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Human T-Cell Leukemia Virus Type I-Induced Proliferation of Human Immature $CD2^{+}CD3^{-}$ Thymocytes

VERONIQUE MAGUER, HUGUETTE CASSÉ-RIPOLL, LOUIS GAZZOLO, AND MADELEINE DUC DODON*

Immuno-Virologie Moléculaire et Cellulaire, UMR30, Centre National de la Recherche Scientifique/Université Claude Bernard Lyon I, Faculté de Médecine A. Carrel, 69372 Lyon Cedex 08, France

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The mitogenic activity of human T-cell leukemia virus type I (HTLV-I) is triggering the proliferation of human resting T lymphocytes through the induction of the interleukin-2 (IL-2)/IL-2 receptor autocrine loop. This HTLV-I-induced proliferation was found to be mainly mediated by the CD2 T-cell antigen, which is first expressed on double-negative lymphoid precursors after colonization of the thymus. Thus, immature thymocytes express the CD2 antigen before that of the CD3-TCR complex. We therefore investigated the responsiveness of these $CD2^{+}CD3^{-}$ immature thymocytes and compared it with that of unseparated thymocytes, containing a majority of the $CD2^{+}CD3^{+}$ mature thymocytes, and that of the $CD2^{-}CD3^{-}$ prothymocytes. Both immature and unseparated thymocytes were incorporating [^{3}H]thymidine in response to the virus, provided that they were cultivated in the presence of submitogenic doses of phytohemagglutinin. In contrast, the prothymocytes did not proliferate. Downmodulation of the CD2 molecule by incubating unseparated and immature thymocytes with a single anti-CD2 monoclonal antibody inhibited the proliferative response to HTLV-I. These results clearly underline that the expression of the CD2 molecule is exclusively required in mediating the proliferative response to the synergistic effect of phytohemagglutinin and HTLV-I. Immature thymocytes treated with a pair of anti-CD2 monoclonal antibodies were shown to proliferate in response to HTLV-I, even in the absence of exogenous IL-2. We further verified that the proliferation of human thymocytes is consecutive to the expression of IL-2 receptors and the synthesis of IL-2. These observations provide evidence that the mitogenic stimulus delivered by HTLV-I is more efficient than that provided by other conventional mitogenic stimuli, which are unable to trigger the synthesis of endogenous IL-2. Collectively, these results show that the mitogenic activity of HTLV-I is able to trigger the proliferation of cells which are at an early stage of T-cell development. They might therefore represent target cells in which HTLV-I infection could favor the initiation of the multistep lymphoproliferative process leading to adult T-cell leukemia.

Since its detection, the first human pathogenic retrovirus has been considered as the etiological agent of adult T-cell leukemia (ATL) and was later found to be linked to the development of a neurological disease, i.e., tropical spastic paraparesis or human T-cell leukemia virus type I (HTLV-I)-associated myelopathy (12, 13, 26, 37). So far, no difference has been detected between HTLV-I isolated from patients suffering either from ATL or tropical spastic paraparesis or HTLV-I-associated myelopathy, underlining that the same virus is causing two different diseases (4, 20, 21). Furthermore, proviral expression is very high in circulating mononuclear cells from tropical spastic paraparesis or HTLV-I-associated myelopathy patients and directly linked to the progression of the disease, whereas it is silent in leukemic cells from ATL patients (3, 14, 24, 38). This observation suggests that early events are of main importance in unravelling the pathophysiological mechanisms involved in the leukemic process. The conclusion can therefore be drawn that time and route of infection should be considered to explain the emergence of these two different diseases linked to the same virus. Of particular interest are reports that ATL development is preferentially linked to neonatal infection, via mother's milk, thus suggesting that infection of specific target cells infected at this time may be directly correlated with the development of the leukemia several decades later (24).

* Corresponding author.

As indicated by previous studies, the role of HTLV-I gene products may be of importance in stimulating the proliferation of quiescent target T cells. Thus, we have shown that HTLV-I virions concentrated from conditioned media of HTLV-I-producing T cells behave as mitogens for human T lymphocytes by triggering the autocrine interleukin-2 (IL-2) receptor/IL-2 growth loop (9, 11). In addition, mitogenic stimulation by noninfectious HTLV-I virions purified by sucrose density centrifugation was shown to induce the production of large quantities of human immunodeficiency virus type 1 (HIV-1) by human peripheral blood leukocytes infected with human immunodeficiency virus type 1 (39). Furthermore, an HTLV-producing T-cell line was shown to induce an IL-2-dependent early proliferation of T cells (18). Finally, HTLV-I-infected T-cell clones were found to stimulate noninfected cells (36). Taken together, these observations indicating that infection of these target cells was not a prerequisite for T-cell activation revealed the mitogenic activity of HTLV-I for T lymphocytes. This initial proliferation may favor the intervention of the transactivator Tax protein in activating proviral transcription as well as in upregulating genes associated with T-cell activation (2, 28–30, 34). Finally, we and others have demonstrated that HTLV-I is interfering with the activation pathway mediated via the CD2 molecule but not with the pathway initiated by the CD3–T-cell receptor complex (8, 17, 36).

After colonization of the thymus by $CD2^{-}CD3^{-}$ lymphoid precursors which proliferate and rearrange their T-cell receptor gene, CD2 is one of the first surface antigens ex-

pressed on these cells. In the $CD2^+CD3^-$ immature thymocytes, α and β CD3 mRNAs are translated but not yet expressed on their membrane (16). Therefore, we decided to investigate the susceptibility of these immature thymocytes to the mitogenic activity of HTLV-I.

Here, we present evidence that HTLV-I virions in the presence of a nonmitogenic cosignal are able to trigger the proliferation of $CD2^+CD3^-$ immature thymocytes and that this proliferation is consecutive to the stimulation of the autocrine loop IL-2/IL-2 receptor. These results, which again underline the pivotal role of the CD2 pathway in HTLV-I-mediated T-cell activation, suggest that immature thymocytes represent target cells in which HTLV-I infection could favor the induction of events leading to the development of the leukemic disease.

MATERIALS AND METHODS

MAbs and reagents. Monoclonal antibodies (MAbs) directed to the CD3, the CD8, the CD4 (IOT4a), the CD1a, and the CD19 cell surface antigens and nonreactive MAbs of the relevant isotype (immunoglobulin G1 [IgG1] or IgG2a) were all purchased from Immunotech (Luminy, France). Other MAbs, anti-CD8, anti-CD25 (IL-2 receptor), and anti-CD1a were purchased from Dako (Copenhagen, Denmark), together with the MAbs anti-CD2-PE (phycoerythrin), anti-CD4-PE, and anti-CD3-fluorescein isothiocyanate. Antibodies to the CD2 epitopes (D66, X11, O275) used in previous studies were kindly provided by A. Bernard (Nice, France) (8). Human anti-CD25 blocking antibody (p55 chain of the human IL-2 receptor, IOT14a) was obtained from Immunotech and was used at a final concentration of 4 μg/ml.

Human recombinant IL-2 was obtained from Boehringer (Mannheim, Germany) and was used at a final concentration of 25 U/ml. Phytohemagglutinin (PHA) was obtained from Welcome (Dratford, Great Britain) and was used at a final concentration of 3 to 20 μg/ml. Cyclosporin A (CsA) was obtained from Sandoz Pharmaceuticals (Basel, Switzerland), was dissolved in ethanol (10 mg/ml), and was diluted in RPMI medium to a stock solution of 50 mg/ml. CsA was used at a final concentration of 0.2 μg/ml.

Virus preparation. The 48-h culture media of the HTLV-I-producing C91/PL cells seeded at 2.5×10^5 cells per ml (27) were collected, centrifuged at low speed, and then concentrated by centrifugation for 2 h at $32,000 \times g$ with a type 70 Ti rotor in an L6 centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The pellet was resuspended in an appropriate volume of RPMI medium to obtain a 20-fold-concentrated viral preparation, 50 μl of which was added to each well. This preparation was shown to be mitogenic for human resting peripheral T lymphocytes (11), as indicated by thymidine incorporation values ranging from 20,000 to 30,000 cpm.

Cell preparations. Thymic tissue was obtained from normal children ranging in age from 5 months to 2 years and undergoing reparative cardiac surgery at the Neuro-Cardiologic Hospital (G. Champsaur, Lyon, France). A single cell suspension, prepared by teasing thymus fragments over a stainless steel mesh, was then centrifuged over Ficoll-Hypaque and extensively washed. Immature thymocytes were prepared as described by Deans et al. (6). The thymocyte suspension (10^8 cells per ml) was incubated in a mixture of anti-CD3, anti-CD4, anti-CD8 (2 ng/ml), anti-CD1a (1 ng/ml), and anti-CD19 (0.2 ng/ml) MAbs for 30 min at 4°C. After two extensive washings, cells were suspended in a solution of sheep anti-mouse IgG-coated magnetic beads (Dynabeads M450; Dynal, Oslo, Norway) at a bead/cell ratio of 2/1. After incubation and gentle mixing for 30 min at 4°C, the beads were pelleted with a magnet and the supernatant containing the negatively selected cells was collected. When necessary, one or two other cycles with magnetic beads were performed. To isolate the prothymocyte population, the thymocyte suspension (10^8 cells per ml) was first treated with anti-CD3 MAb (1 ng/ml) for 30 min at 4°C and, after washing, was incubated with magnetic beads at a bead/cell ratio of 2/1, and then unbound cells (2×10^6 cells per ml) were incubated with anti-CD3 (2 ng/ml) and anti-CD8 (1 ng/ml) MAbs (one bead per cell). Finally, cells were incubated with anti-CD8 and anti-CD4 (2 ng/ml) MAbs (two beads per cell).

Proliferation assays. Thymocytes were seeded in triplicate at a ratio of 3×10^5 cells per well of a 96-well flat-bottomed microdilution plate (Costar) in 150 μl of RPMI 1640 supplemented with 10% fetal calf serum, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 5 mM L-glutamine, 100 U of penicillin per ml, and 50 μg of streptomycin per ml in the absence or presence of the different stimuli. Proliferation was assessed 4 days later. Cells were pulsed during the last 6 h with 0.5 μCi of [^{3}H]thymidine per well (specific activity, 6.7 Ci [248 GBq]/mmol; NEN, Boston, Mass.) and then were collected with an automatic cell harvester (Skatron, Lier, Norway). Radioactivity was measured in a liquid scintillation counter. Data are reported as means of triplicate cultures ± standard deviation.

Flow cytometry analysis of cell surface molecules. Thymocytes were stained for 1 h at 4°C with the MAb described above. They were then washed and fixed with 2% paraformaldehyde. Cell surface fluorescence was analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.). Integrated fluorescence of the gated population was measured and data from 5,000 analyzed events were collected.

Assay for IL-2 activity. Thymocytes were cultured for 4 days in 24-well plates in the presence of the relevant stimulus. The culture medium was first centrifuged at 13,000 rpm for 10 min and then was assayed for IL-2 content by its ability to sustain the proliferation of the murine IL-2-dependent cell line CTLL-2 (15). Units of IL-2 were measured by using a human recombinant IL-2 as a standard.

RESULTS

HTLV-I induces human thymocyte proliferation in the presence of submitogenic doses of PHA. Prior studies have indicated that HTLV-I particles obtained after centrifugation of culture media of HTLV-I-producing T-lymphoblastoid cells are able to induce the proliferation of human peripheral blood T lymphocytes, even in the absence of accessory cells (11). In addition, we have shown that HTLV-I virions failed to stimulate proliferation of human thymocytes. However, the combination of HTLV-I virions and submitogenic doses of PHA was found to trigger the proliferation of these cells (10). We have confirmed these data and have shown that the thymocyte proliferative response to both HTLV-I and PHA became evident by day 2, peaked on day 4, and persisted until day 6 (Fig. 1A). Proliferation induced by HTLV-I alone or PHA alone was never significantly higher than background values. As expected, PHA at a concentration of 100 μg/ml was by itself sufficient to activate thymocytes. The effects of several submitogenic PHA concentrations (from 3 to 20 μg/ml) were determined. As shown in Fig. 1B, it was found that the optimal thymocyte proliferation induced by

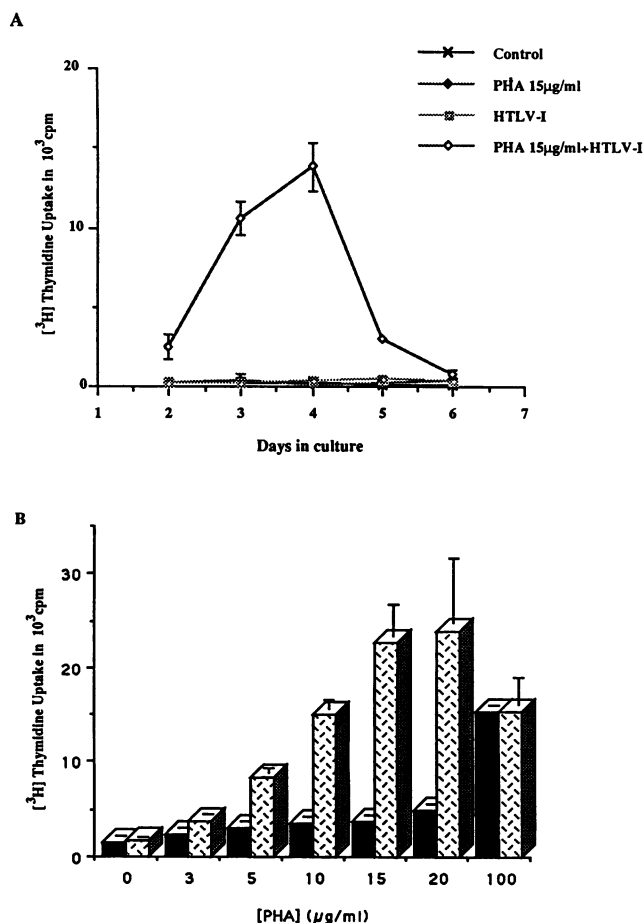

FIG. 1. Synergistic effect of PHA and HTLV-I on human thymocyte proliferation. (A) Kinetics analysis of thymocyte proliferation. Thymocytes, prepared as described in Materials and Methods, were seeded (3×10^5 cells per well of a 96-well plate) and then were incubated in the presence of HTLV-I and/or PHA at a final concentration of 15 μg/ml for 6 days. Proliferative responses were measured by [^{3}H]thymidine incorporation for 6 h. (B) Concentration-dependent effect of PHA on DNA synthesis by thymocytes stimulated by HTLV-I. Thymocytes (3×10^5 cells per well) were cultured for 4 days in the presence of the indicated concentrations of PHA either alone (solid bars) or in presence of HTLV-I virions (hatched bars). [^{3}H]thymidine uptake was assessed on day 4 of the culture during the last 6 h. Results are expressed as mean counts per minute ± standard deviations of triplicate cultures and are representative of more than six experiments with thymic cells from four different donors.

HTLV-I and measured on day 4 was observed in the presence of 15 μg/ml, as indicated by a sixfold increase in thymidine incorporation. These experiments clearly indicate that HTLV-I is able to stimulate thymocyte proliferation when these cells are cultivated with submitogenic doses of PHA.

To document this synergistic effect, additional experiments were then performed, in which thymocytes were cultured either with PHA or with HTLV-I for 2 days, at which time either HTLV-I or PHA, respectively, was added. Proliferative responses were then measured on day 4 after the onset of the culture and compared with those obtained when both HTLV-I and PHA were added simultaneously to the cultures. These results (Table 1) clearly indicated that

TABLE 1. Effect of time of addition of PHA or HTLV-I on thymocyte proliferation[a]

Stimulus addition on[b]:		Mean [^{3}H]thymidine incorporation[c]	
Day 0	Day 2	cpm ± SD	SI[d]
Medium	Medium	109 ± 56	1
Medium	HTLV-I	60 ± 8	1
HTLV-I	Medium	251 ± 126	2
Medium	PHA	213 ± 30	2
PHA	Medium	426 ± 40	4
PHA	HTLV-I	10,204 ± 676	94
HTLV-I	PHA	12,256 ± 3,760	115
HTLV-I + PHA	Medium	8,290 ± 241	76
Medium	HTLV-I + PHA	4,341 ± 1,268	40

[a] Human thymocytes, prepared as described in Materials and Methods, were seeded at 3×10^5 cells per 150 μl per well of a 96-well microdilution plate.
[b] PHA at a final concentration of 15 μg/ml and/or HTLV-I was added to the thymocyte cultures at the indicated times.
[c] Thymocyte proliferation was monitored on day 4 by measuring the [^{3}H]thymidine uptake during the last 6 h of the culture. Experiments were done in triplicate.
[d] SI, stimulation index, represents the ratio of the thymidine incorporation measured in stimulated cultures with respect to that measured in the unstimulated cultures.

the delayed addition of either PHA or HTLV-I did not significantly alter the extent of the proliferative thymocyte response to the synergistic effect of both stimuli.

It has been shown that thymocytes are able to weakly proliferate in response to IL-2 (31, 32). We therefore tested the ability of this lymphokine to act synergistically with the virus. The experiment was performed in a fashion similar to the one described above: the virus was added either 2 days before IL-2 or 2 days after the cells had been incubated with IL-2. As shown in Table 2, thymocytes were able to proliferate only when IL-2 was added at the culture onset. Under these conditions, HTLV-I added either at the same time or 2 days after IL-2 led to a 6.7- or a 3.4-fold increase in thymocyte proliferation, respectively. Because no proliferation was observed when the addition of IL-2 was delayed and because the time of addition of HTLV-I did not matter, these results suggest that a human thymocyte subset expressing the IL-2 receptor is responsive to the synergistic effect of the lymphokine and of the virus.

TABLE 2. Effect of time of IL-2 or HTLV-I addition on thymocyte proliferation[a]

Stimulus addition on[b]:		Mean cpm of [^{3}H]thymidine incorporation ± SD[c]
Day 0	Day 2	
IL-2	Medium	624 ± 166
IL-2	HTLV-I	2,124 ± 71
HTLV-I	IL-2	395 ± 60
HTLV-I + IL-2	Medium	4,174 ± 385
Medium	HTLV-I + IL-2	121 ± 51

[a] Human thymocytes, prepared as described in Materials and Methods, were seeded at 3×10^5 cells per 150 μl per well of a 96-well microdilution plate.
[b] IL-2 at a final concentration of 25 U/ml or HTLV-I was added to the thymocyte cultures at the indicated times.
[c] Thymocyte proliferation was monitored on day 5 by measuring the [^{3}H]thymidine uptake during the last 6 h of the culture. Experiments were done in triplicate.

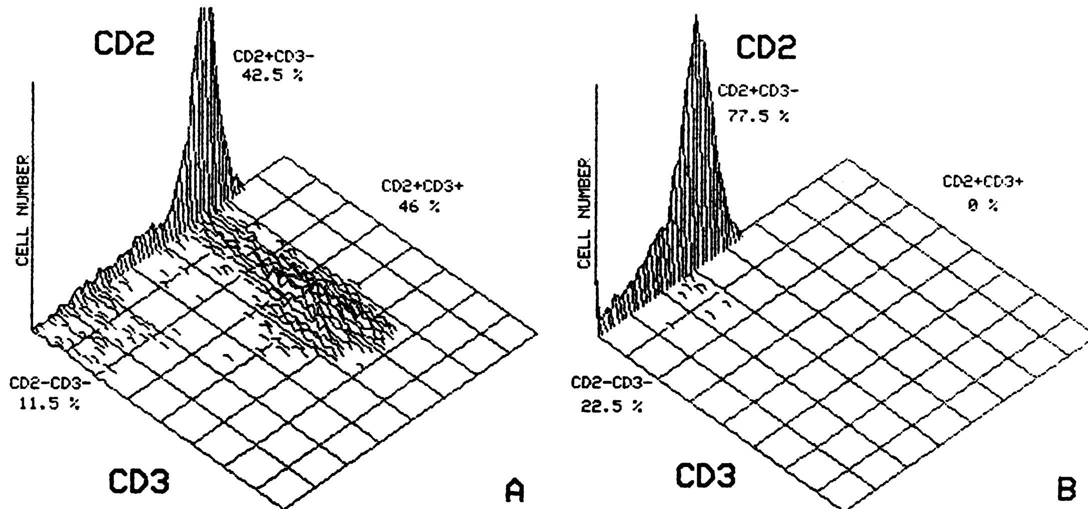

FIG. 2. Two-color immunofluorescence cell surface analysis of the distribution of CD2 and CD3 antigen on unseparated and immature thymocytes. Thymocytes were stained with anti-CD2-PE and anti-CD3-fluorescein isothiocyanate. (A) Unseparated thymocytes. (B) Immature thymocytes selected after incubation with the appropriate MAbs, and immunomagnetic beads, as described in Materials and Methods.

HTLV-I induces the proliferation of $CD2^+CD3^-$ immature thymocytes. The experiments described above were performed with unseparated thymocytes. In this thymocyte population, three subsets were identified when the expression of either CD2 or CD3 antigens on the surface of these cells was analyzed by flow cytometry. As shown in Fig. 2A, in the unseparated population, 46% of the thymocytes were expressing both CD2 and CD3; 42.5% were only expressing CD2: they were then referred to as $CD2^+CD3^-$ immature thymocytes; and 11.5%, referred to as prothymocytes, were $CD2^-CD3^-$ cells. The question of whether immature $CD2^+CD3^-$ thymocytes will also proliferate in response to the mitogenic activity of HTLV-I was then raised. To answer it, unseparated thymocytes were then fractionated by negative selection with specific MAbs and anti-IgG-coated magnetic beads. A $CD2^+CD3^-$ immature thymocyte subset (in which $CD2^+CD3^+$ cells could not be detected) (Fig. 2B) and a subset characterized by the presence of a majority of $CD2^-CD3^-$ (about 68%) prothymocytes were thus isolated.

These three thymocyte populations—unseparated, mature, and prothymocytes—were then incubated in the presence of both PHA and HTLV-I. Controls included the same cultures that received either HTLV-I or PHA. As shown in Fig. 3, thymidine incorporation (1,476 ± 436 cpm) in the cultures containing a majority of prothymocytes showed that this population could not be stimulated when incubated in the presence of HTLV-I and PHA. In contrast, not only unseparated thymocytes, as expected, but also the $CD2^+CD3^-$ immature thymocytes were found to proliferate in response to the mitogenic activity of HTLV-I in the presence of PHA. The level of thymidine incorporation was about twofold less in the immature thymocytes than in the unseparated thymocyte cultures (7,229 ± 2,012 versus

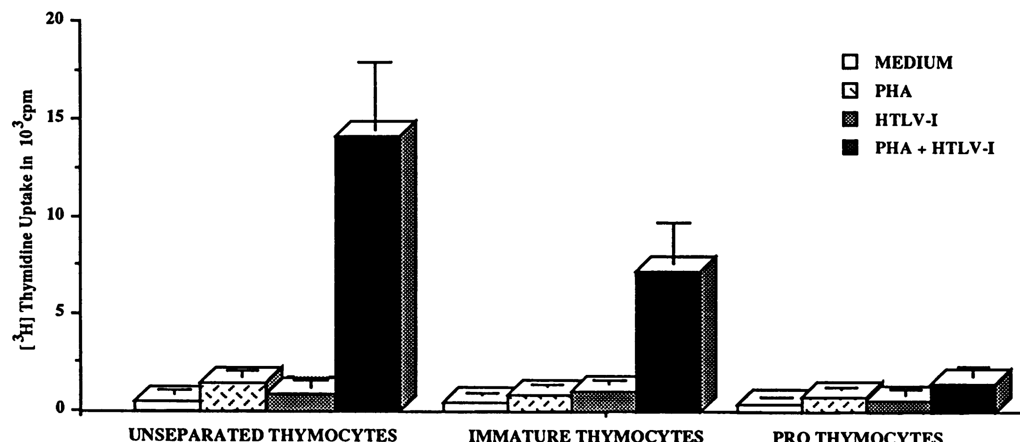

FIG. 3. Synergistic effect of PHA and HTLV-I virions on different thymocyte subpopulations. Thymocytes (3×10^5 cells per well) were cultured for 4 days in complete medium, or in the presence of HTLV-I alone, PHA alone at a final concentration of 15 μg/ml, or HTLV-I and PHA. After 4 days in culture, [^{3}H]thymidine uptake was measured during the last 6 h. Results are expressed as mean counts per minute ± standard deviations of triplicate cultures and are representative of four experiments with thymic cells from three different donors.

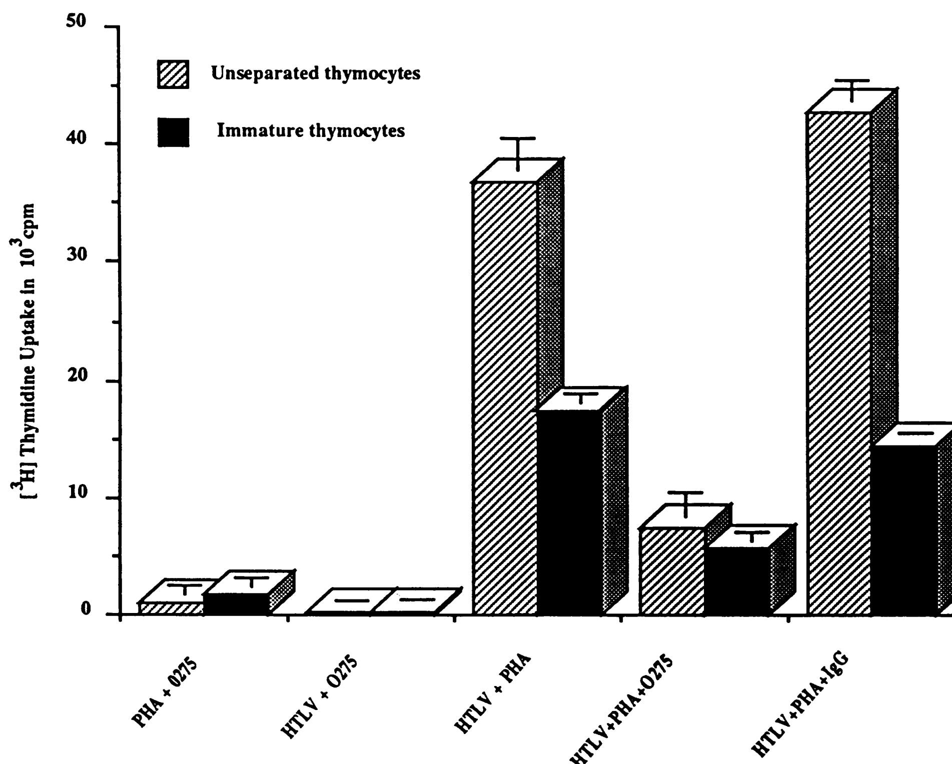

FIG. 4. Effect of the anti-CD2 (O275) MAb on HTLV-I-induced thymocyte proliferation. Unseparated and immature thymocytes (3×10^5 cells per well) were cultured for 4 days. PHA was added at a final concentration of 10 μg/ml, and O275 was used at a final dilution of 1/500,000. [^{3}H]thymidine incorporation was performed during the last 6 h. Results are expressed as mean counts per minute ± standard deviations of triplicate cultures and are representative of three experiments with thymic cells from two different donors.

14,175 ± 3,405 cpm), indicating that the presence of $CD2^+CD3^+$ cells in the latter population was conferring a higher responsiveness to the mitogenic activity of HTLV-I and PHA. More importantly, these results demonstrate that the presence of a functional CD2 molecule at a time when CD3 is not yet functional appears to be necessary and sufficient to mediate the proliferation of thymocytes in response to HTLV-I.

To further verify the critical involvement of the CD2 molecules in this event, thymocytes were incubated with PHA and HTLV-I, in the presence of the O275 MAb directed against one epitope of the CD2 antigen, which was previously shown to inhibit the HTLV-I-mediated proliferation of peripheral blood T lymphocytes (8). Under these conditions, thymidine incorporation was decreased by threefold in unseparated thymocytes and fivefold in immature thymocytes over that observed with thymocytes incubated with PHA and HTLV-I (Fig. 4).

We next tested the ability of IL-2 to act synergistically with the virus to induce immature thymocyte proliferation. Virus was added either 2 days before IL-2 or 2 days after the cells had been incubated with IL-2. As shown in Table 3, immature thymocytes were able to proliferate as long as IL-2 was added at the culture onset. Under these conditions, HTLV-I added at the same time led to a threefold increase in thymocyte proliferation. In contrast, when it was added 2 days later, thymocyte proliferation was not significantly increased. This observation (correlated with that found with unseparated thymocytes) suggests that a small subset of $CD2^+CD3^-$ immature cells bearing the IL-2 receptor is responding to the synergistic effect of IL-2 and virions when added together at the culture onset.

Collectively, these results provide evidence that HTLV-I-induced proliferation is linked to the expression of the CD2 molecule during thymocyte maturation.

Effect of CD2 triggering of thymocytes on the mitogenic activity of HTLV-I. Previous studies have shown that immature thymocytes exposed to various mitogenic stimuli are always requiring the addition of IL-2 in order to proliferate

TABLE 3. Effect of time of IL-2 or HTLV-I addition on immature thymocyte proliferation[a]

Stimulus addition on[b]:		Mean cpm of [^{3}H]thymidine incorporation ± SD[c]
Day 0	Day 2	
IL-2	Medium	2,997 ± 471
IL-2	HTLV-I	3,596 ± 870
HTLV-I	IL-2	1,708 ± 788
HTLV-I + IL-2	Medium	7,847 ± 1,430
Medium	HTLV-I + IL-2	418 ± 110

[a] Human immature thymocytes, prepared as described in Materials and Methods, were seeded at 3×10^5 cells per 150 μl per well of a 96-well microdilution plate.

[b] IL-2 at a final concentration of 25 U/ml or HTLV-I was added to the thymocyte cultures at the indicated times.

[c] Thymocyte proliferation was monitored on day 5 by measuring the [^{3}H]thymidine uptake during the last 6 h of the culture. Experiments were done in triplicate.

TABLE 4. Effect of anti-CD2 MAbs on thymocyte proliferation in response to HTLV-I virions[a]

Stimulus (final dilution)[b]	Virus	Mean cpm of [^{3}H]thymidine incorporation in[c]:	
		Unseparated thymocytes	Immature thymocytes
Anti-CD2 (1/10,000)	−	391	92
	+	403	102
Anti-CD2 (1/400)	−	8,658	1,501
	+	40,670	10,084
None	−	183	106
	+	135	112

[a] Thymocytes, prepared as described in Materials and Methods, were seeded at 3 × 10^5 cells per 150 μl per well of a 96-well microdilution plate.
[b] Anti-CD2 MAbs (D66 and X11) were used at the indicated final dilution of ascites fluids and were added at the onset of the cultures.
[c] Thymocyte proliferation was monitored on day 4 by measuring the [^{3}H]thymidine uptake during the last 6 h of the culture. Experiments were done in triplicate.

(5, 7, 25, 33). That was particularly observed with a pair of anti-CD2 MAbs (directed to two distinct epitopes of the CD2 molecule) which were inducing IL-2 receptor expression on immature thymocytes but were unable to induce IL-2 secretion (5). We next determined whether HTLV-I could synergize with anti-CD2 MAbs and trigger the proliferation of human immature thymocytes. Our results (Table 4) show that when anti-CD2 MAbs were added at a final dilution of 1/10,000, unseparated and immature thymocytes failed to proliferate even after addition of HTLV-I. However, when anti-CD2 MAbs were added at a lower dilution (1/400), both subsets were weakly proliferating. Furthermore, the addition of HTLV-I virions increased this proliferation by a factor of 4.7 for the unseparated thymocytes and by a factor of 6.7 for the immature thymocytes. These results underline that triggering the CD2 molecules of immature thymocytes is able to reveal the mitogenic effect of HTLV-I on these cells.

HTLV-I triggers the autocrine IL-2/IL-2 receptor loop in immature thymocytes. When incubated with high PHA concentrations, immature thymocytes were found to proliferate consecutively in response to the induction of the autocrine IL-2/IL-2 receptor loop (5). We therefore verified whether the proliferation of immature thymocytes incubated with submitogenic doses of PHA and with HTLV-I virions may be a consequence of the induction of this autocrine mechanism. HTLV-I, when added alone, failed to trigger IL-2 receptor (CD25) expression, while PHA (5 μg/ml) alone is able to induce it on about 10% of these cells. Furthermore, neither HTLV-I nor PHA, when added separately, induced IL-2 synthesis (Fig. 5). However the simultaneous addition of the lectin and of the virus led to the expression of IL-2 receptor on about 60% of the cells 4 days later and the synthesis of 4.5 U of IL-2 by the same cultures. These results clearly indicate that HTLV-I virions, in the presence of PHA, are able to trigger immature thymocyte proliferation by upregulating the expression of IL-2 receptors and by inducing IL-2 synthesis.

To further demonstrate that the autocrine IL-2/IL-2 receptor loop is directly involved in the mitogenic activity of HTLV-I mediating the proliferation of CD2-triggered thymocytes, we first added to the cultures a MAb to the α chain of the IL-2 receptor (anti-CD25). As illustrated in Fig. 6, this antibody inhibited the proliferation of thymocytes when these cells were incubated with HTLV-I in the presence of a

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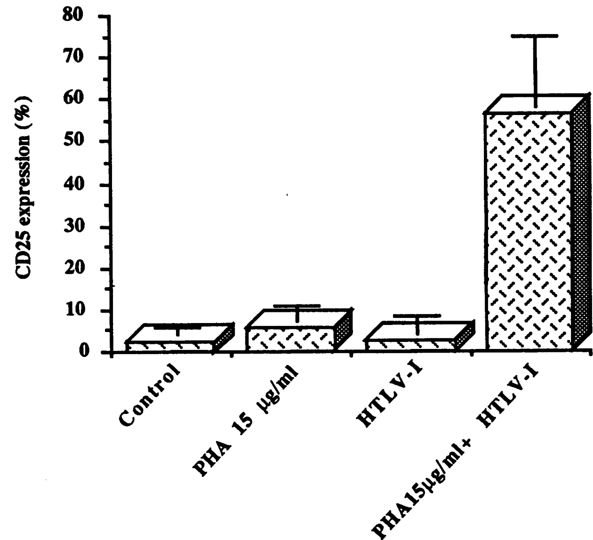

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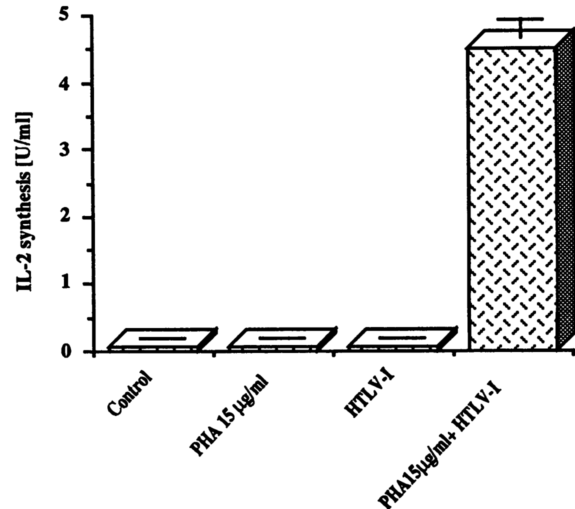

FIG. 5. Effect of HTLV-I and/or PHA on CD25 expression and IL-2 synthesis by immature thymocytes. (A) Immature thymocytes (3 × 10^5 cells per well) were incubated in the presence of PHA at a final concentration of 10 μg/ml and/or HTLV-I. After 4 days of culture, cells were stained and expression of CD25 was analyzed by flow cytometry. (B) Culture media were tested for IL-2 content with the murine CTLL-2 cell line, as described in Materials and Methods. Results are expressed as mean counts per minute ± standard deviations of triplicate cultures and are representative of more than four experiments with thymic cells from three different donors.

MAb to CD2. Next, thymocytes triggered by the same mitogenic stimuli were incubated in the presence of CsA, known to inhibit IL-2 synthesis. As shown in Fig. 6, thymocyte proliferation was found to significantly decrease. In addition, thymidine incorporation was increased when exogenous IL-2 was added to the CsA-treated cultures. Similar results were obtained with PHA–HTLV-I costimulations (data not shown). Taken together, these results clearly

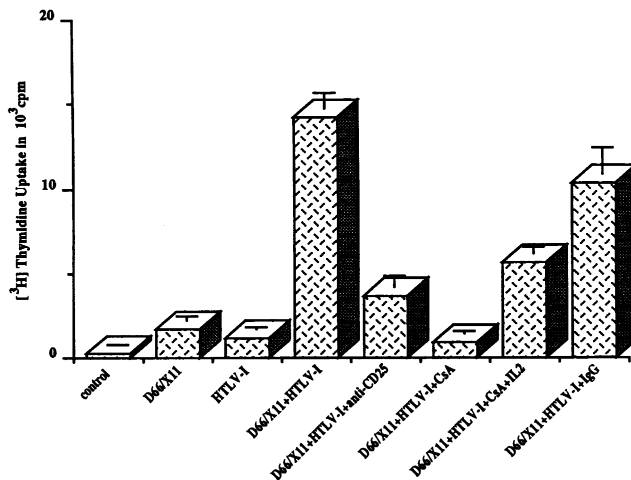

FIG. 6. Effect of the anti-CD25 MAb and CsA on HTLV-I-induced immature thymocyte proliferation. Immature thymocytes (3×10^5 cells per well) were cultured for 4 days. A pair of anti-CD2 MAbs (directed to two distinct epitopes of the CD2 molecule, D66 and X11) were added at a final dilution of 1/400, anti-CD25 MAb was used at a final dilution of 4 μg/ml, and CsA was added at a final concentration of 0.2 μg/ml. [^{3}H]thymidine incorporation was performed during the last 6 h. Results are expressed as mean counts per minute ± standard deviations of triplicate cultures and are representative of five experiments with thymic cells from three different donors.

indicate a direct involvement of the autocrine IL-2 receptor/IL-2 growth loop in the HTLV-I-induced proliferation of immature thymocytes.

DISCUSSION

The experiments described above document the response of human thymocytes to the mitogenic activity of HTLV-I. We have indeed previously demonstrated that HTLV-I is triggering the CD2-mediated proliferation of human resting peripheral T lymphocytes through the induction of the IL-2/IL-2 receptor loop (9, 11). Recent observations have shown that this mitogenic activity is restricted to virus-producing T cells and is mediated through the CD2/LFA-3 pathway (17, 36). These reports further indicate that the mitogenic activity of HTLV-I may not be linked to a direct effect of envelope proteins, but rather may be linked to cellular membrane proteins either associated with virions (1) or concentrated by ultracentrifugation of conditioned culture media.

The main goal of the present study was to evaluate how the mitogenic activity of HTLV-I is triggering the proliferation of cells of the T-lymphoid lineage at different steps of their maturation. The results show that HTLV-I is able to trigger human thymocyte proliferation, provided that the thymocytes were cultivated in the presence of submitogenic doses of PHA. Furthermore, experiments performed with unseparated thymocytes, $CD2^+CD3^-$ immature thymocytes, and $CD2^-CD3^-$ prothymocytes reveal that the expression of a functional CD2 molecule on the membrane of thymocytes is exclusively required to allow the proliferation of these cells in response to mitogenic signals delivered by PHA and HTLV-I. Thus, in the presence of the lectin and of the virus, both unseparated and immature thymocytes were incorporating thymidine while the prothymocytes were unresponsive. These results confirm and extend our previous data demonstrating that T-cell activation triggered by HTLV-I is a CD2-mediated event. We have shown that modulation of the CD2 molecule and not that of the CD3–T-cell receptor complex from the membrane of peripheral blood T lymphocytes rendered these cells unresponsive to the mitogenic effect of HTLV-I (8). The observation that immature thymocytes which possess functional CD2 molecules at a time when the CD3–T-cell receptor pathway is not yet functional have the ability to proliferate in response to HTLV-I and PHA clearly underlines the mandatory role of the CD2 pathway in this event. Furthermore, incubation of immature and unseparated thymocytes with a single anti-CD2 antibody inhibits cell proliferation induced by HTLV-I and PHA, probably as a consequence of the downmodulation of the CD2 molecule. We have further shown that HTLV-I is able to induce the proliferation of immature thymocytes incubated in the presence of a pair of appropriate anti-CD2 antibodies. These results suggest that HTLV-I is indeed complementing activation signals mediated by stimuli added at submitogenic concentrations and thus is able to induce the autocrine IL-2/IL-2 receptor growth loop, even in the absence of exogenous IL-2.

The $CD2^+CD3^-$ thymocytes represent one of the earliest human T-cell subsets, and numerous studies have been performed to understand the precise signals leading to their growth and differentiation (5, 16, 31, 33). In particular, these studies have shown the ability of PHA, when added at a very high concentration, to induce the expression of IL-2 receptors and autocrine IL-2 synthesis. In contrast, anti-CD2 MAbs were only able to induce expression of IL-2 receptors and exogenous IL-2 is required to trigger proliferation of these immature thymocytes (5). Pierres et al. have shown that a subset of immature thymocytes can be activated to proliferate through the CD2 or the CD28 pathway only in the presence of exogenous IL-2 (25). Finally, Denning et al. have demonstrated that in presence of the LFA-3 antigen, the natural ligand of CD2, and of one single anti-CD2 antibody, the addition of IL-2 is again required to induce the proliferation of immature thymocytes (7). Our present observations clearly underline that HTLV-I virions are upregulating the expression of IL-2 receptors and provide evidence that this virus displays the unique ability to induce IL-2 synthesis by immature thymocytes. These conclusions are supported by results of experiments in which the addition of one stimulus is delayed with respect to the addition of the other stimulus. As shown in Table 1, thymocytes proliferated when PHA and HTLV-I were either added at the same time or not. These results therefore suggest that each of these stimuli is able to complement signals delivered by the other, HTLV-I increasing the expression of IL-2 receptors triggered by PHA, and the lectin increasing the synthesis of IL-2 triggered by the virus.

We have further shown that HTLV-I acts synergistically with IL-2 to induce a lymphokine-dependent proliferation. However, proliferation of either unseparated (Table 2) or immature thymocytes (Table 3) was not observed when the addition of IL-2 was delayed, suggesting that some cells of these thymocyte populations are short-lived and are expressing IL-2 receptors. Nevertheless, this observation stresses the hypothesis that HTLV-I is acting on this autocrine pathway involved in thymocyte proliferation. Finally, it is interesting that a subpopulation of phenotypically immature thymocytes binds Epstein-Barr virus and that this virus acts synergistically with IL-2 to induce a lymphokine-dependent proliferation (35).

The unique interaction of HTLV-I virions with human immature thymocytes should be correlated with the involvement of this human retrovirus in the development of ATL. Several epidemiological studies have clearly demonstrated that HTLV-I is the etiological agent of ATL, a lymphoproliferative disease, and of tropical spastic paraparesis or HTLV-I-associated myelopathy, a neurological disorder. They have further indicated that individuals infected early in life have a higher risk to develop ATL than tropical spastic paraparesis or HTLV-I-associated myelopathy (24). Therefore, one critical event which could constitute a prerequisite to the development of ATL late in life could be infection of the thymus by HTLV-I early in life and, more importantly, infection of the immature thymocytes, which are a critical subset in T-cell maturation. As such, mitogenic activation by HTLV-I and cellular gene transactivation by the Tax protein may interfere with the proliferation and maturation of human immature thymocytes, thus favoring the emergence of cells among which leukemic cells are recruited. Furthermore, thymus infection at the neonatal stage may induce a state of immunologic nonresponsiveness linked to intrathymic replication of the virus, as has been demonstrated in mice exposed as neonates to Gross murine leukemia virus (22) or to lymphocytic choriomeningitis virus (19). Therefore, HTLV-I infection of the thymus of neonates may explain the long latent period extending from infection, revealed by the presence of proviral DNA (detected by polymerase chain reaction), to the appearance of antiviral antibodies several years later (23).

Finally, it should be remembered that the thymus is a lymphoepithelial organ (16). Cell-cell adhesion plays an important role in delivery of proliferation and differentiation signals to thymocytes in the course of the close association of thymocytes with thymic epithelial cells (33). It is therefore tempting to speculate that infection of cortical epithelial cells and immature thymocytes may interfere with T-cell development. In this perspective, an understanding of the HTLV-I-induced leukemogenesis might benefit from an analysis of the molecular and cellular events after infection of the thymic cells by this human retrovirus.

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