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(Citation)
The Kobe journal of the medical sciences,37(2):57-79

(Issue Date)
1991

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/E0034086

Kobe J. Med. Sci. 37, 57/79, April 1991

INHIBITION OF CELLULAR PROLIFERATION IN HUMAN T LYOPHOTROPIC VIRUS TYPE I(HTLV-I)-INFECTED T CELLS FROM PATIENTS WITH HAM BY STEROID HORMONES AND CYCLOSPORIN A

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INDEXING WORDS

HTLV-I; HTLV-I-associated myelopathy (HAM); glucocorticoid, $1,25(OH)_2D_3$; Cyclosporin A

SYNOPSIS

The effects of steroid hormones and Cyclosporin A(CsA) on de novo DNA synthesis in four human T lymphotropic virus type I (HTLV-I)-infected T cell lines from patients with HTLV-I-associated myelopathy (HAM) were investigated. These T cell lines were characterized by helper/inducer phenotypes and expressed IL-2-receptor(Tac) and HLA-DR antigen in high percentages.

Three of the four cell lines had their de novo DNA synthesis inhibited by steroid hormones (dexamethasone and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)) and CsA in a dose dependent manner. The inhibitory effects arose 24 hours

Received for publication : February 25, 1991
Authors' names in Japanese : 山田博規, 中尾實信, 藤田拓男

after initiation of incubation with either steroid hormones or CsA. The forth cell line was not inhibited by these reagents. It also revealed through the use of an *in vitro* assay utilizing the human IL-2 dependent cell line,Sez 627,that none of these T cell lines secrete IL-2 in detectable volumes.

In order to clarify the mechanism of the inhibitory effects of steroid hormones and CsA, among these 4 cell lines H-89-59,insensitive to the reagents , and H-109,sensitive to them,were used for northern blotting analysis. C-*myc* gene expression was demonstrated both in H-89-59 and H-109. $1,25(OH)_2D_3$ and CsA supressed c-*myc* expression in H-109 as well as cell proliferation. In H-89-59,however ,neither expression of c-*myc* nor cell proliferation was supressed. On the other hand,the level of HTLV-I gene transcripts was not changed by these agents.

Hence,inhibition of cellular proliferation by these reagents was not caused by the inhibition of HTLV-I p40(x) gene but by inhibition of transcriptional factors such as c-*myc* products.

INTRODUCTION

Human T lymphotropic virus type I (HTLV-I) has been impricated as the cause of an aggresive and often fetal neoplasm of $CD4^+$ lymphocytes termed adult T cell leukemia (ATL) , a disease endemic in the south-west Japanese islands and Caribbean islands.[23,24,27,32] In addition, Geissman et al.(1985) reported that most patients with tropical spastic paraparesis (TSP) had antibodies to HTLV-I in their sera.[5] Osame et al.(1985) found enough patients in Japan with a similar type of myelopathy to propose a new clinical entity termed HTLV-I-associated myelopathy(HAM).[22] Since then, approximately 450 patients with HAM have been registered in a nation-wide study by Japanese scientists.[20]

Of note in terms of clinical findings in HAM patients is a favorable response to glucocorticoid. On the other hand, steroid hormones are known to affect cell proliferation and functional differentiation in a variety of cells, including HTLV-I-infected T cell lines from ATL patients.[9] In previous studies, we pointed out certain phenotypic resemblances between ATL cells and phytohemagglutinin (PHA)-activated T cells, i,e. expression of IL-2-receptors (IL-2R) and HLA-DR antigen as well as sensitivity to the srteroid hormone, $1,25(OH)_2D_3$.[10,14,15,18] It is of interest that in those studies, $1,25(OH)_2D_3$ supressed cell proliferation and de novo DNA synthesis of certain HTLV-I positive T cell lines from ATL patients as well as that of PHA-activated T cells. For these reasons, it is logical to be curious about the effects of steroid hormones on HTLV-I-infected T cell lines from HAM patients.

It has been demonstrated that the most notable features of HTLV-I infected leukemic T cell lines seem to be their constitutive high level expression of IL-2R[4,6,28] with only occasionl simultaneous production of IL-2.[1,25,29,30] In order to investigate the similarities and differences between HTLV-I infected T cell lines from patients with HAM (HAM T cell lines) and HTLV-I infected T cell lines from patients with ATL (ATL T cell lines) or PHA-activated T cells, we examined the sensitivity of four HAM T cell lines to the steroid hormones, dexamethason and $1,25(OH)_2D_3$, and CsA an eleven amino acid cyclic peptide derived from Fungi imperfecti. A potent immunosupressive agent, CsA, unlike other agents, lacks significant myelotoxicity and appears to specifically inhibit T cell-mediated immune responses, probably inhibiting IL-2 activity.[2,7,8] Clarification of the mechanisms of any inhibitory effects of steroid hormones and CsA on HTLV-I-infected T cells should, therefore, aid in elucidation of the pathogenic mechanism

involved in HTLV-I-induced T cell transformation.

MATERIALS AND METHODS

Chemicals

$1,25(OH)_2D_3$ was a gift from the Teijin Institute for Biochemical Research(Tokyo,Japan). Dexamethasone was purchased from Sigma Chemical Co.(St.Louis,MO). CsA and recombinant IL-2 were kindly provided by Dr.J.F.Borel(Sandoz Ltd., Basel, Switzerland) and Shionogi Pharmaceutical Co.(Osaka,Japan),respectively. $1,25(OH)_2D_3$, dexamethason and CsA were dissolved in ethanol and ,as stock solutions ,were stored at -20°C and diluted with RPMI-1640 medium (Flow Laborotories,Inc.,Rockville,MD) prior to use. The final concentration of ethanol was less than 0.1%. Addition of these amounts to the culture produced no detectable effects on the cells.

Cells and Cell culture

H-89-59,H-89-79,and H-89-85 are human HTLV-I-infected T cell lines established from the peripheral blood of HAM patients. H-109 is a HTLV-I-infected T cell line from the cerebrospinal fluid of a HAM patient.[16,26] These cells were cultured in RPMI-1640 mdium supplemented with 20% heat-inactivated fetal calf serum(FCS,Flow Laboratory,Inc.) and 100μg/ml kanamycin in a humidified atmosphere of 5% CO_2 in air. The cells were seeded in culture flasks(25cm^2; Corning Glass Works,Corning,NY) and cultured in the presencee of various concentrations of reagents.

Flow-cytometry

Cells were stained by using an indirect

immunofluorescence technique as described previously.[13] One million cells from each cell line were incubated with 0.5μg of the monoclonal antibodies, Leu 2a (anti-CD8 antibody), Leu 3a (anti-CD4 antibody) (Beckton Dickinson, Mountain View, California). OKT 3 (anti-CD3 antibody), OKT 11 (anti-CD2 antibody) OK Ia1 (anti-HLA-DR antibody) (Ortho Diagnostic System Inc., Rartian, New Jersey) and Tac (anti-IL-2R antibody) (kindly provided by Dr Uchiyama, Kyoto University, Kyoto, Japan) in an ice bath for 30 min. After washing with phosphate buffered saline (PBS), the cells were incubated with fluorescence isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Beckton Dickinson) in an ice bath for 30 min and then washed extensively. The cells were finally resuspended with PBS. The antigen-positive cells were counted utilizing a fluorescence-activated cell sorter (FACS) system (Model 440, Beckton Dickinson). Ten thousand cells were scattered per sample. Values were expressed as the ratio of cells with a fluorescent intensity above that of control cells stained without monoclonal antibodies to the total cell population. The absolute number of specific antigen-positive cells in each cell line was calculated by multiplying the total cell number by the percentage of antigen-positive cells present.

Effects of 1,25(OH)$_2$D$_3$, Dexamethasone and CsA on de novo DNA synthesis

H-89-59, H-89-79, H-89-85 and H-109 cells (2×10^5 cells/well) were cultured for 48 hours in 200μl of complete medium in the presence of various concentrations of 1,25(OH)$_2$D$_3$, dexamethasone and CsA in a 96-well tissue culture plate (Falcon, Beckton Dickinson, Labware Oxnard, Ca) in a humidified atomosphere of 5% CO_2 in air unless otherwise specified. Cells were pulsed with 0.2μCi

methyl-[^{3}H]-thymidine(TdR)(6.7Ci/m mol,New England Nuclear Products.Boston,MA) for the final 6 hr, harvested on glass fiber(Whatmann,England) using a cell harvester(Nunc,Denmark) and counted using a liquid scintillation counter(Packard Instruments Co., Dones Grone,IL) as previously described.[11,15]

Statistical differences were established by student-t test.

Kinetics of effects of steroid hormones and CsA

To investigate the time course effects of steroid hormones and CsA,H-109 cells (2×10^5) were cultured for 6,12,24 and 48 hour in 200μl of complete medium in the presence of each agents,then pulse labeled,harvested and counted using the methods described above.[11,15] Statistical differences were estimated by student-t test.

IL-2 assay

Based on the assay of Namiuchi et al.,[19] cells of the cloned human IL-2-dependent T-cell line,Sez-627 (kindly provided by Dr.M.Maeda,Kyoto University,Kyoto,Japan) were washed extensively and incubated with serially diluted supernatants of the H-89-59,H-89-85 and H-109 cell lines in 100μl of culture medium with 5% FCS at 37°C in 96 Farcon multiple microwells. After 24 hours,0.2μCi of methyl[^{3}H]-TdR per 200μl was added for another 6 hours. Cells were harvested and counted using the methods described above.

IL-2 titers were expressed as the reciprocal of the supernatant dilution which induced 50% maximal response in reference to human recombinant IL-2 as a standard.

Statistical differences were estimated by student-t test.

Preparation of total RNA

H-89-59 and H-109 cells ($1X10^8$) were harvested at 48 hours after exposure to the reagenmts. The cells were immediately homoginized in a syringe with 1.2 ml of GTC solution(4M Guanidium thyocyanate,0.5% sodium n-mercaptoethanol,0.1% antifoam). The homogenates centrifuged in HITACHI rotor at 45000 rpm for 12 hours at 15 °C through CsCl cushion(5.7M CsCl,0.1M EDTA). The RNA pellets were dissolved in 400μl of 10mM Tris -5mM EDTA-1% SDS and transferred to Eppendorf tubes. The solution was extracted by ethanol precipitation(o.1 volume of 3M sodium acetate,ph 4.8; 2.5 volumes of 100% ethanol) overnight at -20°C. The RNA was pelleted in a microfuge at 15000 rpm for 15 min at 4 °C,washed in 200μl of 70% ethanol ,dried under vacuum and pellet was dissolved in 400μl H_2O. Optimal density A_{260}/A_{280} was measured.A typical yield was 200μg RNA from $1X10^8$ cells. The RNA was stored at -20° C.

Northern Blot Analysis

Total cellular RNA(15μg/lane) was denatured in 0.65% formamide,7% folmaldehyde by heating at 60°C for 15 min,followed by 10 min on ice fractionated by electrophoresis on 1.2% agarose gel containiong 2% formaldehyde,1XMOPS buffer and blotted onto nylon membrane filters(BIODINE;Paul Ultra Filtration Cort.,NY).

Hybridization was performed at 40° C over 16 hr in 50% formamide,2XDenhardt's solution 2XSSC and salmon sperm DNA. After hybridization,filters were washed in 2XSSC-0.5%SDS at room temperature twice and at 55° C with 0.1XSSC-0.5% SDS twice. Autoradiography was performed at -80° C with Fuji Film.

The DNA probes were nick translated by Amersham's kit.

C-DNA for c-myc and HTLV-I were provided by Japanese gene bank.

RESULTS

Phenotype of HAM Cell Lines

Although certain differences exist between each of the cell line, HAM cell lines were basically characterized by antigenic phenotypes of helper/inducer T cells and moderate to high levels of IL-2R expression. HLA-DR antigen was also expressed at a high ratio in these cells (Table 1).

Table I

FACS analysis of cell surface antigenic phenotypes of HAM T cell lines.

	CD3	CD4	CD8	CD2	IL-2R	HLA-DR
H-89-59	3.2	76.5	2.7	19.5	89.7	94.6
H-89-79	92.0	96.1	2.7	99.6	92.1	N.D.*
H-89-85	5.1	43.7	0.4	94.0	38.8	80.8
H-109	3.9	79.0	1.0	N.D.*	99.6	67.0

Cells were stained with monoclonal antibodies using an indirect immunofluorescence technique. Antigen-positive cells were counted using a FACS system.

*N.D.; not done.

Effects of Steroid Hormones and CsA on de novo DNA Synthesis in HAM cell lines

Figure 1a illustrares the effects of steroid hormones

and CsA on de novo DNA synthesis in H-89-59 cell line. Basically,these agents caused no effects in this cell line. Figures 1b,1c,1d show the effects of steroid hormones and CsA on de novo DNA synthesis in the H-89-79,H-89-85 and H-109 cell lines. Contrary to the H-89-59 cell line, each of these three agents inhibited de novo DNA synthesis of these 3 cell lines in a dose dependent manner. Among the steroid hormones,$1,25(OH)_2D_3$ was more effective than dexamethasone at a concentration of 10^{-8}M.

Hence,heterogeneity in the effects of steroid hormones and CsA on these HTLV-I-infected T cell lines from HAM patients was obserbed.

Kinetics of de novo DNA Synthesis in the inhibitory effects of these agents

The kinetics of de novo DNA synthesis in H-109 cells after incubation with steroid hormones and CsA is illustrated in Figure 2. The inhibitory effect arose 24 hours after the start of incubation. There was no discrepancy regarding the timing of the inhibitory effect among these agents.

IL-2 assay

Figure 3 shows the IL-2 activity of culture supernatants of HAM T cell lines. There was no detectable activity in the supernatants of each cell lines,H-89-59,H-89-85 and H-109,using the assay which utilized the human IL-2 dependent cell line,Sez 627.

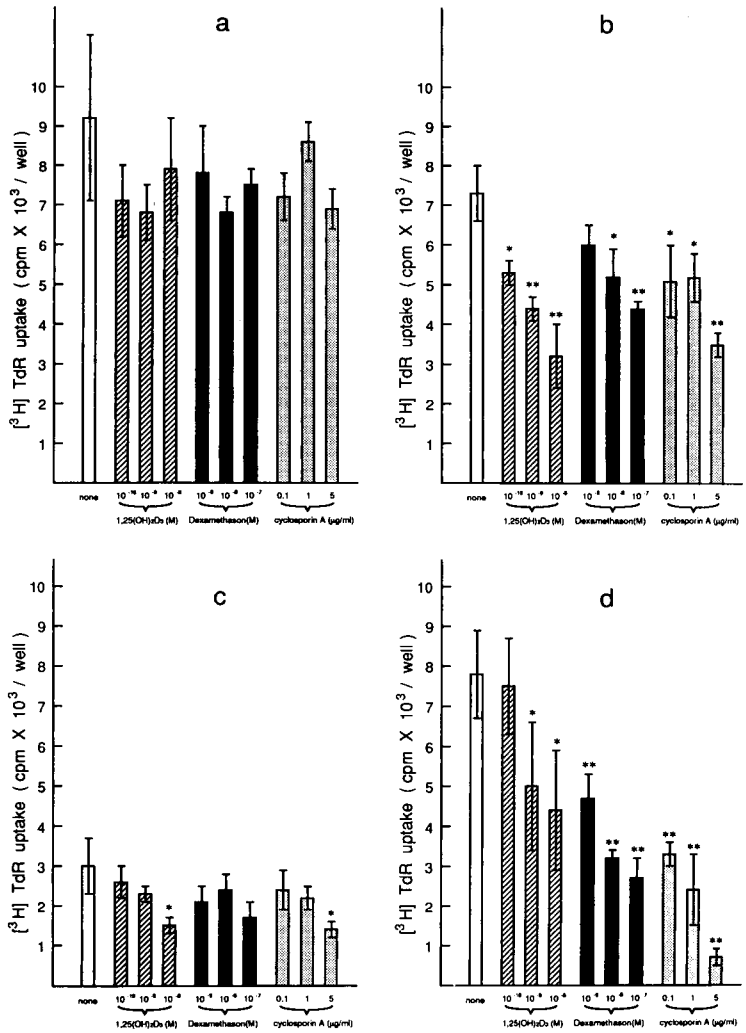

Figure 1.De novo DNA synthesis of H-89-59(a),H-89-79(b), H-89-85(c) and H-109(d) cells cultured with various agents. Cells ($2\times10^5/200\mu l$)were seeded in the presence of 0.1% ethanol (control) $1,25(OH)_2D_3$, dexamethason and CsA , and cultured for 48 hours. Methyl-[^{3}H] TdR uptake was determined by pulsing 0.2μCi [^{3}H]-TdR for the final 6 hours. Values indicated are mean±SD of triplicate assays. Stastical difference (vs.control) is shown by arterisks: * $p<0.05$, ** $p<0.01$.

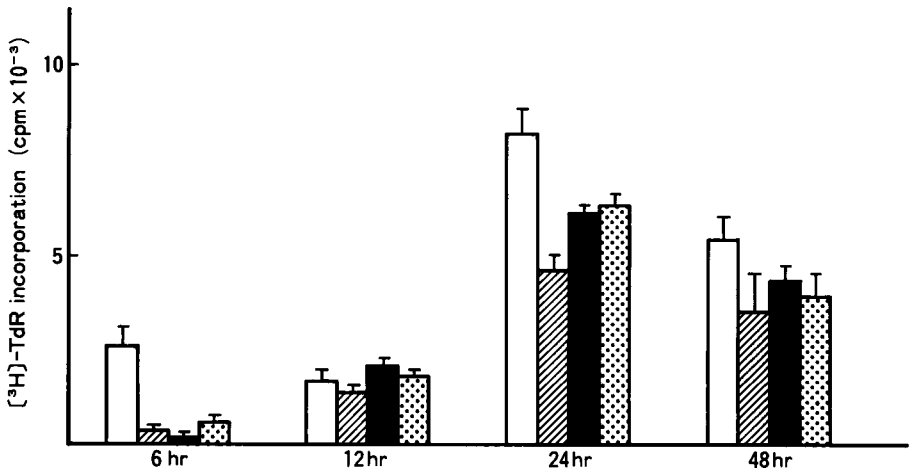

Figure 2.Time course study of de novo DNA synthesis in H-109 cells cultured with various agents. 0.1% ethanol(control), 1,25$(OH)_2D_3$ 10^{-8} M, dexamethason 10^{-7}M , CsA 1μg/ml . Values are means ± SD of triplicate assays. Statistical difference (vs. control) is shown by asterisks: * $p<0.05$, ** $p<0.01$.

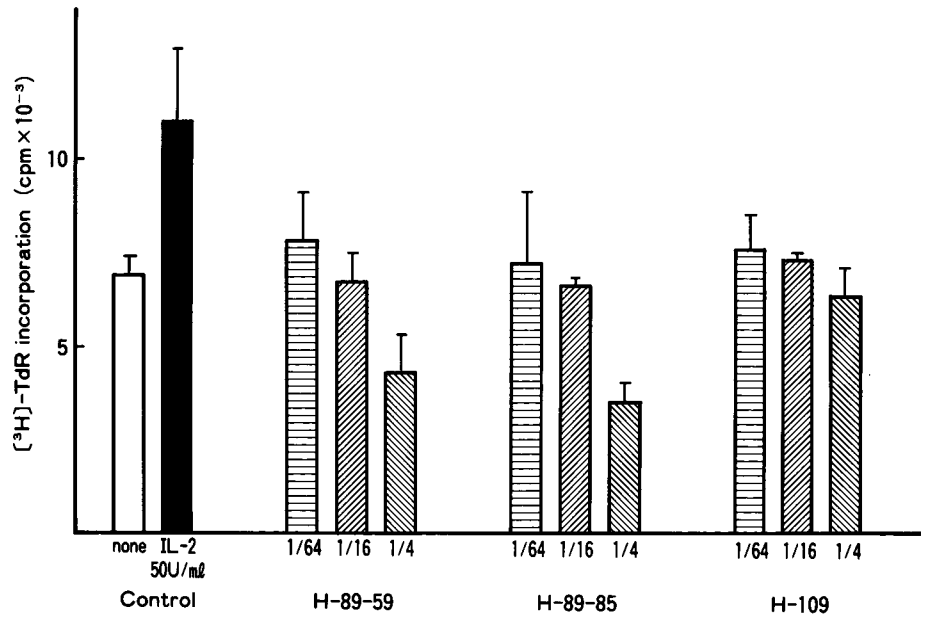

Figure 3.Il-2 activity was assayed by measuring methyl-[³H] TdR incorporation using the human IL-2-dependent T-cell line.Sez 627,as a target cell. Sez 627 cells were incubated with serially diluted supernatants of the H-89-59,H-89-85 and H-109 cell lines in 100 l of culture medium with 5% FCS at 37 C. After 24 hour, 0.2 Ci of methyl-[³H] TdR per 200 l was added for another 6 hours. Cells were harvested using a cell harvester and counted using a liquid scintillation counter. Statistical difference(vs. control) is shown by asterisks:* $p<0.5$.

Effects of steroid hormones and CsA to gene expression

C-myc mRNA was expressed in both H-89-59, insensitive to the reagents and H-109, sensitive to them. In H-109, the level of c-myc expression was inhibited by steroid hormone and CsA wheras in H-89-59 they did not (Fig.4).

HTLV-I RNA was also expressed in both cell line. However the level of HTLV-I gene expression was not affected by these reagents in both cell line (Fig.5).

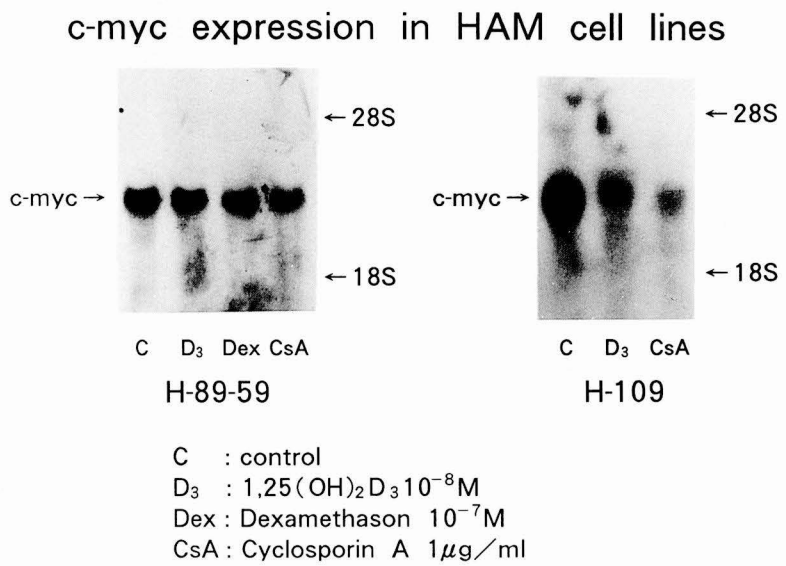

Figure 4. Northern blot analysis of total RNA from H-89-59 and H-109 cells probed with c-myc specific c-DNAs. The lane indicates as described in the figure.

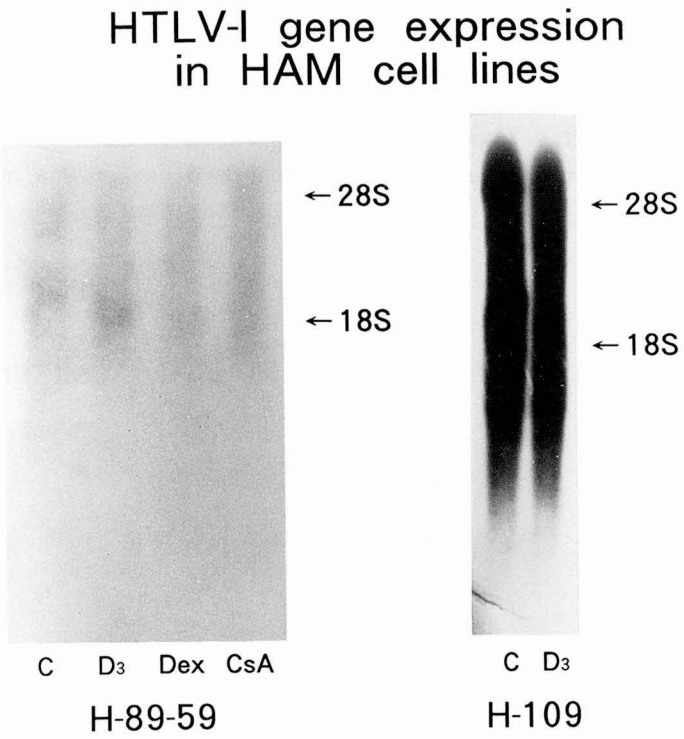

Figure 5. Northern blot analysis of total RNAs from H-89-59 and H-109 cells probed with HTLV-I specific c-DNAs. The lanes indicate ; c:control, D_3:1,25$(OH)_2D_3$ 10^{-8} M, dex:dexamethason 10^{-7} M , CsA:Cyclosporin A 1ng/ml.

DISCUSSION

Both DNA and RNA viruses are capable of immortalizing proliferation of human lymphocytes. For incetance, Epstein-Barr viruses and HTLV-I viruses can immmortalize human B and T cells,respectively. Furthermore,co-culture of irradiated HTLV-I-positive T cells with normal human lymphocytes is known to cause cellular proliferation of in vitro HTLV-I-infected T cells.[7]

Although there are several similarities between in vitro HTLV-I-infected T cells and fresh leukemic T cells from ATL patients,these HTLV-I-infected T cells do differ from leukemic cells.[19] T cell abnormalities in HAM patients are not leukemic changes and are controllable by glucocorticoids. Thus,it is of importance to elucidate the molecular and genetic differences between HTLV-I-infected T cells in HAM patients and leukemic T cells in ATL patients.

Human T cells,activated with mitogens, antigens or antibodies to the T_3-T_i complex(CD3 protein-T cell receptor complex), acquire a cascade of new receptors,including those for stimulatory factors such as IL-2,transferrin,or insulin-like growth factors(type 1,type 2), or those for inhibitory factors such as $1,25(OH)_2D_3$ or glucocorticoids.[1,3,13,25,31] These receptors are expressed only in low levels ,if at all,on resting T cells. It seems likely therefore,that they are associated with the regulatory mechanisms of T cell proliferation. In addition,activated T cells secrete many lymphokines such as IL-2, γ-intrrferon and so on.

In contrast to normal resting T cells ,HTLV-I-infected fresh leukemic T-cells and T cell lines from patients with ATL continuously express IL-2R without production of IL-2.[29] In a previous study ,we demonstrated the increased sensitivity of a HTLV-I-infected-T cell line from a patient

with ATL (ATL cell line) to the steroid hormone, $1,25(OH)_2D_3$, may have been associated with phenotypic resemblances between activated T cells and ATL cell lines.[8] HTLV-I-infected leukemic T cells may have receptors not only for stimulatory factors but also for inhibitory factors such as steroid hormones as demonstrated in PHA-activated T cells.

In this study, we investigated 4 HTLV-I-infected T cell lines from patients with HAM. All of 4 T cell lines are $T4^+$ and express IL-2R and HLA-DR antigen. These phenotypes are similar to PHA-activated $CD4^+$ T cells and ATL T cell lines. These 4 HAM T cell lines ,however,exhibite heterogeneous sensitivity to dexamethasone, $1,25(OH)_2D_3$, and CsA. H-89-79,H-89-85 and H-109 are inhibited in their de novo DNA synthesis by all three reagents whreas H-89-59 is not. This could reflect the possibility that HTLV-I infection may cause heterogeneous stages in T cell immortalization or transformation. Moreover and differing from ATL T cell lines, these cell lines are not monoclonal but oligoclonal cell populations,although they are certainly infected by HTLV-I.[16,26] In this regard, it is possible that these cell lines represent various stages of T cell transformation by HTLV-I.

The fact that ,among these 4 HAM T cell lines,H-89-59 is not inhibited by $1,25(OH)_2D_3$, dexamethasone or CsA whereas the other 3 cell lines are,seem to suggest the presence of heterogeneous processes in cell transformation by HTLV-I and T cell activation by antigens.

Even the H-89-59 cell line, which is insensitive to steroid hormones and CsA,has a high percentage of IL-2R but no detectable IL-2 production. These results suggest that H-89-59 cell line may be an intermediate type of T cell transformation by HTLV-I. In this type of T cell transformation,immortalized T cells have receptors for

stimulatory factors but are insensitive to inhibitory factors such as steroid hormones and CsA. In contrast,activated T cells are sensitive to both stimulatory and inhibitory factors and express the corresponding receptors.[13] The other three cell lines could be more similar to activated T cells.

The effect of CsA on these 4 HAM T cell lines is similar to that of steroid hormones. Although the mechanism of the inhibitory effect of CsA on T cells is not completely clarified, it may involve the signal pathways of IL-2 activity in certain later steps.[2] Hence,it is important to clarify the kinetics of the inhibition of de novo DNA synthesis by both CsA and steroid hormones in these HAM T cell lines. Since the inhibitory effect arose at nearly the same time using three agents,a common mechanism may exist. In order to elucidate the precise pharmacological actions of these three reagents on HAM T cells,furthur studies are presently underway in our laboratory.

It is worthwhile to focus attention on whether the growth inhibition of these reagents is directly related to the regulatory mechanisms of HTLV-I gene. The transcriptional activating mechanism of p40(x) produced by the HTLV-I gene existing characteristic pX region has been thought to be one of the major biological effects. In a previous study on a HTLV-I-infected ATL cell line,KH-2 as well as both dexamethason and $1,25(OH)_2D_3$ remarkably inhibited cellular proliferation but did not reduce the mRNA levels of HTLV-I ,IL-2R and T cell receptor gene.[12] Unexpectedly,these steroid hormones supressed c-myc mRNA levels in KH-2 cells.[12] For these reasons we investigated the effects of steroid hormones and CsA on the mRNA levels of proliferation-associated genes in HAM T-cell lines. Cellular proliferation of H-109 was remarkably inhibited by $1,25(OH)_2D_3$ but the mRNA level of HTLV-I was not reduced.

Whereas $1,25(OH)_2D_3$ supressed c-myc mRNA level in H-109. On the other hand,in H-89-59 cell line steroid hormones and cyclosporin A did not inhibit cellular proliferation and did not reduce the mRNA level both of c-myc and HTLV-I. This suggest that these three agents inhibit cellular proliferation not by inhibiting the p40(x) expression but some transcriptional factors such as c-myc.

In the pathogenesis of HAM or ATL among HTLV-I infected persons is not throughly elucidated,since there are a number of nonsymptomatic HTLV-I carriers.
Additional co-factors,therefore,are thought to be important in these HTLV-I causing disorders. Regarding this point,it seems logical to investigate the molecular and genetic characteristics of these HAM T cell lines so as to clarify the method of T cell immortalization and transformation by HTLV-I in comparison with that of T cell activation by PHA ,antigens or antibodies to T_3-T_i complex.

ACKNOWLEDGEMENTS

We are most grateful to Dr.Takahiko Saida for providing HAM T-cell lines,Dr.Michiyuki Maeda for providing Sez 627 cell line
and Dr. Takashi Uchiyama for giving the monoclonal antibody Tac.

We thank Mr.D.Mrozek for his critical reading.

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