may be categorized into at least 2 groups by the ability of HTLV-I-expression in their ATL cells. In our present study, HTLV-I expression was inducible in about half the ATL cases, and the other half showed irreversible viral silencing in their ATL cells. Although the irreversible silencing of HTLV-I may be due to various genomic changes in ATL cells,[24,25] HTLV-I expression is not completely silent in the other inducible type of viral suppression. This is supported by previous and recent reports that HTLV-I mRNA is detectable by RT-PCR in fresh ATL cells.[27,48] The inducible type of suppression is commonly seen in PBMC from HTLV-I-carriers and HAM/TSP patients.[22,49] Despite such suppression of viral expression *in vivo*, HTLV-I Tax-specific CTL are highly activated in HAM/TSP patients and some HTLV-I-carriers,[16,19] implying the presence of sufficient levels of antigen-presentation *in vivo* for priming and maintaining CTL. This is consistent with the observation in our present study that sub-detectable amounts of viral expression induced HTLV-I-specific T cell response *in vivo* but not fully activated Tax-specific CTL line *in vitro*. Such marginal levels of viral expression may partly explain how HTLV-I persists *in vivo* in the presence of HTLV-I-specific CTL. Nevertheless, active HTLV-I-specific CTL responses are associated with tumor-free state in human[16,21,29] and limited proviral loads in rats,[38] still suggesting contribution of HTLV-I-specific CTL to controlling expansion of HTLV-I-infected cells *in vivo*. It remains to be clarified where and when HTLV-I-specific CTL can affect infected cells *in vivo*.

Our results indicated that, in respect of the ability of viral expression, ATL has diversity even within the acute type ATL. In about half the ATL cases, ATL cells retained the ability of viral expression. Among these patients, fresh ATL cells from one case could induce Tax-specific helper T cell response *in vivo* despite their undetectable viral expression in *in vitro* assays. These imply that ATL cells may express low but sufficient levels of Tax or Tax-induced antigens to be recognized by T cells *in vivo*.

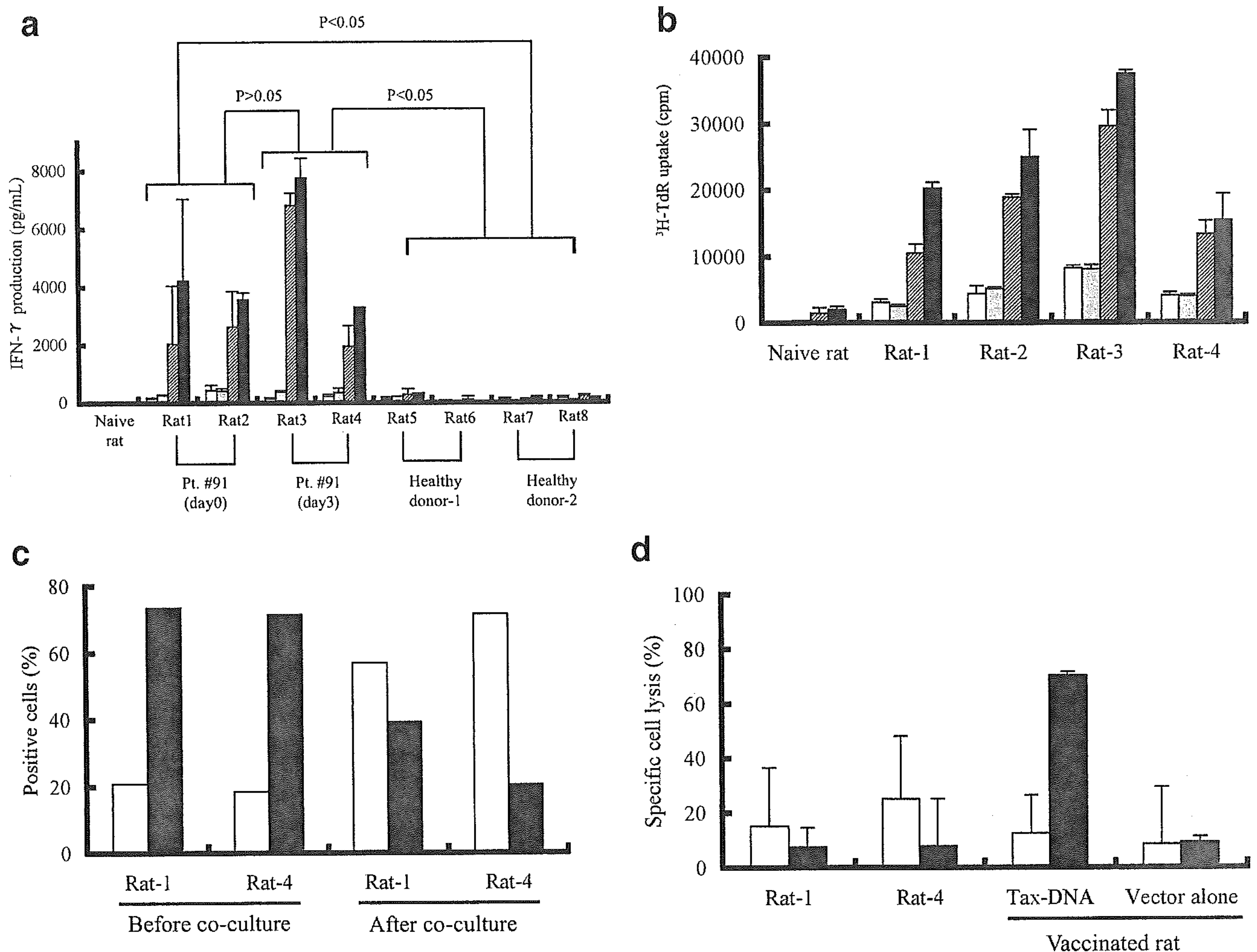

FIGURE 7 – *In vivo* induction of HTLV-I-specific T cell response by inoculation of fresh ATL cells in rats. Immunocompetent naive rats were subcutaneously immunized with either ten million of formalin-fixed ATL cells from Patient 91 that had been uncultured (Rats 1, 2) or 3 day-precultured (Rats 3, 4), or with the same number of formalin-fixed uncultured PBMC from 2 healthy donors (Rats 5–8), twice with a 2-week interval. One month after second immunization, spleen cells were collected. (*a*) IFN-γ production of spleen T cells from these rats were measured by ELISA after incubation without (open bar) or with formalin-fixed syngeneic G14 (gray bar), G14-Tax (hatched bar) or FPM1 (closed bar) cells for 6 days. Similar results were obtained also at the third day of incubation when half the medium was changed. (*b*) Proliferation of spleen T cells from immunized rats was monitored by a [^{3}H]-TdR-uptake assay after 3 days of incubation with various stimulator cells that were set up similarly to (*a*). (*c*) CD4 (open bar) or CD8 (closed bar) positive cell number in the spleen T cells from Rats 1 and 4 was evaluated by flow cytometry before and after 7 days of co-cultivation with formalin-fixed G14-Tax. Values indicate percent positive against viable cell number. (*d*) Cytotoxicities of spleen cells from Rats 1 and 4 were measured after 7 days of pre-culture with formalin-fixed G14-Tax. Similarly pre-cultured spleen cells from rats vaccinated with plasmids containing *tax* cDNA (Tax-DNA) or vector plasmids alone served as positive and negative controls, respectively. These effector cells were mixed with [^{3}H]-TdR-labeled G14 (open bar) and G14-Tax (closed bar) cells at the effector/target cell ratio of 10. After 6 hr incubation, cells were harvested and radioactivities remaining in the target cells were measured. Specific cell lysis were calculated (see Material and Methods) and expressed as the mean ± SD of triplicate cultures.

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Review Article

Tumor immunity against adult T-cell leukemia

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Human T-cell leukemia virus type-I (HTLV-I) causes adult T-cell leukemia (ATL) in a small population of infected individuals after a long incubation period. Although the process of clonal evolution of ATL cells may involve multiple steps, ATL cells from half of the ATL cases still retain the ability to express HTLV-I Tax, a key molecule of HTLV-I leukemogenesis. A recent finding of reactivation of Tax-specific cytotoxic T lymphocytes (CTL) in ATL patients after hematopoietic stem cell transplantation suggests the presence of Tax expression *in vivo* and potential contribution of the CTL to antitumor immunity. This is consistent with the results of a series of animal experiments indicating that Tax-specific CTL limit the growth of HTLV-I-infected cells *in vivo*, although the animal model mimics only an early phase of HTLV-I infection and leukemogenesis. Establishment of an insufficient HTLV-I-specific T-cell response and an increased viral load in orally HTLV-I-infected rats suggests that host HTLV-I-specific T-cell response at a primary HTLV-I infection can be a critical determinant of persistent HTLV-I levels thereafter. These findings indicate that Tax-targeted vaccines may be effective for prophylaxis of ATL in a high-risk group, and also for therapy of ATL in at least half the cases. (*Cancer Sci* 2005; 96: 249–255)

It is estimated that approximately one million people are infected with human T-cell leukemia virus type I (HTLV-I) in Japan. Although most HTLV-I carriers are asymptomatic throughout their lives, 1–5% of infected subjects develop adult T-cell leukemia (ATL),[1–3] and another small fraction of HTLV-I carriers develop a chronic progressive neurological disorder termed HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and other inflammatory disorders.[4,5]

Adult T-cell leukemia is characterized by tumor cells with mostly $CD4^+$ and $CD25^+$ mature T-lymphocyte phenotypes, onset during middle age or later, immune suppression and poor prognosis.[6] There are four clinical subtypes of ATL: acute, lymphoma, chronic and smoldering types, based on Shimoyama's diagnostic criteria.[7] Monoclonal integration of HTLV-I provirus in ATL cells indicates that ATL arises from a single HTLV-I-infected cell that undergoes malignant phenotypic progression.[8] However, oligoclonal expansion of HTLV-I-infected cells *in vivo* is also observed in HAM/TSP patients and some asymptomatic HTLV-I carriers,[9] suggesting that HTLV-I-infected cells generally have proliferative potential.

The HTLV-I viral protein Tax is a multifunctional protein, interacting with many cellular proteins regulating cell growth and apoptosis resistance. Tax activates Nuclear factor κ B (NFκB), cAPM response element binding protein (CREB), serum response factor (SRF), activator protein 1 (AP-1), and represses p53 or other tumor suppressor proteins either by direct or indirect mechanisms, partly accounting for HTLV-I-induced leukemogenesis.[10]

However, the level of HTLV-I expression in freshly isolated peripheral ATL cells is extremely low. This paradoxical observation provides controversy concerning the role of Tax in HTLV-I leukemogenesis. It has been reported that fresh ATL cells exhibit constitutive activation of NFκB,[11] one of the transcription factors induced by Tax, while Tax is undetectable in these cells. This implies that either subdetectable levels of Tax or some other mechanism substituting for Tax function activates NFκB in ATL cells.

Expression of viral antigen

It has been noted that HTLV-I expression is inducible in ATL cells from some ATL patients after several hours of culture.[12] A recent study using flow cytometry indicated that similar induction of HTLV-I Tax and Gag antigens occurs in ATL cells in approximately half of the ATL cases tested (Fig. 1).[13] Earlier studies demonstrated that HTLV-I mRNA is detectable at low levels in ATL cells without culture, and is also detectable in ATL lymph nodes *in situ*.[14,15] Moreover inoculation of uncultured formalin-treated ATL cells into naive rats resulted in induction of a HTLV-I-specific T-cell response.[13] These observations suggest that ATL cells in approximately half of all ATL cases may express very low levels of HTLV-I antigens, which are further enhanced by *in vivo* culture. Similar transcriptional repression of HTLV-I expression *in vitro* and its induction in *in vitro* culture have been observed in the peripheral blood mononuclear cells (PBMC) of HAM/TSP patients and HTLV-I-carriers.[16–18]

The mechanism of this transient transcriptional repression of HTLV-I in the peripheral blood is unknown. Methylation of the CpG motif found in the 5′-long-terminal repeat (LTR) may be partly involved, but do not fully explain the phenomenon.[19–21]

Adult T-cell leukemia cells from the other half of cases fail to express HTLV-I antigens even after *in vitro* culture. This irreversible silencing of HTLV-I could be due to various genomic changes in the HTLV-I provirus of ATL cells, such as deletions at the 5′-LTR and *gag/pol* regions.[22,23]

Thus, ATL cases may be categorized into two groups in the context of their HTLV-I expression in ATL cells; HTLV-I expression is inducible in approximately half of all ATL cases, while irreversible in the other half of cases. The inducible type of viral suppression in the PBMC may be a common phenomenon in HTLV-I-infected individuals irrespective of the disease.

Anti-tumor immunity in HTLV-I infection

Cytotoxic T-lymphocyte response in human HTLV-I-infected individuals. Although no consistent differences have been observed among HTLV-I strains isolated from ATL and HAM/TSP patients,[24,25] immunological studies have found a clear difference in HTLV-I-specific T-cell responses between these

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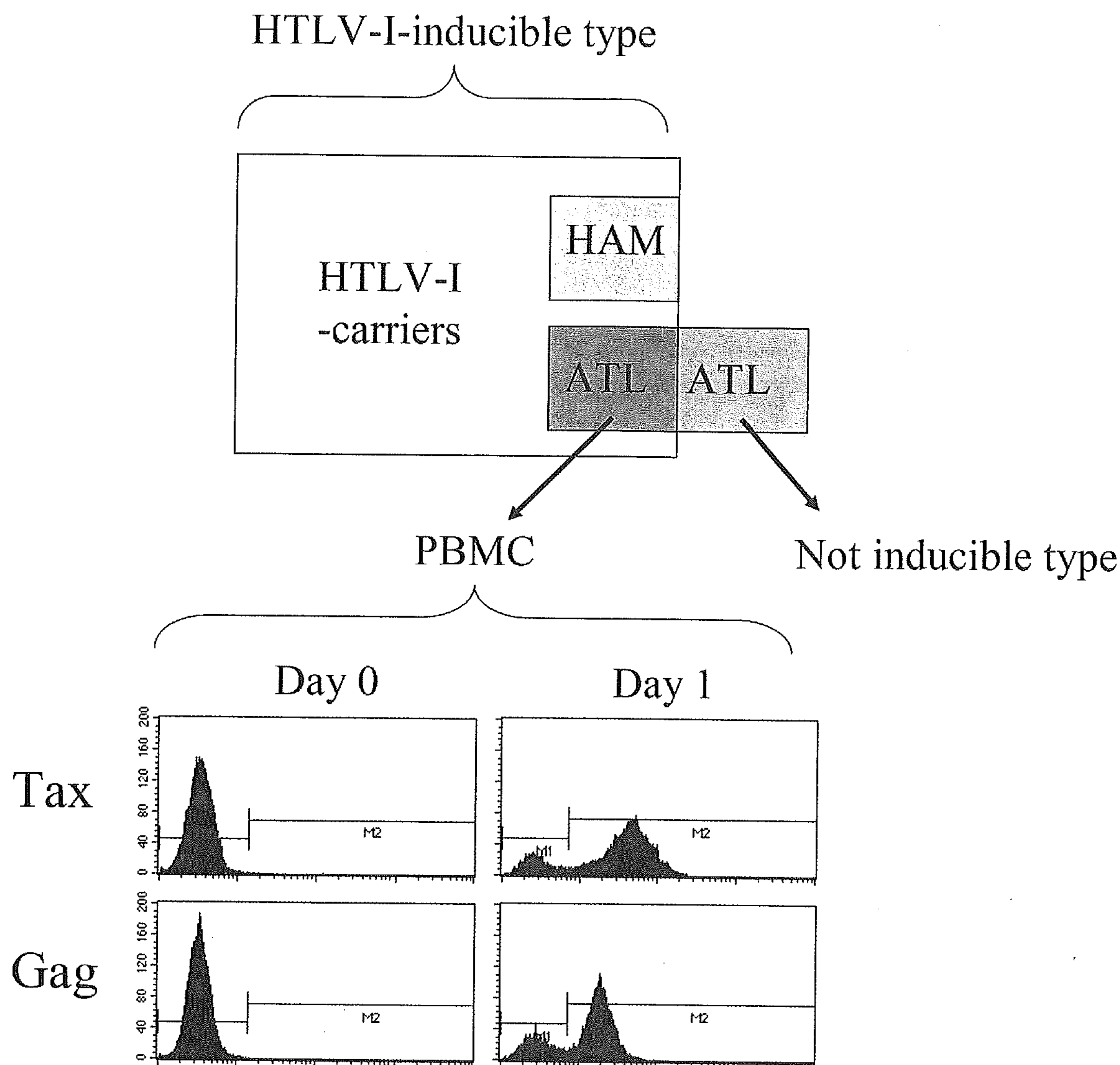

Fig. 1. Induction of human T-cell leukemia virus type-I (HTLV-I) antigens in adult T-cell leukemia (ATL) cells. ATL cells isolated from peripheral blood of approximately half of the ATL cases expressed a significant amount of HTLV-I Tax and Gag antigens in 1-day culture, as detected by flow cytometry.[13] A similar phenomenon is seen commonly in asymptomatic HTLV-I-carriers and HTLV-1-associated myelopathy/tropical spastic paraparesis patients. HTLV-I antigens were not inducible in the other half of the ATL cases.

two diseases. HTLV-I-specific cytotoxic T-lymphocytes (CTL) are highly activated in HAM/TSP patients and are sometimes readily detectable in PBMC without any stimulation *in vitro*. Similar CTL can be induced in PBMC culture from many asymptomatic HTLV-I carriers when stimulated with autologous HTLV-I-infected cells *in vitro*. However, HTLV-I-specific CTL are only rarely induced in ATL patients.[26–29] A recent report demonstrated that HTLV-I-specific CTL are present in ATL patients but expand insufficiently,[30] suggesting involvement of some immune suppression or tolerance.

Human T-cell leukemia virus type I core, envelope, polymerase and Tax proteins are recognized by HTLV-I-specific CTL.[28,31,32] In addition, oligopeptides of Tof and Rof were shown to induce CTL from HTLV-I-infected individuals.[33] Among these antigens, HTLV-I Tax, a critical viral protein for T-cell immortalization, is the most popular target for HTLV-I-specific CTL found in HTLV-I-infected individuals.[28,31] HTLV-I Tax-specific CTL are capable of killing short-term cultured ATL cells expressing viral antigens *in vitro*.[34]

Experimental tumor vaccine. To understand the influence of host immunity to HTLV-I leukemogenesis *in vivo*, a series of experiments using rat models for HTLV-I-infected T-cell lymphomas were carried out. In these models, a syngeneic HTLV-I-transformed T-cell line underwent phenotypic evolution to cause fatal lymphomas in immune-suppressed rats.[35] However, this cell line did not cause tumors in immune-competent rats. Immunological analysis revealed that the antitumor effects in immune-competent rats were mediated by CTL predominantly directed to HTLV-I Tax.[36] It is intriguing that the major target of HTLV-I-specific CTL is Tax both in rats and humans.

Antitumor effects of Tax-specific CTL were further confirmed by vaccine experiments, in which T-cells from immune-competent rats vaccinated with Tax-encoded DNA could eradicate fatal T-cell lymphomas in athymic rats when transferred.[37] Similar results were obtained by vaccination with oligopeptides corresponding to the major CTL epitope.[36]

Immune response in post-hematopoietic stem cell transplantation ATL patients

Although the rat models described above may mimic some aspects of HTLV-I leukemogenesis, they differ from full-blown

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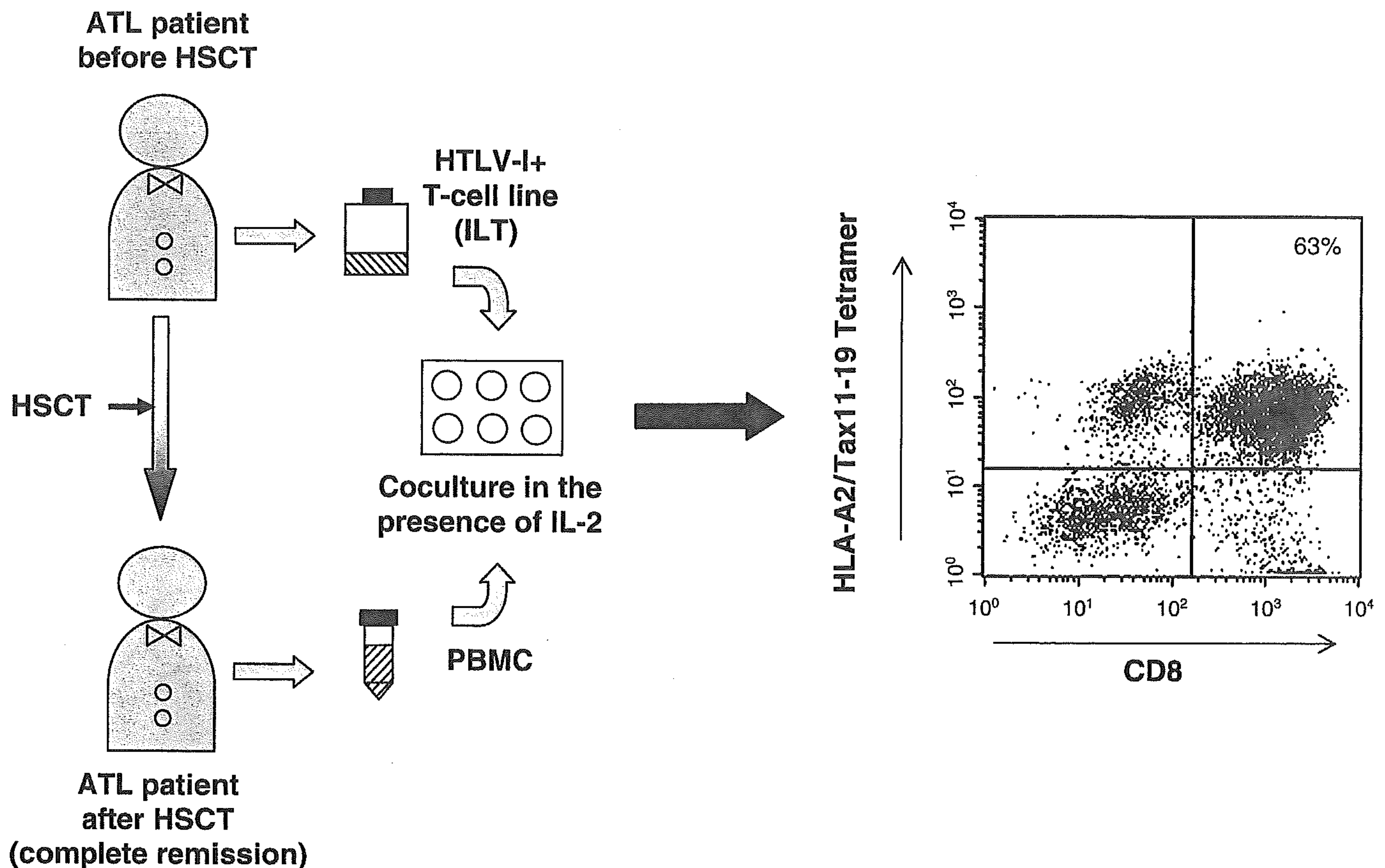

Fig. 2. Induction of Tax-specific cytotoxic T lymphocytes (CTL) from an adult T-cell leukemia (ATL) patient receiving hematopoietic stem cell transplantation (HSCT) from a human leukocyte antigen (HLA)-identical donor. A spontaneous human T-cell leukemia virus type-I-infected T-cell line (ILT) was established from an ATL patient before HSCT. These cells were formalin-treated, then co-cultured with peripheral blood mononuclear cells from the same ATL patient after HSCT. After several weeks of culture, 63% of the cells were HLA-A2-restricted Tax11-19-specific $CD8^+$ CTL as detected with phycoerythrin-conjugated tetramers.[39]

ATL in humans as ATL develops in immune-competent individuals following over 40 years of incubation, whereas rat lymphoma consists of HTLV-I-transformed cells and develops only in immune-suppressed hosts.

Allogeneic hematopoietic stem cell transplantation (HSCT) has been used to treat ATL and achieved long-lasting complete remission in some ATL patients.[38] Graft-versus-host (GVH) or graft-versus-leukemia (GVL) responses are presumed to contribute to antitumor effects in these patients. Because the GVH/GVL response is mediated primarily by T cells, we investigated T-cell responses in ATL patients who obtained complete remission following non-myeloablative allogeneic peripheral blood HSCT from human leukocyte antigen (HLA)-identical sibling donors (Fig. 2).[39] In that study, as the target of the GVH response, mitogen-stimulated IL-2-dependent T-cell lines (ILT) were established from ATL patients before HSCT, which express antigens originating from the recipient. These cells were also infected spontaneously with HTLV-I. When the PBMC from the same patients after HSCT were stimulated in culture with formalin-treated ILT cells *in vitro*, $CD8^+$ CTL capable of killing ILT cells proliferated vigorously. Further analysis revealed that most of these CTL predominantly recognized a limited number of Tax epitopes; Tax 11-19 restricted by HLA-A2 in one patient and Tax 301-309 restricted by HLA-A24 in another. However, PBMC from these ATL patients before HSCT did not show such CTL responses.

Similar oligoclonal expansion of Tax 11-19-specific CTL was reported previously in HLA-A2 + HAM/TSP patients.[40] This phenomenon is explained by a highly activated host CTL response against abundant HTLV-I antigens in HAM/TSP patients. Thus, the Tax-specific CTL expansion observed in the post-HSCT ATL patients implies that these patients may be in a similar status to HAM/TSP patients in the context of their activated levels of T-cell response and/or Tax antigen presentation *in vivo*. Significant reduction in the proviral load in these patients following HSCT might be partly due to such a strong anti-Tax CTL response.

Various minor histocompatibility antigens (mHA) have been postulated to act as the target of the GVH/GVL response in HSCT.[41] In cultures of post-HSCT ATL patients, a minor population of CTL induced against ILT cells was directed to an unknown antigen other than Tax, probably related to the GVH response. The role of the anti-Tax CTL response in relation GVL effects remains to be clarified. However, the strong HTLV-I-specific response observed in the patients after complete remission suggests that HTLV-I-specific CTL as well as the GVH effectors might participate in the maintenance of remission.

Immunological risk factors for ATL development

The insufficient HTLV-I-specific T-cell response observed generally in ATL patients could be either a consequence of ATL or a risk associated with ATL development. If this were a risk associated with ATL, a wide survey of HTLV-I-specific T-cell responses among HTLV-I carriers would be useful to identify a high-risk group, to whom prophylactic strategies should be applied.

Epidemiological risk factors for ATL. In cohort studies of HTLV-I carriers, it appears that the risk factors for ATL might include

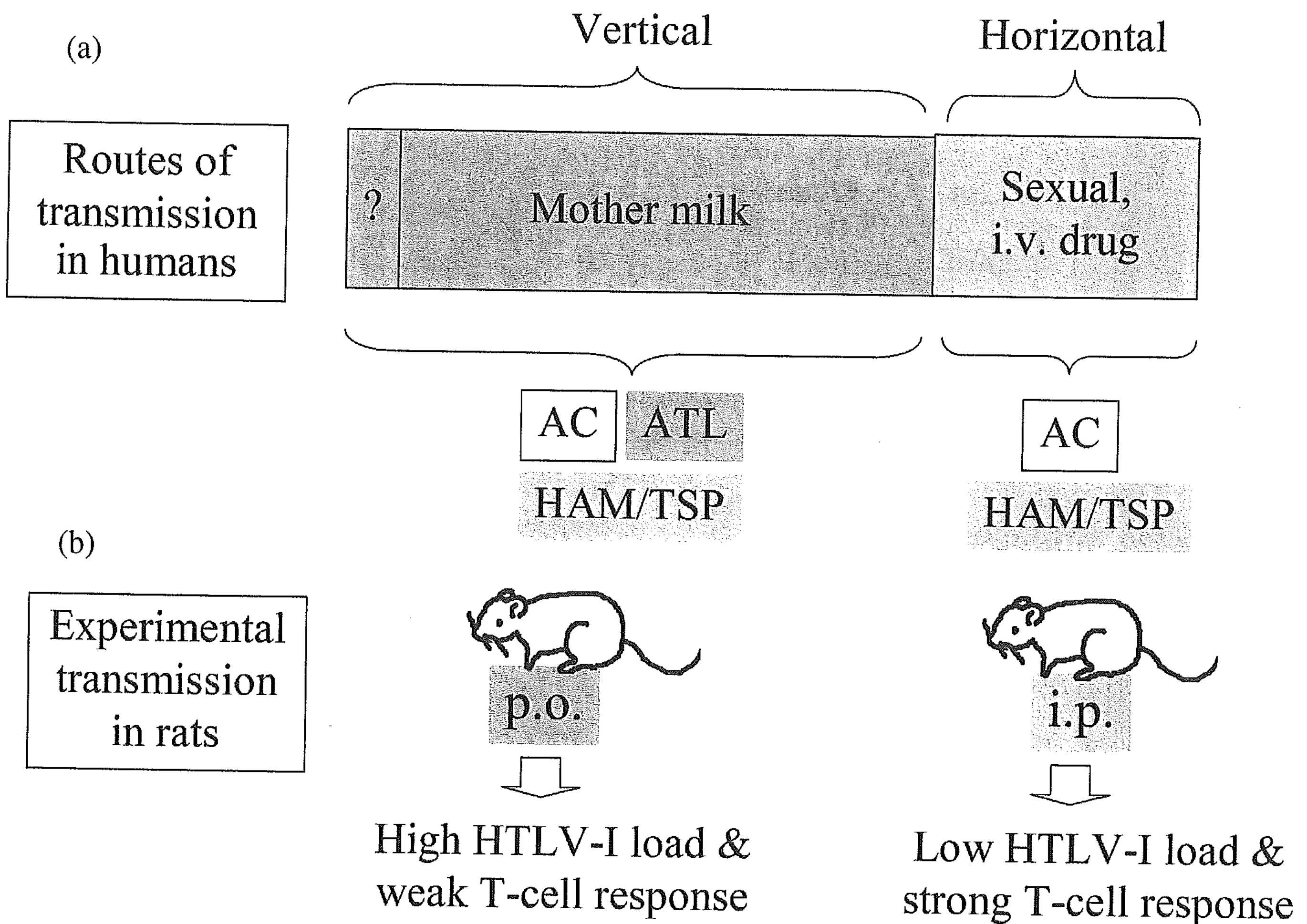

Fig. 3. Relationship between the routes of human T-cell leukemia virus type-I (HTLV-I) infection and diseases in humans or outcome in rat experiments. (a) Adult T-cell leukemia arises from a vertically infected population, whereas HTLV-1-associated myelopathy/tropical spastic paraparesis arises from both vertically and horizontally infected populations. Irrespective of the route of infection, most of the infected individuals are asymptomatic HTLV-I-carriers (AC). (b) Adult rats infected orally with HTLV-I had an increased viral load and a weak HTLV-I-specific T-cell response, whereas intraperitoneally infected rats had a low viral load and a strong HTLV-I-specific T-cell response.(48,49)

vertical HTLV-I infection, and increasing numbers of abnormal lymphocytes.(3,42,43) HTLV-I transmits from mother to child mainly through breast milk, and from male to female by sexual contact. Blood transfusion or intravenous drug use also causes HTLV-I transmission.(44–46) Among these, mother-to-child transmissions are the major natural route in Japan. The higher incidence of ATL in males is attributed to the relatively higher ratio of vertical infection in males. The presence of typical HLA haplotypes for ATL in an endemic area(47) also implies that vertical infection might transmit some determinants of HTLV-I leukemogenesis.

Oral infection as a determinant of insufficient T-cell response in rats. In a rat model, the routes of infection strongly affect HTLV-I-specific immunity (Fig. 3).(48) Among immune-competent adult rats infected with HTLV-I through various routes, rats inoculated orally showed very low levels of HTLV-I-specific T-cell response, whereas significant responses were detected in rats infected through other routes.(49) In contrast, HTLV-I proviral load in the spleen cells, examined several months after infection, was significantly higher in orally infected rats. Because HTLV-I proviruses are associated with infected cells, the increase in proviral load indicates the increase in infected cell number.

Together with the fact that oral infection through mothers' milk is a major route of vertical HTLV-I infection in humans,(44) the results of the rat experiments of oral HTLV-I infection strongly suggest that the epidemiological risks of ATL (i.e. vertical HTLV-I infection and high viral load) link to the immunological risk (i.e. low T-cell responses to HTLV-I).

Balance between host immunity and HTLV-I in natural HTLV-I infection

Positive or negative correlation between host immunity and the virus. In the rat experiment described above, there was an inverse correlation between HTLV-I proviral load and HTLV-I-specific T-cell proliferation,(49) indicating that HTLV-I-specific T-cell responses might contribute to limiting expansion the of HTLV-I-infected cells *in vivo*, and that oral infection may be a reason for insufficient T-cell immunity to HTLV-I.

Infants born to HTLV-I-carrying mothers are fed approximately 1×10^8 HTLV-I-infected cells before weaning,(45) and a number of infantile carriers stay seronegative for HTLV-I for a certain period of time,(50) probably due to some immunological tolerance during this period. Most of these children show seroconversion by the age of 3 years.(51) Although T-cell immune responses to HTLV-I in children are not known, many adult HTLV-I carriers show HTLV-I-specific CTL responses, suggesting that the T-cell response might recover spontaneously later in life, just as happens in vertical hepatitis B virus infection.

In contrast to the results of rat experiments, HTLV-I proviral load in human adult HTLV-I carriers correlate positively with

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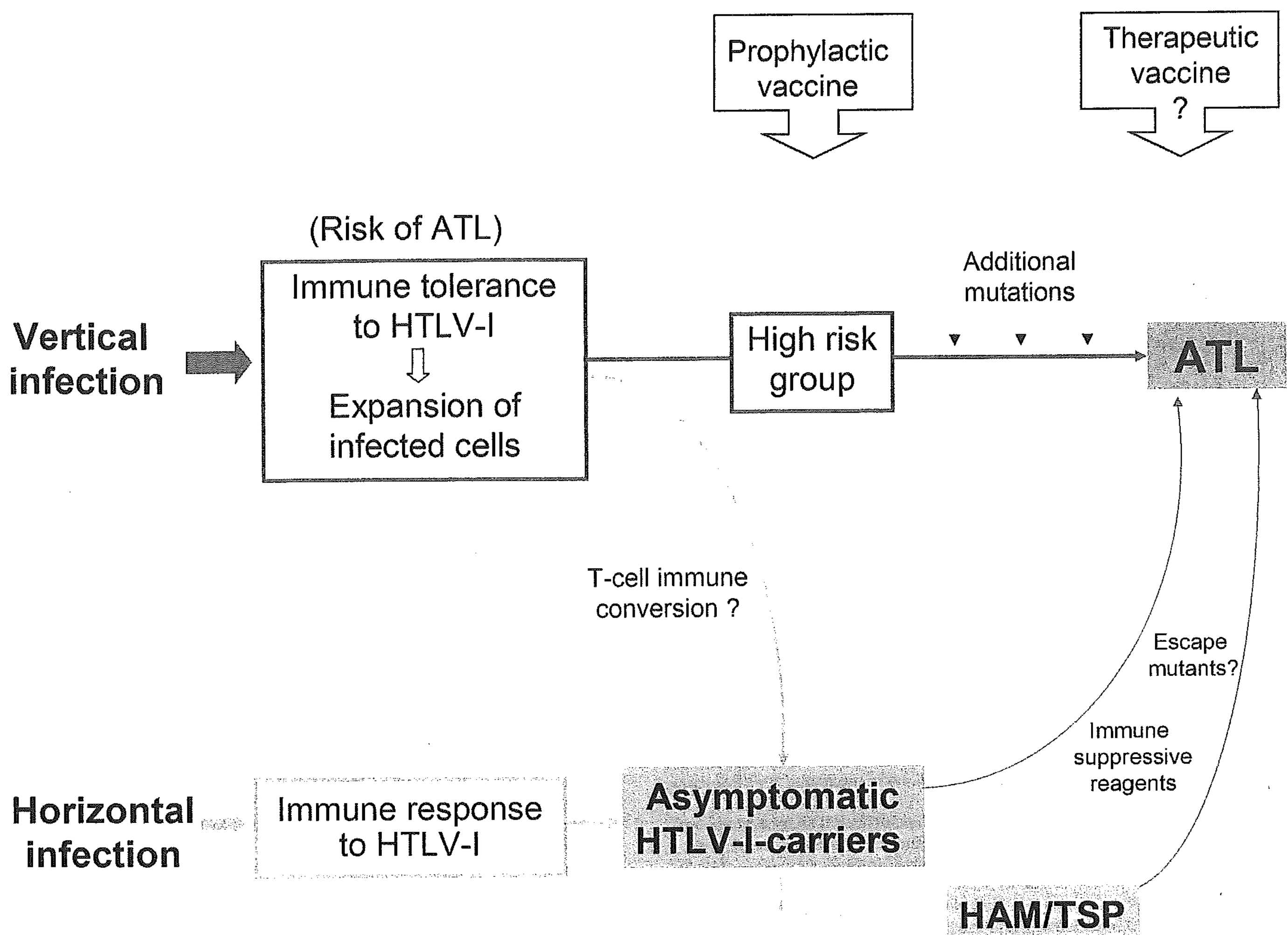

Fig. 4. Hypothesis on the relationship among host T-cell immunity, risk of adult T-cell leukemia (ATL), and the route of infection in humans. Vertically infected human T-cell leukemia virus type-I (HTLV-I) carriers harbor risks of ATL (i.e. insufficient HTLV-I-specific T-cell response and expansion of infected cells). HTLV-I-specific T-cell responses eventually recover spontaneously in most of these carriers and the risk of ATL decreases. However, if a small population remains in the high-risk group, insufficient T-cell immunity in these individuals may allow clonal evolution of infected cells toward ATL. HTLV-I carriers infected through horizontal routes would have a lower risk of ATL.

the HTLV-I-specific T-cell response.[52,53] This discrepancy between rats and humans may be partly explained by the difference in the period of HTLV-I infection; several months in the rat experiments but many years in human HTLV-I carriers. If the T-cell response recovered after a long period of insufficient response to HTLV-I, the magnitude of the recovered response would correlate positively with the elevated levels of pre-existing proviral load *in vivo*.

Hypothetical relationship among host immunity, disease development and the route of infection. Figure 4 gives a schematic demonstration of our current hypothesis on the immunological risks of ATL in the natural course of HTLV-I infection. Vertically infected HTLV-I carriers harbor risks of ATL (i.e. insufficient HTLV-I-specific T-cell response and expansion of infected cells). However, such risks may be reduced in many HTLV-I carriers by spontaneous recovery of the HTLV-I-specific T-cell response. If there is a small group of adult HTLV-I carriers still showing insufficient T-cell responses to HTLV-I despite an abundant viral load, this might be a high-risk group for ATL, to whom prophylactic vaccines targeting Tax may be beneficial.

Favorable levels of T-cell response and a lower risk of ATL are expected in individuals infected through horizontal routes, although a small fraction of this group might develop HAM/TSP. The genetic determinants of HAM/TSP are not known. In Japan, many HAM/TSP cases arise from the vertically infected population, suggesting that T-cell immune conversion has occurred at some stage. The oligoclonal expansion of HTLV-I-infected cell clones often seen in HAM/TSP patients indicates that these clones might have been in the process of leukemogenesis.[9] Nevertheless, the incidence of ATL among HAM/TSP patients is limited, probably due to the activated host HTLV-I-specific T-cell immunity. In this respect, administration of immunosuppressive reagents to HAM/TSP patients might increase the risk of ATL development. In addition, the post-HSCT patients with T-cell immune conversion should be followed up carefully, although development of HAM/TSP in post-HSCT ATL patients has not been reported so far.

Prophylaxis and therapy for ATL

The findings of the immunological studies described above suggest that Tax-targeted vaccines may be beneficial for prophylactic use against the high-risk group. For this purpose, a handy method to detect HTLV-I-specific T-cell immune response would be required for a wide survey among HTLV-I carriers.

A number of combination chemotherapy protocols have been applied, and the median survival time of a recent protocol

(JCOG9303) was 13 months.[54] In addition, several kinds of experimental therapies have also been applied to ATL. A small number of ATL patients respond to intravenous administration of anti-CD25 monoclonal antibody.[55] The combination therapy of azidothymidine (AZT) and interferon α achieved a high response rate but did not prevent relapse of ATL.[56,57] The mechanisms for the antitumor effects of these antiretroviral drugs are not clear, as HTLV-I proliferation occurs mainly by proliferation of infected cells, not by viral replication. A recent study indicated that AZT and interferon α suppress NFκB activity and induce TRAIL expression, respectively.[58] A combination of arsenic and interferon α[59] and some other NFκB-targeted therapies have been proposed.

Recently, allogeneic but not autologous HSCT achieved long-lasting complete remission in some ATL patients.[38,60] However, there is also a risk of GVH disease, which is sometimes lethal. If Tax-specific CTL induced in post-HSCT ATL patients makes any contribution to GVL effects, Tax-targeted immunotherapy might be worth trying either with or without HSCT, and selective GVL effects would be expected. Indication of Tax-targeted immunotherapy, however, should be limited to those cases whose ATL cells retain the ability to express Tax.

Conclusion

Although the peripheral HTLV-I-infected cells do not express detectable levels of HTLV-I antigens, they retain the ability to express Tax in most HTLV-I-infected individuals, including asymptomatic HTLV-I carriers, HAM/TSP patients and approximately half of all ATL cases. Immunological findings support the contribution of Tax-specific CTL to antitumor immunity in these hosts, encouraging immunological prophylaxis and therapy for ATL.

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