In vitro Induction of Cytotoxic T Lymphocytes against HTLV-I-infected T-Cells from Adult T-Cell Leukemia Patients, Asymptomatic HTLV-I Carriers and Seronegative Healthy Donors

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We investigated an *in vitro* method to produce cytotoxic T lymphocytes (CTLs) against HTLV-I-infected T-cells using peripheral blood mononuclear cells (PBMC) of adult T-cell leukemia (ATL) patients, asymptomatic HTLV-I carriers (AC) and seronegative healthy donors. The PBMC were restimulated repeatedly for 4 weeks with HLA-matched HTLV-I-infected T-cells which had been pretreated at 56°C for 30 min to inactivate infectious HTLV-I. The culture medium included 10–100 units/ml of recombinant lymphokines (rIL-1, rIL-2, rIL-4, rIL-6 and rIL-7) and 10% fetal calf serum in RPMI-1640 medium. The cytotoxic activity was measured against HLA-matched HTLV-I-infected T-cell lines after CD4^{+} or CD8^{+} cells were positively panned from the cultured PBMC. The PBMC of ATL, AC and healthy donors were able to produce either CD4^{+} or CD8^{+} CTLs against HTLV-I-related antigens (*env*, *gag*, p21[x], p27[rex] and p40[tax]) as well as the antigen(s) of as-yet unknown specificity expressed on HTLV-I-infected T-cells. All the CTLs recognized the specific antigens in the context of either class I or class II HLA types. These results indicated that ATL patients, AC and healthy donors were immunocompetent to generate CTLs against HTLV-I-infected T-cells and probably against HTLV-I-transformed T-cells.

Key words: Anti-HTLV-I CTL — *In vitro* induction of CTL — ATL — Asymptomatic HTLV-I carrier — HTLV-I seronegative healthy donor

Adult T-cell leukemia (ATL) is a T-cell malignancy associated with human T-cell leukemia virus type-I (HTLV-I) infection.[1] The prognosis of ATL is known to be very poor due to opportunistic infections and multiple organ failures.[2] The currently available therapeutic measures for ATL are anti-leukemic chemotherapy, irradiation therapy and application of biological response modifiers, but complete remission is rarely obtained.[3] Thus, new therapeutic measures for ATL are urgently necessary.

Immune response to HTLV-I infection has been observed among asymptomatic HTLV-I carriers (AC)[4] and HTLV-I-associated myelopathy (HAM) patients,[5–8] whose cytotoxic T lymphocytes (CTLs) were thought to inhibit outgrowth of HTLV-I-infected T-cells and their leukemic transformation.[9] It was postulated that HTLV-I-specific CTLs might be a major immunological effector to prevent HTLV-I carriers from developing ATL.[10] Thus, the CTLs against HTLV-I-infected T-cells may be a good candidate for the immunotherapy of ATL.

In the present study we investigated an *in vitro* method to induce HTLV-I-specific CTLs from peripheral blood mononuclear cells (PBMC) of ATL patients, AC and healthy donors, and analyzed the antigenic epitopes recognized by the CTLs.

MATERIALS AND METHODS

Subjects Two cases of ATL, 4 cases of AC and one HTLV-I seronegative healthy donor were the subjects of this study (Table I). All subjects were Japanese of 40–72 years old. AC-1, AC-2, AC-3 were spouses of ATL patients and AC-4 was the spouse of an HAM patient. ATL and HAM patients were diagnosed by clinico-pathological criteria according to Shimoyama *et al.*[2] and Osame *et al.*,[11] respectively.

Blood samples and cryopreservation Heparinized peripheral blood (25–35 ml) was drawn from the subjects and the PBMC were prepared by density gradient centrifugation on Mono-Poly Resolving Medium ($d = 1.114$) at 400g for 30 min at 22°C and processed for cryopreservation in liquid nitrogen as follows. The PBMC

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Table I. CTL Donors of This Study

Donors	Age/Sex	HLA haplotypes	HTLV-I PA titer[a]	Clinical status
HD-1	40/M	A24Cw-B52DR15DQ1 A24Cw-B52DR15DQ1	<16	HTLV-I seronegative healthy donor
AC-1	65/F	A24Cw-B52DR15DQ1 A24Cw-B52DR9 DQ3	1024×	asymptomatic HTLV-I carrier
AC-2	56/M	A24Cw-B52DR15DQ1 A24Cw-B51DR9 DQ3	16384×	asymptomatic HTLV-I carrier
AC-3	71/F	A24Cw-B52DR15DQ1 A24Cw7B7 DR8 DQ1	2048×	asymptomatic HTLV-I carrier
AC-4	72/M	A24Cw-B52DR15DQ1 A24Cw-B52DR15DQ1	1024×	asymptomatic HTLV-I carrier
ATL-1	66/F	A24Cw-B52DR15DQ1 A2 Cw3B61DR4 DQ3	2048×	ATL (chronic type)
ATL-2	62/F	A24Cw-B52DR15DQ1 A2 Cw1B54DR4 DQ4	256×	ATL (chronic type)

a) Serum antibody titers of HTLV-I were measured by means of the particle agglutination test.

(3×10^7 cells) were suspended in 3 ml of ice-cold 30% fetal calf serum (FCS)-RPMI-1640 medium supplemented with 2 mM of L-glutamine, 50 U/ml of penicillin, and 50 μg/ml of streptomycin (30% FCS-RPMI-1640), and 3 ml of ice-cold 20% dimethylsulfoxide (DMSO) in 10% FCS-RPMI-1640 medium was added dropwise (one drop/3–5 s) to make a final concentration of 10% DMSO and 20% FCS in RPMI-1640. This mixing procedure was carried out manually or by use of a Biocryomixer S2Y-90 (Nippon Freezer Co. Ltd.). One ml aliquots of the mixed PBMC suspension were transferred to 2 ml cryotubes, wrapped with plenty of defatted cotton wool, and slowly frozen at $-80°$C overnight in a Biofreezer (Nippon Freezer Co. Ltd.) or quickly frozen in a programmed freezer, Profreeze SNY-88 (Nippon Freezer Co. Ltd.). The frozen PBMC were transferred to a liquid nitrogen tank for cryopreservation until used. When the cryopreserved PBMC were required for experiments, they were defrozen by rapid thawing at 37°C and immediately washed with 10 ml of 10% FCS-RPMI-1640. The defrozen PBMC showed more than 95% viability as judged from the trypan blue dye exclusion test.
HLA typing Serological HLA typing was performed by the standard NIH microcytotoxicity test[12] using T and B lymphocytes separated from the cryopreserved PBMC by a nylon wool column method.[13] HLA typing sera were standardized according to the 11th International Histocompatibility Workshop.[14] HLA haplotypes of the subjects were determined by HLA types from more than 4 relatives.
Establishment of HTLV-I-infected T-cell lines An HTLV-I-infected T-cell line was established from PBMC of an HTLV-I seronegative healthy donor (HD-1) by co-culture with mitomycin C-treated MT-2 cells (MMC-

MT-2 cells).[15, 16] In brief, the MMC-MT-2 cells were prepared by treating MT-2 cells with mitomycin C (MMC) at a concentration of 50 μg/1$\times$10^6 cells/ml in 10% FCS-RPMI-1640 for 30 min at 37°C and washed twice with 10% FCS-RPMI-1640. The PBMC (5×10^6 cells) were co-cultured with 5×10^6 MMC-MT-2 cells in 10 ml of 10% FCS-RPMI-1640 supplemented with 2 μg/ml of PHA-P and 100 U/ml of recombinant interleukin-2 (rIL-2) (PHA-IL2-RPMI-1640) in humidified 5% CO_2-95% air. The cultures were continued for 60 days by replacing the medium every 3 days. The blastoid T-cells grown in the cultures were separated by Percoll density gradient (30%, 40% and 55%) centrifugation at 600g for 30 min at room temperature.[17] The cells floating on the interface between 40% and 55% Percoll were collected and cultured as an HTLV-I-infected T-cell line, IHD-1.

Beside IHD-1, 2 other HTLV-I-infected T-cell lines were established from 2 patients with lymphoma-type ATL by long-term culture in PHA-IL2-RPMI-1640 medium. They were shown to be infected with HTLV-I and were designated IATL-3 and IATL-4.
Analysis of HTLV-I antigens on HTLV-I-infected T-cell lines HTLV-I antigens expressed on IHD-1, IATL-3 and IATL-4 cells were examined by immunofluorescent antibody staining (IF test).[18] We used murine monoclonal antibodies against HTLV-I; anti-*gag* p19 (GIN-14), anti-*env* gp21 (F-10) and anti-p40^{tax} (Lt-4), as described.[19–21] The profiles of HTLV-I antigens expressed on the cells are summarized in Table II.
Heat-inactivation of infectious HTLV-I The IHD-1 cells were used as antigen-presenting cells to induce HTLV-I-specific CTLs. To prevent HTLV-I infection of the CTLs during *in vitro* cultivation, the MMC-treated IHD-1 cells suspended in 10% FCS-RPMI-1640, were incubated at

Table II. Properties of HTLV-I-infected T-Cell Lines

HTLV-I-infected T-cell lines	HLA haplotypes	HTLV-I antigens[a] (%)		
		p19	gp21	p40[tax]
IHD-1	A24Cw-B52DR15DQ1	92.0	25.8	68.8
	A24Cw-B52DR15DQ1			
IATL-3	A31Cw3B35DR15DQ1	94.7	76.7	8.7
	A26Cw1B54DR4 DQ4			
IATL-4	A24Cw7B 7DR1 DQ1	100.0	96.0	5.7
	A26Cw1B59DR9 DQ3			
MT-2	A24Cw-B51DR15DQ1	100.0	100.0	100.0
	A- Cw3B61DR4 DQ3			

a) Expression of HTLV-I antigens examined with monoclonal antibodies (p19: anti-*gag* p19, gp21: anti-*env* gp21, p40[tax]: anti-p40[tax]).

various temperatures from 4°C to 90°C for 30 min in a water bath. The infectivity of HTLV-I was tested by cocultivating 1×10^6 cells of normal PBMC (HD-1) with the same number of heated IHD-1. After 4 weeks of cultivation, the PBMC of HD-1 were examined for the expression of HTLV-I antigens by IF test. The heat-inactivated IHD-1 (5×10^4 cells) were tested for immunogenicity to elicit mixed lymphocyte reaction (MLR) with 5×10^4 autologous PBMC (HD-1) in 0.2 ml of RPMI-1640 medium supplemented with 10% heat-inactivated pooled human serum (10% HS-RPMI-1640) in 96-well round-bottomed plates for 6 days as described.[5] The effect of heat treatment on HLA antigen expression of IHD-1 was analyzed by flow cytometry using an Epics Profile II (Coulter Electronics, Healeth) with anti-HLA ABC (W6/32) and anti-HLA DR (L243).

Induction of HTLV-I-specific CTLs IHD-1 cells were heat-treated at 56°C for 30 min. Then 5×10^6 heat-treated IHD-1 cells were co-cultured with 5×10^6 PBMC of the CTL donors for 7 days in 50 ml culture flasks containing 10 ml of 10% HS-RPMI-1640 medium and the medium was replaced with 10% FCS-RPMI-1640 supplemented with 100 U/ml of rIL-2 (TGP-3: Takeda Chemical Industries), rIL-1, rIL-4 and rIL-6 and 10 U/ml of rIL-7. The cultures were restimulated with 5×10^6 heat-treated IHD-1 cells and HLA-matched feeder cells (PBMC of HD-1) every 7 days. After 4 weeks of cultivation, the activated T-cells were separated by 40–55% Percoll density gradient centrifugation and CD4^{+} and CD8^{+} cells were positively panned out by the immunobeads method.[22]

CTL assay was performed by the 4 h ^{51}Cr-release method[23] in a microplate using ^{51}Cr-labeled targets of either HTLV-I-infected T-cell lines or HTLV-I/vaccinia recombinant virus-infected B-cell lines of HD-1 which were transformed with Epstein-Barr virus (EBV-B-cells) as described.[7] The HTLV-I/vaccinia recombinant

viruses expressed HTLV-I envelope (LO5-*env*), core (LO5-*gag*) or *pX* proteins (LO5-p21[x], LO5-p27[rex], LO5-p40[tax]) as confirmed by IF tests with anti-HTLV-I antisera. Spontaneous release of ^{51}Cr was less than 20% of the maximal release in triplicate experiments. Negative control targets (LO5-infected, HLA-matched B-cells and HLA-unmatched T-cells) produced 0%–9.8% cytolysis, so cytolysis of more than 10% was defined as significant CTL activity.

RESULTS

Effect of heat treatment on HTLV-I infectivity and immunogenicity of HTLV-I-infected T-cell line The HTLV-I-infected T-cell line produces infectious HTLV-I *in vitro*. To prevent infection of the CTL precursors with HTLV-I, we inactivated infectious HTLV-I by heat treatment at various temperatures for 30 min. The infectivity of HTLV-I decreased at more than 40°C and was completely lost at 56°C (Fig. 1a). However, the immunogenicity of IHD-1 cells after having been heated at 56°C remained at 74% of that of unheated controls (Fig. 1b). Flow cytometry revealed that the heated IHD-1 cells showed 14% loss of HLA class I and 10% loss of class II antigenicity (Fig. 1c).

These results indicated that heat treatment at 56°C for 30 min was sufficient to inactivate all HTLV-I infectivity while leaving intact the immunogenicity to stimulate anti-HTLV-I CTLs *in vitro*.

***In vitro* induction of HTLV-I-specific CTLs from healthy donor, asymptomatic HTLV-I carriers and ATL patients** The healthy donor (HD-1) was able to induce only CD4^{+} CTL which recognized target cells of IHD-1, MT-2 and IATL-3 in the context of HLA-DR15 (Fig. 2; HD-1, Table II). CD8^{+} CTLs were not obtained from this healthy donor (HD-1).

The AC donors (AC-1–4) induced CD4^{+} CTLs which recognized target cells of IHD-1, MT-2 and IATL-3 in the context of HLA-DR15, and CD8^{+} CTLs which recognized IHD-1, MT-2 and IATL-4 in the context of HLA-A24 (Fig. 2; AC-1–4, Table II).

The ATL patient (ATL-1) induced both CD4^{+} and CD8^{+} CTLs which recognized the target cells in the context of HLA-DR15 and HLA-A24, respectively (Fig. 2; ATL-1, Table II). However, no CTLs were induced from ATL-2 (Fig. 2; ATL-2).

Antigenic epitopes recognized by the CTLs induced *in vitro* Antigenic epitopes recognized by the CTLs were analyzed by the use of EBV-B-cells infected with HTLV-I/vaccinia recombinant viruses. The CD4^{+} CTLs from HD-1, AC-1 and ATL-1 recognized HTLV-I antigens with varying degrees of cytolysis against *env*, *gag*, p21[x], p27[rex] and p40[tax] epitopes (Fig. 3a). The CD8^{+} CTLs from AC-1 and ATL-1 recognized p40[tax] and *gag*

a.

b.

c.

anti-HLA-ABC (W6/32)

14% loss

anti-HLA-DR (L243)
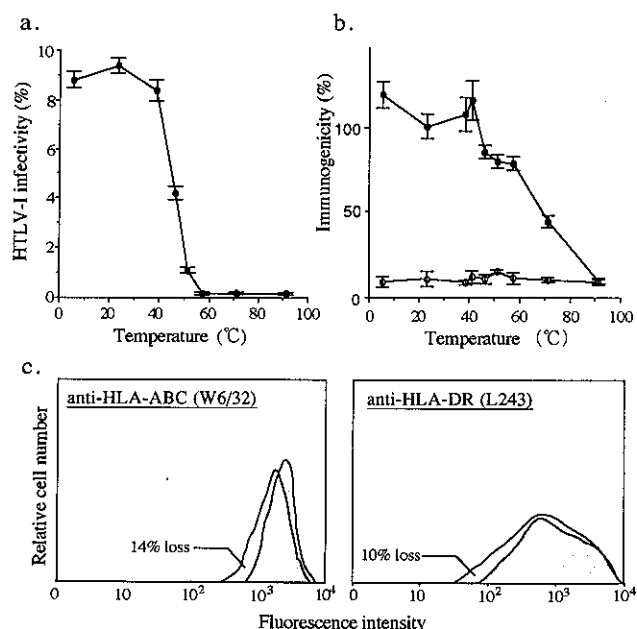
10% loss

Fluorescence intensity

Fig. 1. Effect of heat treatment of HTLV-I infectivity and immunogenicity of HTLV-I-infected T-cell line. a) HTLV-I infectivity. The PBMC of HD-1 were cultured in PHA-IL2-RPMI-1640 medium with MMC-treated IHD-1 which had been treated for 30 min at various temperatures (4°C, 22°C, 37°C, 45°C, 50°C, 56°C, 70°C and 90°C). After 4 weeks of cultivation, activated HD-1 cells were collected by Percoll centrifugation, and immunofluorescent staining was conducted with anti-HTLV-I monoclonal antibody (GIN-14). HTLV-I infectivity was expressed as percent of HTLV-I antigen-positive cells by counting 500 cells under an inverted fluorescence microscope. b) Immunogenicity. The MMC-treated IHD-1 (●) and HD-1 (○) were treated for 30 min at various temperatures (4°C, 22°C, 37°C, 40°C, 45°C, 50°C, 56°C, 70°C and 90°C) and co-cultured with HD-1 responder cells (PBMC) for 6 days in 0.2 ml of 10% HS-RPMI-1640 medium in microplates. The cultures were supplemented with 1 μCi of [^{3}H]TdR for the final 18 h of cultivation and the radioactivity incorporated into the proliferating HD-1 responder cells was measured in a liquid scintillation counter. The immunogenicity was expressed as percent of [^{3}H]TdR uptake with IHD-1 cells treated at 22°C. c) HLA antigen expression. Heat-treated IHD-1 (☐) and non-treated IHD-1 (▨) were stained with anti-HLA-ABC (W6/32) or anti-HLA-DR (L243) monoclonal antibody, and the profiles of HLA antigen expression were analyzed by flow cytometry.

preferentially, although *env* and p27^{rex} were also targeted (Fig. 3b). The pattern of cytotoxicity indicated that the CTLs were polyclonally induced.

The CD8$^+$ CTL of AC-2 recognized a particular antigen(s) of as-yet unknown specificity in the context of HLA-A24 (Fig. 2; AC-2, Fig. 3b; AC-2, Table II). This antigen(s) was not expressed on the EBV-B-cells infected
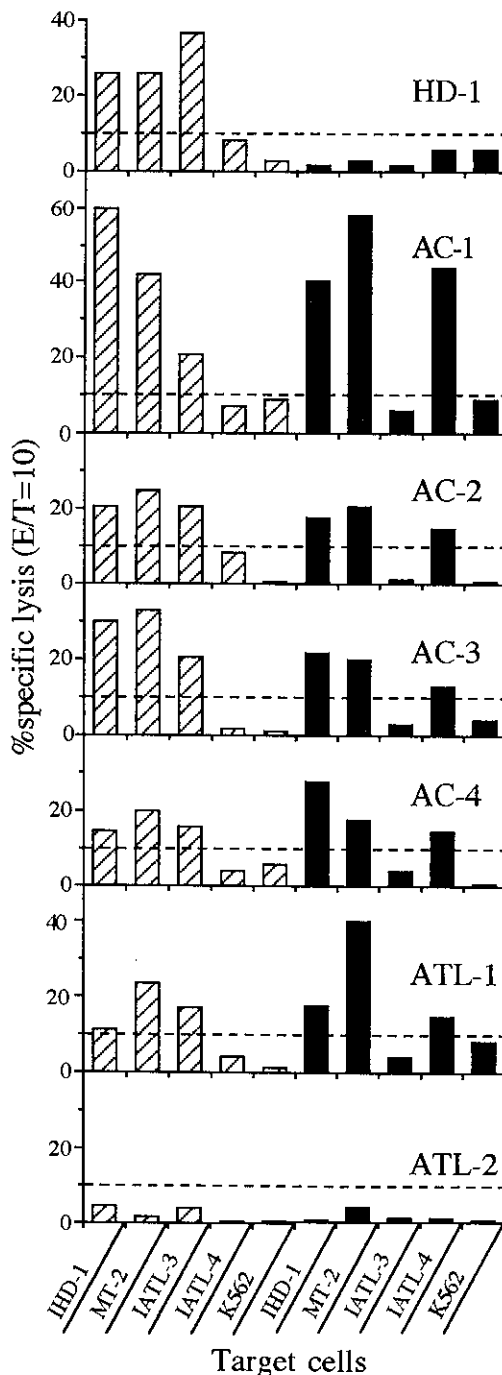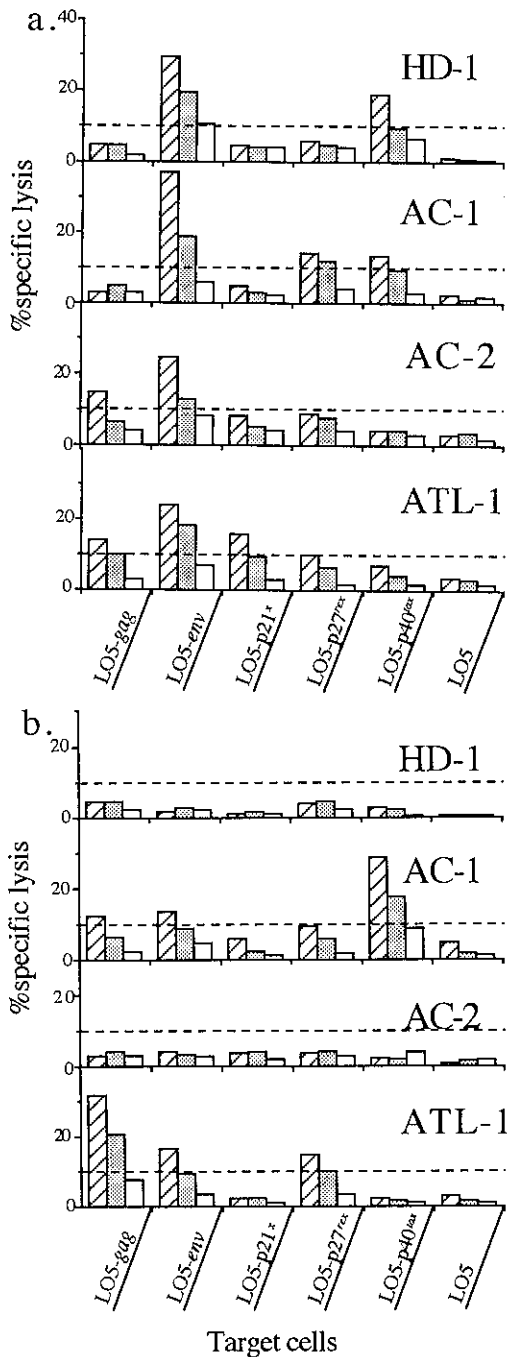

Target cells

Fig. 2. *In vitro* induction of HTLV-I-specific CTLs. The PBMC of a healthy donor, HD-1, asymptomatic HTLV-I carriers, AC-1, AC-2, AC-3 and AC-4, and ATL patients, ATL-1 and ATL-2, were stimulated *in vitro* with the heat-treated IHD-1. After 4 weeks of cultivation, CD4$^+$ or CD8$^+$ cells were panned and the CTL activities were measured by using HTLV-I-infected T-cell lines (IHD-1, MT-2, IATL-3 and IATL-4) and K562 cells as targets. CTL assay was performed at E/T=10. More than 10% specific lysis was regarded as significant. ▨, CD4$^+$ CTL; ■, CD8$^+$ CTL.

Fig. 3. Antigenic epitopes recognized by CTLs. The cytolytic activities of CD4$^+$ or CD8$^+$ CTLs from a healthy donor (HD-1), asymptomatic HTLV-I carriers (AC-1, AC-2) and ATL patients (ATL-1) were measured by using as targets EBV-B-cell lines infected with HTLV-I/vaccinia recombinant viruses (LO5-*gag*, LO5-*env*, LO5-p21^x, LO5-p27^{rex}, LO5-p40^{tax}) and LO5 vaccinia virus. CTL assay was performed at E/T = 30 (▨), 10 (▩) and 3 (☐). More than 10% specific lysis was regarded as significant. a), CD4$^+$ CTL; b), CD8$^+$ CTL.

with HTLV-I/vaccinia recombinant viruses, but was expressed on IHD-1, MT-2 and IATL-4 cells.

The antigenic epitopes recognized by the CTLs of this study are summarized in Table III. It is remarkable that the CD4$^+$ CTLs recognized the *env* epitope preferentially and the CD8$^+$ CTLs recognized a variety of antigenic epitopes such as *env, gag*, p40^{tax} and unknown antigen(s).

DISCUSSION

In vitro induction of CTL requires HLA matching between CTL precursor cells and antigen-presenting cells.[24] In order to establish an *in vitro* method to induce HTLV-I specific CTLs, we selected particular donors with A24Cw-B52DR15DQ1 haplotype, which is the most common haplotype of the Japanese population (Table I).[25]

The CTL stimulator was IHD-1, an HTLV-I-infected T-cell line homozygotic for the A24Cw-B52DR15DQ1 haplotype, which expressed HTLV-I antigens able to induce anti-*gag*, anti-*env* and anti-p40^{tax} CTLs (Tables II and III). The CTL targets were IHD-1, IATL-3, IATL-4 and MT-2 cells which had partially identical HLA antigens with the CTL stimulator (Table II).

CTLs produced *in vitro* are often infected with HTLV-I, which results in the loss of CTL function.[4, 26] To prevent infection of the CTLs with HTLV-I *in vitro*, we explored the optimal conditions of heat treatment to inactivate HTLV-I infectivity while preserving the immunogenicity to stimulate the CTL precursors. The heat treatment of CTL stimulator at 56°C for 30 min was useful for inducing CTLs without HTLV-I infection (Figs. 1 and 2). Indeed, the heat-treated IHD-1 were able to induce CD4$^+$ CTL from the healthy donor and both CD4$^+$ and CD8$^+$ CTLs from AC and one ATL patient. The failure to induce CD8$^+$ CTLs from the healthy donor (HD-1) might be ascribed to the minimal frequency of CD8$^+$ CTL precursors as compared with HTLV-I-infected donors (AC and ATL patients) (Fig. 2; AC-1–4, ATL-1). It was noted that one of the ATL patients failed to induce any CTLs (Fig. 2; ATL-2). This is a typical case of the immunocompromised state of ATL, as described.[10]

Analysis of the antigenic epitopes revealed that the CD4$^+$ CTLs recognize either *env, gag*, p21^x p27^{rex} and p40^{tax} in the context of HLA-DR15, while CD8$^+$ CTLs recognize either *env, gag*, p27^{rex} or p40^{tax} in the context of HLA-A24 (Table III). The CTLs were polyclonally induced to recognize various epitopes of HTLV-I antigens with the same HLA restriction. The high frequency of anti-*env* CD4$^+$ CTLs might be a corollary to the increased level of memory T-cells in the donors with HLA-DR15. The HLA restriction of *env* epitopes with HLA-DR15 was similarly observed in the CD8$^+$ re-

Table III. Antigenic Epitopes Recognized by CTLs in This Study

Donor	Epitopes recognized by CD4$^+$ CTLs	Epitopes recognized by CD8$^+$ CTLs
HD-1	$\boxed{env}$, p40^{tax}	(−)
AC-1	$\boxed{env}$, p27^{rex}, p40^{tax}	$\boxed{p40^{tax}}$, env, gag
AC-2	$\boxed{env}$, gag	yet unknown antigens
AC-3	$\boxed{env}$, gag	$\boxed{env}$
AC-4	$\boxed{env}$	$\boxed{gag}$, $\boxed{env}$
ATL-1	$\boxed{env}$, gag, p21^x	$\boxed{gag}$, env, p27^{rex}
ATL-2	(−)	(−)

The boxed epitopes are predominantly recognized by the CTLs.

sponse against p40^{tax} with HLA-A2 and against *pol* with HLA-A30.[4, 6-8]

Of most interest was the target antigen(s) recognized by the CD8$^+$ CTL from AC-2. The antigen(s) was not *env, gag,* or *pX* antigens of HTLV-I, but was associated with HTLV-I-infected T-cells. The CD8$^+$ CTL might recognize alien antigen(s) as described previously[27] although the *pol* antigen of HTLV-I remains to be excluded as a possibility. The alien antigen(s) might be related to altered surface antigen.[27, 28]

As the prognosis of ATL patients is very poor, a new specific therapy for ATL is desired. The immunotherapy of ATL can be achieved by vaccination of HTLV-I antigens to induce CTLs against HTLV-I-infected T-cells[29] and adoptive transfer of HTLV-I-specific CTLs induced *in vitro*. However, CTL precursors are supposed to be very few in immunocompromised ATL patients, as demonstrated in the case of ATL-2 (Fig. 2; ATL-2), and thus vaccination of HTLV-I antigens may not be effective to provoke HTLV-I-specific CTLs. To circumvent this difficulty, adoptive transfer of HTLV-I-specific CTL might be appropriate for specific immunotherapy of ATL. The present study allows production of HTLV-I-specific CTLs without any deleterious HTLV-I infection using HLA-matched healthy donors as well as asymptomatic HTLV-I carriers, and such an approach could find application in the immunotherapy of ATL.

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