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Inhibition of proliferation and CD25 down-regulation by retinoic acid in human adult T cell leukemia cells

J-I Miyatake and Y Maeda

Third Department of Internal Medicine, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589, Japan

The effects of retinoic acid (RA) on the cell growth and expression of interleukin-2 (IL-2) receptors (IL-2Rα/p55, Tac, CD25) by the human T lymphotropic virus type I (HTLV-I)-positive T cell lines, HUT102 and ATL-2 were investigated. Incubation of these cells for 48 h with either 13-*cis* retinoid acid (13-*cis* RA) or all-*trans* retinoic acid (ATRA) resulted in marked inhibition of cell growth, determined by ^{3}H-thymidine incorporation, and in down-regulation of CD25 expression, determined by flow cytometry. Four HUT102 cell clones were established by limiting dilution, and 13-*cis* RA was shown to inhibit cell growth and CD25 expression in three of these clones (HUT102-M5, -M6 and -M7), but not in the fourth (-M8). RA did not induce growth inhibition or down-regulation of CD25 in the HTLV-I-negative T cell lines (Jurkat and MOLT-4) and in normal lymphocytes that had been stimulated with phytohemagglutinin or phorbol 12-myristate 13-acetate. We have shown that RA markedly inhibited both the cell growth and the expression of CD25 in some HTLV-I-positive T cell clones, but not in normal lymphocytes. These results suggest that RA may be suitable for the treatment of patients with adult T cell leukemia (ATL).
Keywords: retinoic acid; ATL; CD25 expression; anti-proliferative effect

Introduction

Vitamin A and its analogs (retinoids) influence the growth and differentiation of normal and malignant cells,[1] and have been shown to possess anticarcinogenic and antitumor activities *in vitro* and *in vivo*.[2] RA influences the clonal growth of normal human myeloid cells[3] and induces the differentiation of both HL-60 cells (human promyelocytic leukemia cell line) and fresh human acute promyelocytic leukemia (APL) cells into normal granulocytes in morphological study.[4,5]

ATL is a malignancy of mature T cells that is associated with human T lymphotropic virus type I (HTLV-I).[6] IL-2Rα/p55 (Tac, CD25) are overexpressed on the peripheral blood leukemic cells of almost ATL patients as immunological characteristics[7,8] as well as on HTLV-I-transformed T cell lines.[9] The HTLV-I genome contains *gag, pol* and *env* genes, as well as a region referred to as *pX*,[10] which encodes the proteins Tax, Rex, and P21^x respectively.[11] Tax is a nuclear protein that transactivates its cognate long terminal repeat (LTR),[12] immortalizes rat embryo fibroblasts and primary T lymphocytes,[13] and induces apoptosis of W4 cells generated by the transfection of Rat-1 cells with a Tax expression plasmid.[14] The IL-2Rα gene is one of the cellular genes activated by Tax.[15] The expression of CD25 in ATL cell lines is also enhanced by ATL-derived factor (ADF),[16–18] originally defined as an inducer of IL-2Rα and shown to be a homologue of thioredoxin (TRX).[19] Melanoma TRX reductase has been shown to be inhibited by 13-*cis* retinoic acid (13-*cis* RA) in a dose-dependent manner as a result of formation of a thioether enzyme-inhibitor complex. ATRA appears to be incapable of reacting in this way and this is probably due to its stereochemistry.[20] However, it was reported that all-*trans* retinoid acid (ATRA) inhibits inositol phospholipid turnover.[21] In general, the various actions of RA for lymphocytes in the immune response have been reported that RA enhance DNA synthesis of human peripheral blood lymphocytes (PBL) in response to phytohemagglutinin (PHA) in dose-dependent fashion, especially RA-induced stimulation of PBL reactivity to PHA was most evident in T cell-enriched subpopulations.[22,23] Additionally, it was reported that the effect of vitamin A injections on immunological response in mice led to a large increase in the production of antibodies.[24,25]

We have now investigated the effects of RA for ATL cells determined by ^{3}H-thymidine incorporation and the expression of CD25.

Correspondence: Y Maeda
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Materials and methods

Cells and cell culture

Established cell lines used in this study were two HTLV-I positive-T cell lines, HUT102[26] and ATL-2,[27] and two HTLV-I-negative T cell lines, Jurkat and MOLT-4. They were maintained by culturing at 37°C under a humid atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Hazleton Research Products, Lenexa, KS, USA). PBMCs from normal individuals were separated by Ficoll–Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, adjusted to a density of 1×10^6 cells/ml, and stimulated with 0.1% PHA or 5 ng/ml phorbol 12-myristate 13-acetate (PMA) in tissue culture dishes for 48 h. The cells were then washed twice, readjusted to a density of 2×10^5 cells/ml in RPMI 1640 containing 10% FBS, and assayed for the effect of 13-*cis* RA or ATRA on ^{3}H-thymidine incorporation and CD25 expression.

Reagents

PMA, 13-*cis* RA, and ATRA were obtained from Sigma (St Louis, MO, USA), and PHA was from DIFCO (Detroit, MI, USA). Retinoids were dissolved in ethanol and handled as described by Dawson and Hobbs,[28] in each experiment, control incubations were performed with the same concentration of ethanol as that present in the experimental wells. Monoclonal antibodies to CD25 were obtained from Becton Dickinson (Mountain View, CA, USA).

Proliferation assay

Cells (1×10^5 to 2×10^5/ml) were cultured in flat-bottomed 96-well microtiter plates (Corning, NY, USA) with 13-*cis* RA or ATRA (10^{-11}, 10^{-9}, 10^{-7} or 10^{-5} M). ^{3}H-thymidine (1 μCi

per well) (Amersham, Tokyo, Japan) was added and cells were incubated for a further 4 h. Cells were then harvested on to fiberglass filters and processed for liquid scintillation spectroscopy. ^{3}H-thymidine incorporation was expressed in counts per minute.

Determination of CD25 expression

Cells (2×10^5/ml) were incubated with various concentrations (10^{-11} to 10^{-5} M) of 13-*cis* RA or ATRA in 24-well plates (Corning, NY, USA). Cells were then harvested, washed, incubated with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies to CD25 (1 μg/ml) at 4°C for 30 min, washed again with PBS, and analyzed with a flow cytometer (Cytron, Ortho Diagnostic Systems KK, Tokyo, Japan).

Single cloning of HUT102 cells

HUT102 cells were resuspended in RPMI 1640 supplemented with 10% FBS and added to flat-bottomed 96-well microtiter plates at a density of one cell per well in 0.1 ml of medium. After incubation at 37°C under a humid atmosphere containing 5% CO_2 for 2 weeks, selected clones were expanded in RPMI 1640 supplemented with 10% FBS, and assayed for the effects of 13-*cis* RA and ATRA on cell proliferation and CD25 expression.

Results

Growth inhibition and CD25 down-regulation by RA on HUT102 and ATL-2 cells

We initially assessed the effect of 13-*cis* RA or ATRA to HTLV-I-positive T cell lines, HUT102 and ATL-2 cells. Incubation of those cells with various concentrations of 13-*cis* RA or ATRA for 48 h inhibited ^{3}H-thymidine incorporation. When those cells were treated with 13-*cis* RA, as shown in Figure 1a and b, ^{3}H-thymidine incorporation was decreased in a dose-dependent manner. However, ^{3}H-thymidine incorporation was decreased significantly in the treatment with 10^{-5} M ATRA but not 10^{-7} M or 10^{-9} M ATRA. The inhibitory effects of 13-*cis* RA appeared more marked than that of ATRA. To assess the effect of 13-*cis* RA or ATRA to the cell surface antigen, we observed the expression of CD25 by flow cytometry. Incubation of HUT102 and ATL-2 cells for 48 h with 10^{-5} M 13-*cis* RA or ATRA for 48 h also resulted in down-regulation of CD25 expression; the means of four different experiments were indicated (Figure 2a and b). Two peaks were apparent on flow cytometric analysis of HUT102 cells, treated with 10^{-5} M 13-*cis* RA or ATRA, suggesting the existence of sensitive and resistant clones to RA.

Effects of RA on several HUT102 clones

Single HUT102 cells were isolated by limiting dilution and clones HUT102-M5, -M6, -M7 and -M8 cells were established. Both 13-*cis* RA and ATRA inhibited the proliferation of HUT102-M5, -M6 and -M7 cells, but not that of HUT102-M8 cells, in a dose-dependent manner (Figure 3). Incubation of HUT102-M5, -M6 and -M7 cells, but not HUT102-M8 cells, with 13-*cis* RA resulted in down-regulation of CD25 expression; the means of four different experiments were indicated (Figure 4); the effect was particularly marked in HUT102-M7 cells. These results suggested that the different sensitivities to RA were observed among these clones, HUT102-M5, -M6, -M7 cells were sensitive to RA, but HUT102-M8 cells were resistant.

No effect of RA on HTLV-I-negative T cell lines, Jurkat and MOLT-4

Jurkat and MOLT-4 (Figure 5) and MOLT-4 (Figure 6) were incubated with several concentrations of 13-*cis* RA or ATRA

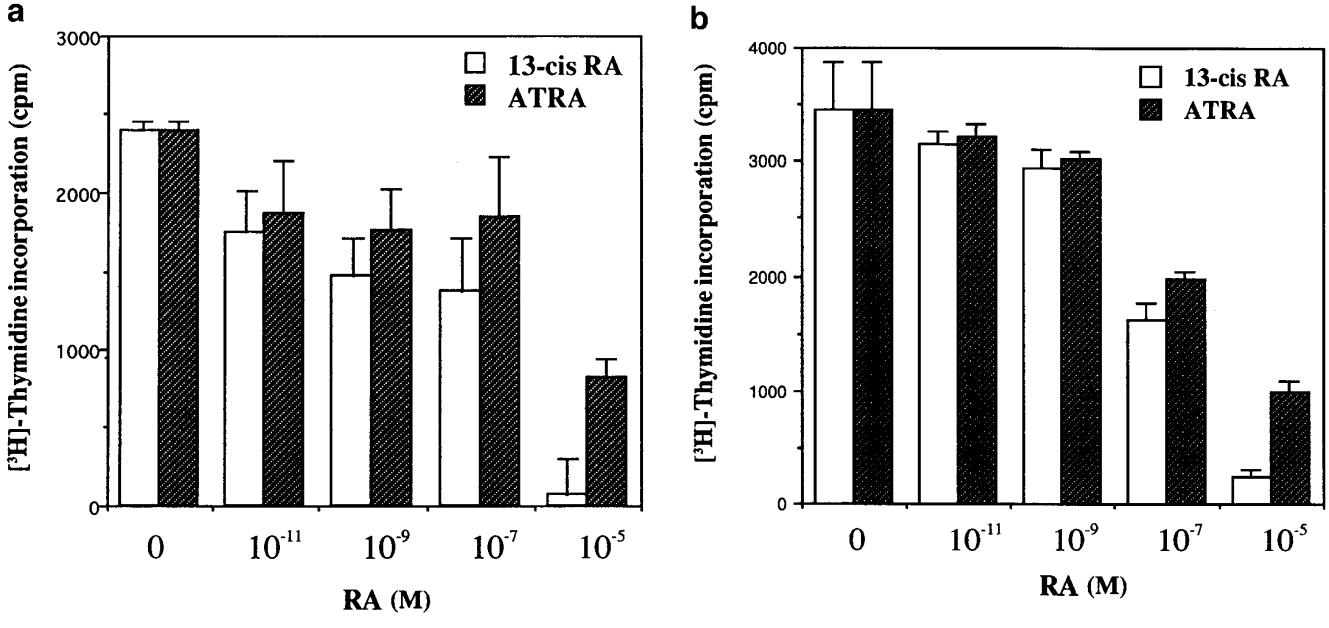

Figure 1 Effects of RA on ^{3}H-thymidine incorporation in HUT102 cells (a) and ATL-2 cells (b). Cells were incubated with the indicated concentrations of 13-*cis* RA or ATRA for 48 h and assayed for ^{3}H-thymidine incorporation. Four different experiments were carried out, and data represent mean ± s.d. in the figure.

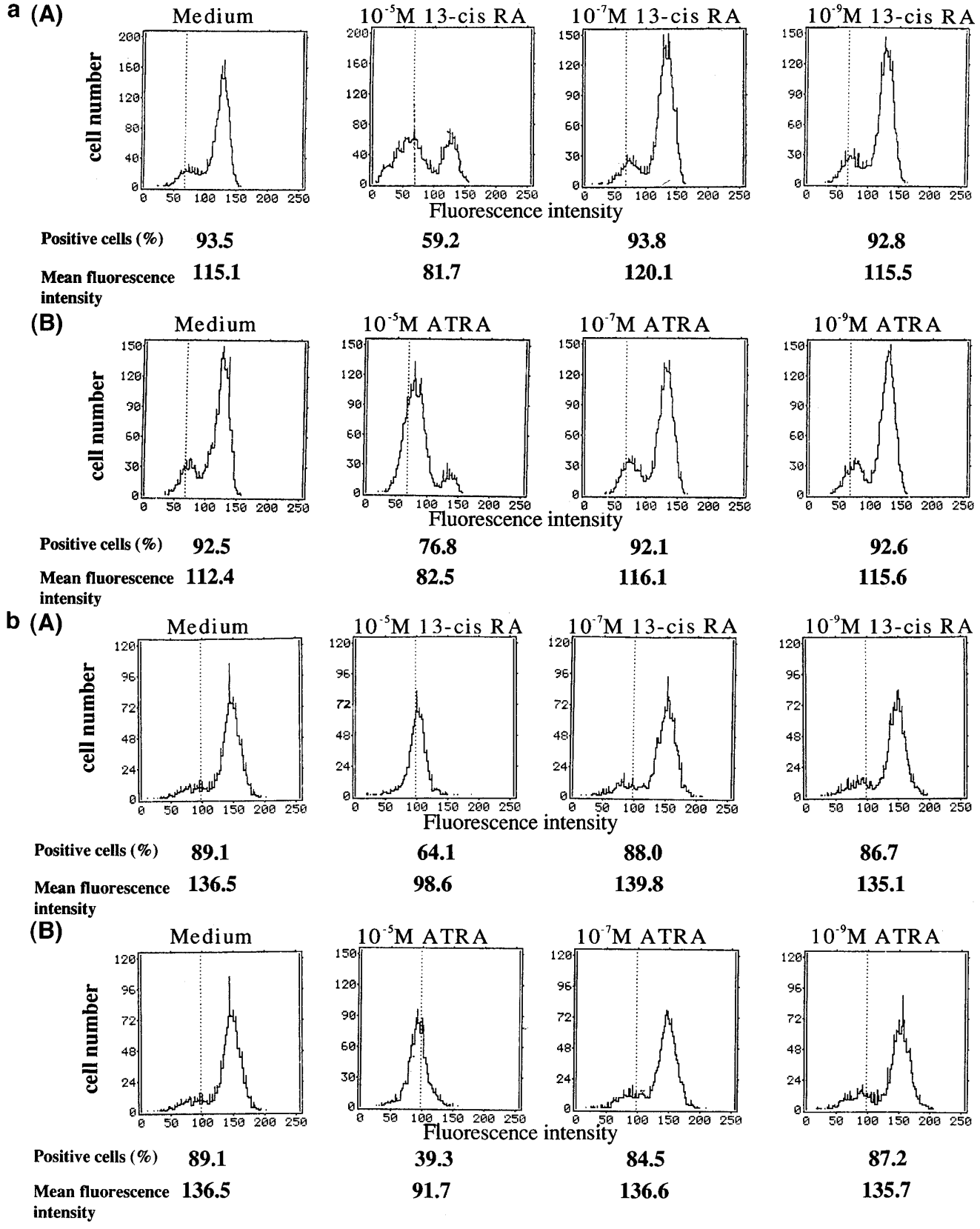

Figure 2 Effects of RA on CD25 expression by HUT102 cells (a) and ATL-2 cells (b). Cells were incubated the indicated concentrations of 13-*cis* RA (A) (% positive: medium = 93.5 ± 10.8, 10^{-5} M = 59.2 ± 7.6, 10^{-7} M = 93.8 ± 9.2, 10^{-9} M = 92.8 ± 8.9 and MFI: medium = 115.1 ± 11.8, 10^{-5} M = 81.7 ± 7.2, 10^{-7} M = 120.1 ± 10.1, 10^{-9} M = 115.5 ± 12.1) or ATRA (B) (% positive: medium = 92.5 ± 11.2, 10^{-5} M = 76.8 ± 6.6, 10^{-7} M = 92.1 ± 11.2, 10^{-9} M = 92.6 ± 8.8 and MFI: medium = 112.4 ± 9.8, 10^{-5} M = 82.5 ± 7.0, 10^{-7} M = 116.1 ± 6.4, 10^{-9} M = 115.6 ± 9.1) for 48 h and then assayed for CD25 expression by flow cytometry. These results represent the mean ± s.d. of four different experiments.

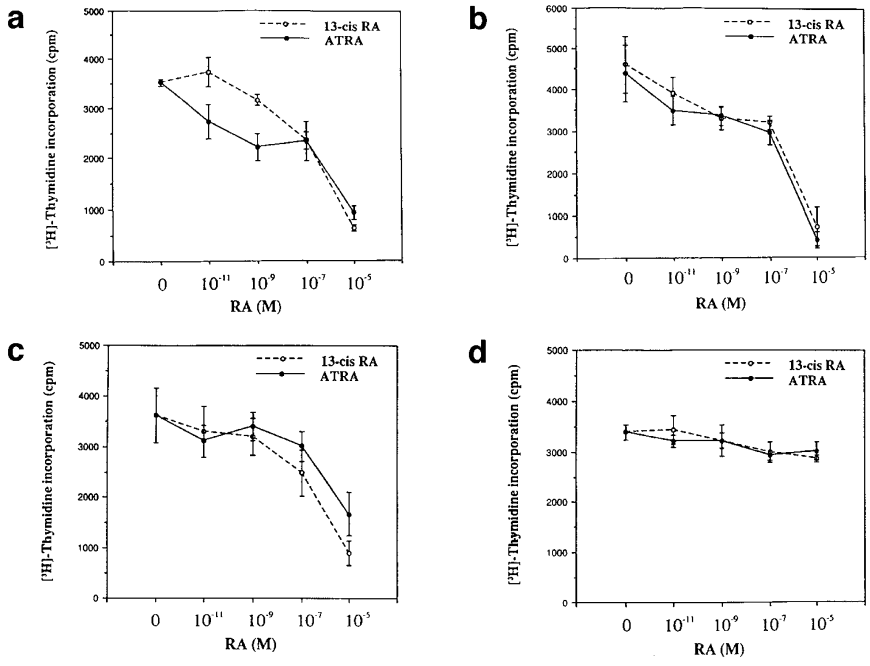

Figure 3 Effects of RA on the proliferation of HUT102 cell clones M5 (a), M6 (b), M7 (c) and M8 (d). Cells were incubated with the indicated concentrations of 13-*cis* RA or ATRA for 48 h and assayed for ^{3}H-thymidine incorporation. These results represent the mean ± s.d. of four different experiments.

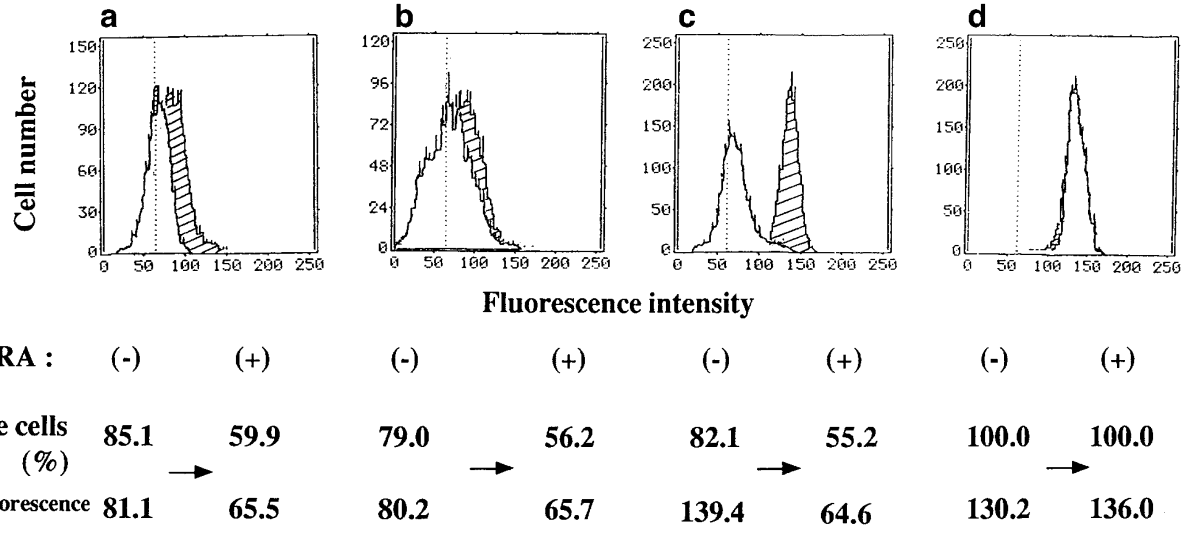

Figure 4 Effects of 13-*cis* RA on CD25 expression by HUT102 cell clones M5 (a) (% positive: medium = 85.1 ± 11.0, 10^{-7} M = 59.9 ± 7.9, MFI: medium = 81.1 ± 9.8, 10^{-7} M = 65.5 ± 10.0), M6 (b) (% positive: medium = 79.0 ± 10.3, 10^{-7} M = 56.2 ± 6.9, MFI: medium = 80.2 ± 9.7, 10^{-7} M = 65.7 ± 13.0), M7 (c) (% positive: medium = 82.1 ± 12.1, 10^{-7} M = 55.2 ± 8.9, MFI: medium = 139.4 ± 9.0, 10^{-7} M = 64.6 ± 12.1), and M8 (d) (% positive: medium = 100.0 ± 10.2, 10^{-7} M = 100.0 ± 8.9, MFI: medium = 130.2 ± 4.9, 10^{-7} M = 136.0 ± 7.8). Cells were incubated for 48 h in the absence (striped peaks) or presence (open peaks) of 10^{-7} M 13-*cis* RA and then assayed for CD25 expression by flow cytometry. These results represent the mean ± s.d. of four different experiments.

for 48 h and assayed for ^{3}H-thymidine incorporation. However, no growth inhibition was observed on either T cell lines.

No growth inhibition and CD25 down-regulation by RA for PHA-stimulated PBMCs

Neither 13-*cis* RA nor ATRA could inhibit the cell growth of normal PBMCs stimulated with 0.1% PHA (Figure 7). CD25 expression by normal PBMCs that had been stimulated with PHA or PMA were not affected by incubation with 13-*cis* RA or ATRA (Figure 8).

Discussion

ATL cells are characterized by the constitutive high level expression of the Tac antigen (IL-2Rα, CD25).[29] The HTLV-I

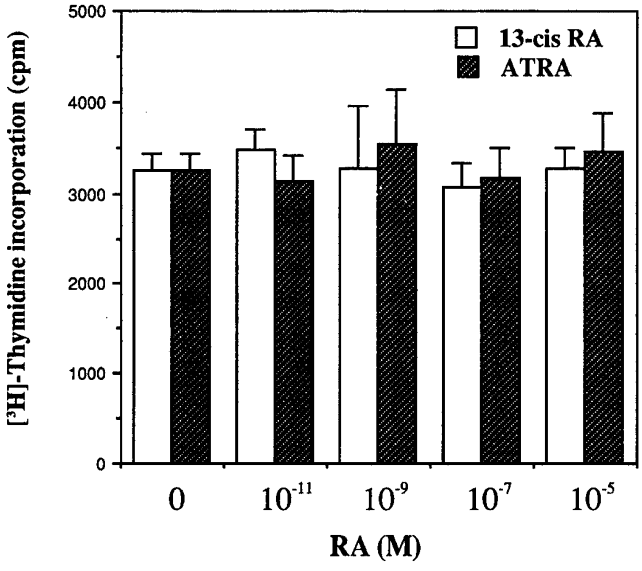

Figure 5 Effects of RA on the proliferation of HTLV-I-negative T cell lines. Jurkat cells were incubated with the indicated concentrations of 13-*cis* RA or ATRA for 48 h and assayed for ^{3}H-thymidine incorporation. Four different experiments were carried out, and data represent mean ± s.d. in the figure.

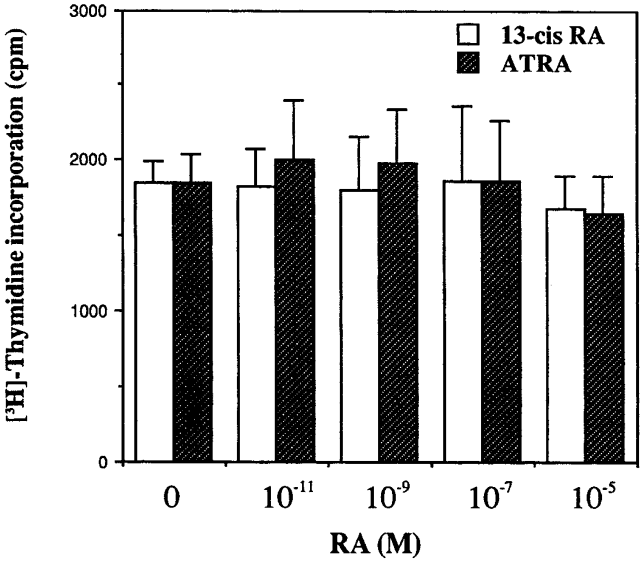

Figure 6 Effects of RA on the proliferation of HTLV-I-negative T cell lines. MOLT-4 cells were incubated with the indicated concentrations of 13-*cis* RA or ATRA for 48 h and assayed for ^{3}H-thymidine incorporation. Four different experiments were carried out, and data represent mean ± s.d. in the figure.

Tax protein activates both the viral LTR and the IL-2Rα gene (CD25), resulting in marked expression of CD25.[12,30] Enhancement of the transcription of the IL-2Rα gene by nuclear factor κB is one of the mechanisms that underlie the high expression of CD25.[15,31–33] The TRX homolog ADF also enhances the expression of IL-2Rα.[19,33,34]

We have now investigated the effects of RA on the cell growth and expression of CD25 by HUT102 cells and ATL-2 cells to shed light on whether this agent might be effective for the treatment of patients with ATL. Both 13-*cis* RA and ATRA inhibited the proliferation and CD25 expression by HUT102

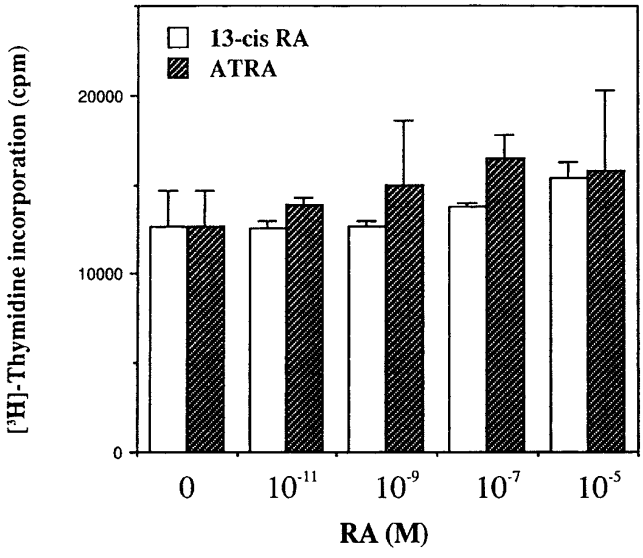

Figure 7 Effects of RA on the proliferation of PHA-stimulated normal human PBMCs. Cells were incubated with the indicated concentrations of 13-*cis* RA or ATRA for 48 h and assayed for ^{3}H-thymidine incorporation. Four different experiments were carried out, and data represent mean ± s.d. in the figure.

cells. The presence of two peaks in the CD25 flow cytometric histogram of HUT102 cells treated with RA suggested the existence of sensitive and resistant cells.

We therefore investigated the effects of RA on four HUT102 cell clones isolated by limiting dilution. Both 13-*cis* RA and ATRA inhibited the growth and CD25 expression by HUT102-M5, -M6, -M7 cell clones but had no effect on the HUT102-M8 clone, confirming differences in the sensitivity to RA among HUT102 cell clones. The most effective concentration of RA was 10^{-5} or 10^{-7} M, and 13-*cis* RA was more effective than ATRA in inhibiting cell growth. Likewise, to investigate whether RA was effective for HTLV-I-negative T cell lines, we tried to observe the effect of RA for cell growth on Jurkat and MOLT-4. However, RA had no effect on the growth by Jurkat and MOLT-4. Similarly, no growth inhibition and down-regulation of CD25 expression were shown in normal PBMCs stimulated with PHA. Although it was reported that RA induced stimulation of lymphocyte reactivity to PHA,[22] the induction of proliferation on the PHA-stimulated lymphocytes by RA was not significant in our study.

The mechanism responsible for the difference in sensitivity of HUT102 cell clones to RA with regard to down-regulation of CD25 is not clear. However, this difference may be attributable to: (1) Differences in the expression of retinoic acid receptors (RARs)[35,36] or retinoid X receptors (RXRs);[37,38] these receptors' expression may be associated with the sensitivity to RA; (2) Differences in the expression of cytotosolic retinoic acid binding proteins (CRABPs), which binds RA before its transfer to the nucleus and acts as an intracellular antagonist of RA action.[39–42] The extent of CRABP expression would be expected to correlate with RA resistance. (3) Differences in the expression of anti-oxidant including ADF. Indeed, our previous study showed that incubation with 13-*cis* RA for 48 h resulted in inhibition of growth for PBMCs and in induction of apoptosis from some patients with ATL, but not for PBMCs from normal individuals.[43] Furthermore, we showed that the sensitivity of cells to RA-induced growth inhibition varied among patients.[44] Thus, there is a possibility that specific target cells of RA may be ATL cells in the peripheral blood.

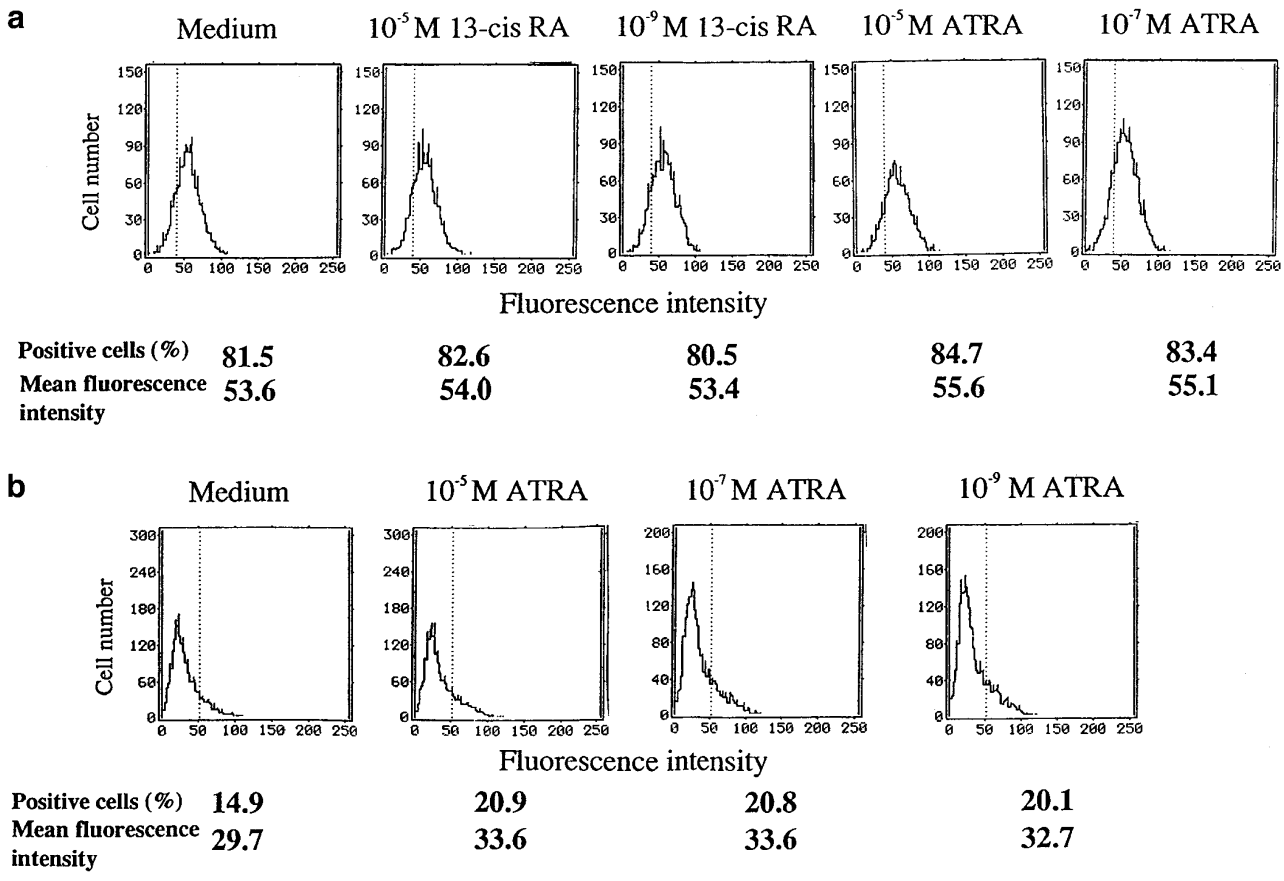

Figure 8 Effects of RA on CD25 expression by normal human PBMCs that had been stimulated with PHA (a) (% positive: medium = 81.5 ± 11.3, 10^{-5} M 13-*cis* RA = 82.6 ± 7.7, 10^{-9} M 13-*cis* RA = 80.5 ± 9.1, 10^{-5} M ATRA = 84.7 ± 5.2, 10^{-7} M ATRA = 83.4 ± 4.2, MFI: medium = 53.6 ± 9.1, 10^{-5} M 13-*cis* RA = 54.6 ± 5.9, 10^{-9} M 13-*cis* RA = 53.4 ± 9.9, 10^{-5} M ATRA = 55.6 ± 5.9, 10^{-7} M ATRA = 55.1 ± 6.1) or PMA (b) (% positive: medium = 14.9 ± 5.6, 10^{-5} M ATRA = 20.9 ± 7.0, 10^{-7} M ATRA = 20.8 ± 6.0, 10^{-9} M ATRA = 20.1 ± 4.8, MFI: medium = 29.7 ± 6.3, 10^{-5} M ATRA = 33.6 ± 9.2, 10^{-7} M ATRA = 33.6 ± 5.8, 10^{-9} M ATRA = 32.7 ± 5.8). Cells were incubated with the indicated concentrations of 13-*cis* RA or ATRA for 48 h and then assayed for CD25 expression by flow cytometry. These results represent the mean ± s.d. of four different experiments.

We have shown that RA markedly inhibited both the growth and CD25 expression of some ATL cell clones. These results suggest that RA may be suitable for the new treatment of patients with ATL.

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