Calcium-Dependent Homotypic Adhesion Through Leukocyte Function-Associated Antigen-1/Intracellular Adhesion Molecule-1 Induces Interleukin-1 and Parathyroid Hormone-Related Protein Production on Adult T-Cell Leukemia Cells In Vitro

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Adult T-cell leukemia (ATL) is a human T-cell leukemia virus type I (HTLV-I)-infected lymphoproliferative disorder that shows a characteristic nodular infiltration into various tissues, hypercalcemia, and subsequent rapid increase of peripheral ATL cell number. ATL cells and HTLV-I-infected T-cell lines also make cluster formation rapidly after the non-stimulative culture. However, the mechanism of the acute proliferation of ATL cells remains to be understood. We report the following novel features of homotypic adhesion via leukocyte function-associated antigen-1 (LFA-1)/intracellular adhesion molecule-1 (ICAM-1) pathway that suggest a role for it in cytokine production and rapid proliferation of ATL cells: (1) ATL cells show clustering in a calcium dependent manner, even at the higher concentration; (2) ATL cells consistently and highly express ICAM-1 and an active form of LFA-1, whereas integrin expression, except for LFA-1, is rather lower compared with that of normal CD4⁺ T cells; (3) ATL cells make conjugate formation within 6 minutes and clustering within 48 hours, both of which are inhibited by the addition of monoclonal antibodies (MoAbs) against LFA-1 and ICAM-1; (4) spontaneous mRNA transcription and protein secretion of both interleukin-1 and parathyroid hormone-related protein are observed consistently in ATL cells, and these productions are inhibited by anti-LFA-1 and anti-ICAM-1 MoAbs but are markedly increased by cross-linking of LFA-1 and ICAM-1 by the immobilized specific MoAbs; and (5) proliferative responses of ATL cells are also inhibited by these MoAbs. We propose that ATL cells proliferate in sequential events: the homotypic and calcium-dependent adhesion through LFA-1/ICAM-1, the signal transduction through these adhesion molecules, the production of cytokines, and the proliferation.

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for the multiple cytokine production, proliferation of ATL cells, and the hypercalcemia in vivo.

MATERIALS AND METHODS

Subjects. Ten patients with ATL were selected in this study. Seven of these patients had the acute type of ATL, 2 had the lymphoma type of ATL, and 1 had the chronic type of ATL (Table 1). The diagnosis for ATL was performed according to the clinical features and hematologic findings, including the morphologic characteristics, 1 cell surface phenotypes of leukemic or lymphoma cells, 2 and serum antibodies against HTLV-I-associated antigens. 3 All of the cases had the monoclonal integration of HTLV-I proviral genome in the leukemic or lymphoma cells. 2 The classification of ATL was performed using the criteria of Shimoyama et al. 3 The clinical data of the patients and the levels of lactate dehydrogenase (LDH; normal, 175 to 400 U/L) and serum total calcium (2.12 to 2.55 mmol/L) are summarized in Table 1. Lymphocytes from four healthy donors and four HTLV-I carriers also were selected on this study as controls.

Preparation of fresh cells. Leukemic cells and peripheral blood (PB) lymphocytes of healthy donors and HTLV-I carriers were collected from heparinized PB and lymphoma cells were from cell suspension of the lymph node material with a centrifugation over a lymphocyte separation medium (LSM; Litton Bionetics, Kensington, MD). The cells were further incubated for 2 hours in anti-CD8 and anti-CD19 monoclonal antibodies (MoAbs) immobilized on plastic culture dishes to remove adherent cells and CD8⁺ T cells and B cells. The purity of CD4⁺ cells was more than 96.8% of the examined cells in all cases.

Cell lines. Established cell lines used in this study are four HTLV-I-positive T-cell lines (MT-1, MT-2, 4 HUT102, and SALT-I), all of which produced IL-1α but not IL-β, as described previously; 5 HTLV-I-negative T-acute lymphocytic leukemia cell lines (Jurkat and MOLT-4) were used as a control. These cell lines were kindly obtained by Dr K. Sagawa (Kurume University Medical School, Kurume, Japan) and Dr T. Hattori (Kumamoto University, School of Medicine, Second Department of Internal Medicine, Kumamoto, Japan). They were maintained by culturing in RPMI 1640 (Nissui Seiyaku Co, Tokyo, Japan) containing 10% fetal calf serum (FCS; Gibco, Grand Island, NY).

Cell culture and observation of homotypic cell adhesion. A total of 5 × 10⁶/mL of freshly isolated ATL cells and normal PB T cells preincubated with both 0.1% of phytohemagglutinin (PHA) and 10 ng/mL of phorbol myristate acetate (PMA) for 24 hours (PHA/PMA T cells) and 1 × 10⁶/mL of cell lines were cultured in 24-well culture plates (1 mL per well) in RPMI1640 medium containing 8% FCS with 1 μg/10⁵ cells of human globulin in the presence or absence of 1 μg/10⁷ cells of relevant MoAbs for 48 hours. Cluster formation was observed by microscopic photograph after 24 and 48 hours of incubation. Calcium-dependent homotypic cell adhesion was examined by the same method using calcium- and serum-free DCCM-1 medium (Biological Industries Co Ltd, Jerusalem, Israel) in the presence of various concentrations of calcium chloride.

Antibodies. The following MoAbs were used as purified Ig: CD54 (ICAM-1) MoAb 84H10 (gift from S. Shaw, National Cancer Institute, Bethesda, MD), 5 CD1 (LFA-1o chain) MoAb TS1/12, CD58 (LFA-3) MoAb TS2/19 (from American Type Culture Collection, Rockville, MD); LFA-1 active form MoAb NK1-L16 (gift from C.G. Figdor, Amsterdam, The Netherlands), 5 CD29 (VLA-β1 chain) MoAb MAβ13 (gift from K. Yamada, National Institute of Dental Research, Bethesda, MD), CD49d (VLA-4o chain) MoAb NIH49d1-1, CD44 MoAb NIH44-I (gift from S. Shaw), 5 CD2 MoAb MT910, CD25 (IL-2 receptor α chain) MoAb ACT-1, CD19 MoAb HD37, CD4 MoAb MT310, and CD8 MoAb DK25 (these last 4 purchased from Dako Japan, Kyoto, Japan).

Immobilization of MoAbs. Ten micrograms per milliliter of MoAbs suspended in phosphate-buffered saline (PBS) was immobilized on 24-well culture plates or 10-cm diameter plastic dishes for 24 hours at 4°C. After the dishes or plates were washed by PBS for three times, these were used as MoAb-immobilized dishes and plates.

Flow cytometric analysis. The following steps were all performed on ice. Prepared cells (2 × 10⁶/mL) were washed twice in fluorescence-activated cell sorter (FACS) media (Hank’s Balanced Salt Solution [HBSS] without calcium and magnesium with 0.5% human serum albumin and 0.1% NaCl). The cells were incubated for 10 minutes with 10 μL of human serum globulin (1 mg/mL; Green Cross, Osaka, Japan) and further incubated with specific MoAb at saturating concentration for mouse antibodies for 30 minutes. After washing, fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG antibodies were added and incubated for 30 minutes. The cells were again washed and incubated with irrelevant MoAb for 15 minutes, and further incubated with phycoerythrin (PE)-conjugated CD4 MoAb for 30 minutes. The cells were washed and were analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

Conjugation assay. Formation of conjugates was measured using a two-color flow cytometer generally as described. 7 Briefly, ATL cells were divided into effector cells and target cells for conjugate formations. Effectors and targets were identical populations. Effector target mix analyzed immediately after mixing in suspension was determined, and this background was subtracted from experimental values, which normally range from 2% to 5%. In assays of MoAb inhibition, saturating concentration of MoAb and human intact globulin to block Fc-binding sites were added before settling and were present continuously thereafter.

Measurement of IL-1β using enzyme-linked immunosorbent assay (ELISA) and c-PTHrP using radioimmunoassay (RIA) on supernatants. Freshly isolated ATL cells (5 × 10⁶/mL) and cell lines (1 × 10⁶/mL) were cultured in MoAb-immobilized or nonimmobilized 24-well culture plates (1 mL per well) in RPMI1640 medium containing 8% FCS. After culture for 48 hours in the presence or absence of soluble MoAbs (10 μg/mL) with intact human globulin (10 μg/mL), the culture supernatants were collected by centrifugation for determination of secreted IL-1β and c-terminal region of PTHrP. IL-1β in the culture supernatants was measured using an IL-1β ELISA kit (Otsuka Pharmaceutical Co Ltd, Tokushima, Japan) and c-terminal region (109-141) of PTHrP was measured using a c-PTHrP RIA kit (Dalichi Isotope Co Ltd, Tokyo, Japan). The sensitivity of these assays is 0.5 pg/mL of IL-1β and 5 pmol/L of c-PTHrP, respectively.

Northern blot analysis. Freshly isolated ATL cells (1 × 10⁶/mL) and cell lines (2 × 10⁶/mL) were cultured in MoAb-immobilized or nonimmobilized culture dishes (4 mL per dish) in RPMI1640 medium containing 8% FCS. In the nonimmobilized dishes, 1 μg/10⁶ cells of human intact globulin and soluble MoAbs (1 μg/10⁶ cells) was added to the medium. After 4 hours of incubation, the cells were collected by centrifugation and the total RNA (20 μg) was electrophoresed in 1% agarose-formaldehyde gel and transferred to Hybond-N nylon filters (Amersham, Arlington Heights, IL). Hy-
Table 1. The Patient Profiles and CD4+ Cell Characteristics of Freshly Isolated ATL Cells

| Cells | WBCs (× 10^3/L) | HLA-ly (%) | LDH (IU/L) | Serum Ca (mmol/L) | CD4 (%) | Type§ | % Positive Cells of CD4+ Cells | CD2 | ICAM-1 | LFA1α | NKL16 | LFA3 | /1 | n4 | CD25 | CD44 |
|-------|-----------------|------------|------------|---------------|---------|-------|--------------------------------|-----|--------|--------|--------|------|-----|-----|------|------|------|
| Normal PBL-T (n = 4) | | | | | | | | | | | | | | | | | | |
| Mean | | | | | | | | | | | | | | | | | | |
| SD | | | | | | | | | | | | | | | | | | |
| PHA/PMA-T (n = 4) | | | | | | | | | | | | | | | | | | |
| Mean | | | | | | | | | | | | | | | | | | |
| SD | | | | | | | | | | | | | | | | | | |
| HTLV-I carrier (n = 4) | | | | | | | | | | | | | | | | | | |
| Mean | | | | | | | | | | | | | | | | | | |
| SD | | | | | | | | | | | | | | | | | | |
| ATL (n = 10) | | | | | | | | | | | | | | | | | | |
| 1 | 83,500 | 86 | 2,928 | 2.68 | 98.9 | Acute | 97.6 | 55.0 | 69.4 | 15.6 | 35.3 | 4.3 | 32.6 | 92.1 | 98.6 |
| 2 | 183,500 | 98 | 1,870 | 3.02 | 99.6 | Acute | 96.3 | 98.8 | 98.8 | 14.4 | 69.4 | 32.4 | 15.6 | 98.2 | 93.8 |
| 3 | 60,400 | 45.5 | 1,450 | 4.45 | 94.5 | Acute | 89.8 | 74.6 | 86.2 | 39.9 | 73.8 | 85.7 | 22.5 | 86.4 | 94.6 |
| 4 | 115,500 | 77.5 | 4,216 | 2.92 | 98.5 | Acute | 85.7 | 37.0 | 80.2 | 20.1 | 61.6 | 6.3 | 7.3 | 90.5 | 90.5 |
| 5 | 3,800 | 5 | 2,769 | 2.35 | 99.6 | Lymphoma | 96.8 | 14.2 | 66.2 | 12.9 | 23.5 | 27.5 | 1.9 | 86.6 | 87.0 |
| 6 | 15,600 | 12 | 597 | 2.25 | 97.8 | Chronic | 90.5 | 23.1 | 75.2 | 9.6 | 42.3 | 55.6 | 49.1 | 87.8 | 94.8 |
| 7 | 9,300 | 3 | 1,258 | 3.45 | 97.4 | Lymphoma | 84.6 | 19.0 | 76.4 | 26.4 | 25.4 | 29.2 | 32.5 | 92.4 | 89.2 |
| 8 | 72,000 | 42 | 4,610 | 3.13 | 97.3 | Acute | 94.8 | 60.8 | 85.2 | 14.1 | 67.6 | 81.5 | 13.2 | 95.3 | 96.7 |
| 9 | 74,000 | 45 | 2,756 | 2.95 | 98.5 | Acute | 82.5 | 70.6 | 67.0 | 18.8 | 76.5 | 28.4 | 88.5 | 98.7 | 98.4 |
| 10 | 26,000 | 58 | 1,311 | 2.28 | 97.7 | Acute | 91.3 | 30.8 | 92.4 | 33.3 | 35.8 | 36.9 | 42.2 | 94.7 | 97.1 |
| Mean | | | | | | | | | | | | | | | | | | |
| SD | | | | | | | | | | | | | | | | | | |
| HTLV-I-negative T-cell lines | | | | | | | | | | | | | | | | | | |
| Jurkat | | | | | | | | | | | | | | | | | | |
| MOLT-4 | | | | | | | | | | | | | | | | | | |
| HTLV-I-positive T-cell lines | | | | | | | | | | | | | | | | | | |
| MT-1 | | | | | | | | | | | | | | | | | | |
| MT-2 | | | | | | | | | | | | | | | | | | |
| HUT102 | | | | | | | | | | | | | | | | | | |
| SALT3 | | | | | | | | | | | | | | | | | | |
| Mean | | | | | | | | | | | | | | | | | | |
| SD | | | | | | | | | | | | | | | | | | |

PHA/PMA T cells were pretreated with 0.1% of PHA and 10 ng/mL of PMA for 24 hours. Student's unpaired t-test was used to compare the results in the normal PBL-T group. Abbreviation: ND, not done.

* The normal range of LDH is 175 to 400 IU/L.
† The normal range of serum Ca (total) is 2.12 to 2.55 mmol/L.
‡ Positive percentages of obtained mononuclear cells used on this study.
§ The type of ATL is determined using Shimoyama's criteria.

P < .05.
Calcium-dependent homotypic cell adhesion (cluster formation) on HTLV-I-positive T-cell line (MT-2), freshly isolated ATL cells (case no. 4), PHA/PMA-activated T cells (PHA/PMA T cells), and HTLV-I-negative T-cell line (Jurkat). The T-cell lines (1 × 10^6/mL), freshly isolated ATL cells, and PHA/PMA T cells (2 × 10^6/mL) were cultured for 48 hours in 1 mL of serum- and calcium-free culture medium (DCCM-1) and various concentration of calcium chloride (the final total Ca concentration is from 0.1 to 4 mmol/L; the normal serum level is 2.12 to 2.55 mmol/L). (A) HTLV-I-positive T-cell line; MT-2 cells. (B) Freshly isolated ATL cells of case no. 4 as a representative pattern of ATL cells. (C) Normal PB T cells preincubated with 0.1% of PHA and 10 ng/mL of PMA for 24 hours (PHA/PMA T cells). (D) HTLV-I-negative T-cell line; Jurkat cells. All data were representative.

Proliferative response. Freshly isolated cells (1 × 10^6/mL) and cell lines (2 × 10^6/mL) were cultured in a 96-well flat-bottomed microwell in either the presence or absence of MoAbs (0.1 or 1 μg/mL) in 200 μL of RPMI1640 medium containing 8% FCS and 10 μL of human intact globulin (10 μg/mL) to block Fc binding sites in triplicate for 24 hours. The cells were pulsed with 0.5 μCi of tritiated thymidine ([3H]-TdR; specific activity, 5 Ci/mmol/L; the Radiochemical Center, Amersham, Buckinghamshire, UK) for the last 6 hours. [3H]-TdR incorporation was measured by a liquid scintillation counter (Aloka, Tokyo, Japan) after harvesting the cells with a cell harvester (Abekagaku Co, Funabashi, Japan). The viability of the cells after 24 hours of treatment was measured using the trypan blue exclusion test.

RESULTS

Calcium-dependent homotypic cell adhesion of ATL cells. One of the most characteristic features of ATL cells is a rapid cluster formation by in vitro culture (Fig 1). We initially assessed the effect of calcium concentration in media on the homotypic adhesion of ATL cells and HTLV-I-infected T-cell lines MT-2, HUT102, SALT-3, and MT-1. All the ATL cells and cell lines except MT-1 generated clustering in a calcium concentration-dependent manner and the maximum formation was observed at 4 mmol/L total calcium (a level sometimes found in the patients), whereas normal PHA/PMA T cells and HTLV-I carrier T cells did not show the cluster formation at a calcium concentration of more than 2 mmol/L. Jurkat and MOLT-4 cell lines did not form clustering at any calcium concentration. The clustering formations of all the cells used were inhibited by the addition of EDTA (see Fig 3).

Increased expression of ICAM-1 on ATL cells using flow cytometric analysis. To assess which cell surface adhesion molecule is involved in the calcium-dependent homotypic adhesion of ATL cells, we performed two-color flow cytometric analysis with several MoAbs using CD4^+ freshly isolated ATL cells, HTLV-I-positive T-cell lines, PB T cells pretreated or not with PHA/PMA from healthy donors, and HTLV-I carrier T cells shown in Table I. Among the MoAbs that were tested, a dramatic difference between all the ATL cells/cell lines, except for MT-1 and normal PB T cells, was observed in ICAM-1 (CD54) and IL-2Ra chain (CD25) expression. They were highly expressed on all the freshly isolated ATL cells (P < .05) and HTLV-I-infected T-cell lines (P < .05) compared with normal CD4^+ PB T cells. MT-1, an HTLV-I-positive T-cell line, did not express ICAM-1 well but did express CD25. The expression of integrin αL (LFA-1α chain, CD11a), which is a counter-receptor for ICAM-1, of LFA-3(CD58), and of CD44 on fresh ATL cells is comparable to that of normal PB T cells. Interestingly,
HOMOTYPIC ADHESION BY LFA-1/ICAM-1 ON ATL

Fig 2. Effects of MoAbs against ICAM-1, LFA-1α, and LFA-3 on early phase of homotypic cell adhesion of (A) MT-2 cells and (B) ATL cells of case no. 3 tested using a conjugation assay after incubation for 6 minutes. The cells are pretreated by 10 μg/mL of human Ig, divided into effector cells (labeled with hydroethidine) and target cells (labeled with sulfofluorescein diacetate) at a 4:1 E:T ratio, added to 1 μg/10⁶ cells of indicated MoAbs (aICAM-1 is anti-ICAM-1 MoAb), and enumerated using a flow cytometric analysis. For details, see the Materials and Methods. Data are reported as the percentage of total targets presented as conjugates. The conjugated percentage is shown in the top, right-hand corner of each panel.

NKI-L16, which is an MoAb against functionally activated LFA-1, was highly expressed on the freshly isolated ATL cells, MT-2, and MT-1 compared with normal PB T cells, PHA/PMA T cells, and HTLV-I carrier T cells. On the other hand, integrin α4 (VLA4-α chain, CD49d; P < .05) and integrin β1 were rather weaker or reduced on ATL cells than on normal PB T cells.

Adhesion molecules involved in homotypic adhesion of ATL cells. MoAbs against a variety of cell surface adhesion molecules were tested for their ability to inhibit homotypic adhesion at the either very early phase conjugate formation (Fig 2) or late phase clustering formation (Fig 3) using PHA/PMA-T cells, all cases of freshly isolated ATL cells, and HTLV-I-infected T-cell lines except for MT-1. Both phases were tested under the condition of the pretreatment with intact human gammaglobulin to avoid nonspecific Fc-bridging. Homotypic conjugates were observed on ATL cells and cell lines within only 6 minutes after the culture at 37°C. Inhibition of the conjugates among ATL cells was consistently observed with MoAb against LFA-1 and ICAM-1. MoAbs specific for LFA-3 and CD25 did not inhibit it.

The cluster formations shown in Fig 1 were also assessed in the presence of these MoAbs at the higher calcium concentration (3 mmol/L). The formations were reduced by EDTA.

Fig 3. Effects of EDTA and MoAbs against ICAM-1, LFA-1α, LFA-3, CD25, and CD44 on late phase of homotypic cell adhesion observed by cluster formation after incubation in RPMI 1640 medium containing 8% FCS and 3 mmol/L calcium for 48 hours. Pretreated by 10 μg/mL of human Ig. A total of 1 × 10⁶/mL of (A) MT-2 cells and (B) ATL cells of case no. 8 or (C) PHA/PMA T cells are used in this study. (a) No MoAb; (b) 1 mmol/L of EDTA; (c) 10 μg/mL of 84H10 (aICAM-1); (d) 10 μg/mL of TS1/22 (aLFA-1α); (e) 10 μg/mL of TS1/22 (aLFA-3); (f) 10 μg/mL of ACT-1 (aL-2Ra; CD25); (g) 10 μg/mL of NIH44-1 (aCD44).
LFA-1α MoAbs always completely inhibited the calcium-dependent cluster formations of ATL cells and the cell lines (Fig 5). None of the MoAbs against LFA-3, CD25, and CD44, which are highly expressed on ATL cells and HTLV-I–positive T-cell lines, reduced the clustering, although they did partially inhibit the clustering of normal PHA/PMA T cells. The remainder of the analysis will focus on the inhibition of homotypic adhesion by MoAbs against LFA-1 and ICAM-1 with pretreatment with intact human gammaglobulin.

Im mobilized LFA-1 and ICAM-1 MoAbs induced IL-1 mRNA expression and IL-1 secretion. It has been reported that freshly isolated ATL cells and cell lines produce multiple cytokines. We have also reported that ATL cells produce IL-1 in a calcium-dependent manner. We assessed whether homotypic adhesion through LFA-1/ICAM-1 is involved in IL-1 production on ATL cells. We examined IL-1 mRNA expression by ATL cells in the presence of LFA-1, ICAM-1, or control MoAbs using Northern blot analysis in 9 of 10 cases of ATL cells (except case no. 6) and HTLV-I T-cell lines. When soluble MoAb against LFA-1 or ICAM-1 was added to the culture, IL-1α and IL-1β mRNA expression was markedly reduced, whereas anti-LFA-3 and CD25 MoAb did not suppress expression significantly (Fig 4Aa and Ba). We also examined IL-1β secretion from freshly isolated ATL cells after the addition of soluble MoAb against LFA-1α or ICAM-1. IL-1β secretion was significantly reduced in all of the 3 cases tested (nos. 3, 4, and 7; data not shown). Contrarily, when LFA-1 or ICAM-1 molecule on ATL cells was cross-linked by immobilized MoAb against LFA-1 or ICAM-1, IL-1β mRNA expression on freshly isolated ATL cells and IL-1α mRNA on MT-2 cells were dramatically enhanced. Immobilized MoAb against LFA-3 and CD25 did not enhance them significantly (Fig 4Ab and Bb).

When ATL cells and ATL cell lines were added to the anti–ICAM-1/LFA-1 MoAb-immobilized dish, they adhered to the dish with cluster formation. We subsequently measured IL-1β secretion from freshly isolated ATL cells using an ELISA system. As shown in Fig 5A, immobilized MoAbs against LFA-1α and/or ICAM-1 strongly amplified IL-1β secretion in all of the cases of fresh ATL cells by the culture for 48 hours. Control immobilized MoAbs, LFA3, VLA-4, and CD25 had no or scarce effects on IL-1 secretion. Of note is that immobilized or cross-linked LFA-1α MoAb tended to be the most potent activator for IL-1 mRNA expression and the protein production.

Im mobilized LFA-1 and ICAM-1 MoAbs induced PTHrP mRNA expression and PTHrP production. Hypercalcemia in ATL is thought to be mainly caused by highly produced PTHrP as well as IL-1, which is a strong bone-resorbing factor. We tested the effect of the LFA-1/ICAM-1 pathway on PTHrP production from ATL cells and cell lines. On the HTLV-I–positive T-cell line MT-2, when soluble MoAb against LFA-1 or ICAM-1 was added to the culture, PTHrP mRNA expression was also markedly reduced (Fig 4Ca). Contrarily, immobilized LFA-1 or ICAM-1 MoAb augmented the PTHrP mRNA expression, whereas MoAb against LFA-3 and CD25 did not (Fig 4Cb). As shown in Fig 5B, immobilized MoAb against LFA-1α or ICAM-1 strongly amplified PTHrP secretion in all the cases of freshly isolated ATL cells by the culture for 48 hours. We also examined PTHrP secretion from freshly isolated ATL cells after the addition of soluble MoAb against LFA-1α or ICAM-1. PTHrP secretion was significantly reduced in all of the three cases tested (nos. 3, 4, and 7; data not shown).

Effects of MoAbs to adhesion molecules on cell proliferation. Finally, we studied the effects of MoAbs against ICAM-1, LFA-1α, LFA-3, CD25, and CD44 on ATL cell proliferation. Although the ATL cells of all 10 cases were tested, only 5 of the cases (no. 2, 3, 4, 7, and 10) showed significant spontaneous elevation of [3H]-TdR uptake. The spontaneous [3H]-TdR uptakes of each case were comparable with each degree of cluster formation (data not shown). Figure 6 showed the inhibitory effects of soluble MoAbs against adhesion molecules on [3H]-TdR uptake of HTLV-I–positive T-cell lines, PHA/PMA T cells, and the ATL cells of the 5 cases after 24 hours of incubation. Cell viability determined by trypan blue exclusion always exceeded 90%. Soluble MoAbs against not only ICAM-1 and/or LFA-1α but also LFA-3 or CD44 equally blocked their proliferation of PHA/PMA T cells. On the other hand, soluble MoAbs against ICAM-1 and/or LFA-1α consistently and strongly inhibited cell proliferation, and the other soluble MoAbs against LFA-3, CD25, and CD44 showed some inhibition on the proliferation of the ATL cells and HTLV-I–positive T-cell lines, except for MT-1. The proliferation of MT-1, an HTLV-I–positive T-cell line, was not affected well with MoAbs against both ICAM-1 and LFA-1. When LFA-1 or ICAM-1 was cross-linked using MoAb through Fc-binding sites, in the absence of human globulin, the proliferation of ATL cells was markedly augmented.

**DISCUSSION**

The rapid and nodular infiltration of ATL cells into several tissues and secondary lymphoid organs and a subsequent rapid increase in the number of peripheral ATL cells and high incidence of hypercalcemia are the most characteristic features of acute type of ATL and often lead to the severe prognosis. In vitro homotypic cell adhesion and cluster formation are the specific characteristics for freshly isolated ATL cells and HTLV-I–infected T-cell lines. As shown in Fig 1, their cluster formation is observed in a calcium-dependent manner at 4 mmol/L, which is two times higher than the normal serum total calcium level (2.12 to 2.55 mmol/L) but is often experienced in ATL patients. We have already reported that ATL cells proliferate well in a higher calcium concentration in vitro. Here, ATL cells of each case spontaneously proliferate in proportion to their cluster formation. However, PHA/PMA T cells neither make cluster formation nor proliferate at higher than 2 mmol/L calcium. Thus, calcium-dependent cluster formation and proliferation appears to be characteristics of ATL cells. The difference of cluster formation between ATL cells and PHA/PMA T cells at a higher calcium level may be caused by modification of intracellular function induced by tax protein encoded by the pX region of the HTLV-I genome. For example, calpain and calpastatin, which are the potent calcium-dependent proteinase and its inhibitor that can modulate protein kinase and...
LFA-1 and ICAM-1 are crucial for homotypic adhesion in ATL cells. LFA-1 possesses three cation-binding sites and its binding to the ligand is completely dependent on calcium concentration in media, supporting our observation that fresh ATL cells and ATL cell lines make calcium-dependent homotypic adhesion through the LFA-1/ICAM-1 pathway.

We assumed that homotypic clustering of ATL cells would be associated with high levels of certain adhesion molecules. Phenotypic analysis of fresh ATL cells and ATL cell lines indicated that many adhesion molecules that belong to the integrin family, such as α4, β1 integrin, and CD2, were rather weakly expressed and that only LFA-1 (αβ) and LFA-3 preserved their expression on the ATL cells compared with CD4+ normal PBL T cells. Of interest is that the expression of active form of LFA-1 was increased on ATL cells, suggesting that LFA-1 on ATL cells is already activated to be able to adhere to its ligand ICAM-1. Actually, all of the ATL cells of the 5 cases that had significant spontaneous proliferation show high expression of NKI-L16 on the cell surface, although it is unknown why fresh ATL cells have constitutional activation of LFA-1 molecules (Table 1).

ICAM-1, the ligand for LFA-1, was moderately or highly expressed on all fresh ATL cells and ATL cell lines, except for MT-1, compared with normal CD4+ T cells, HTLV-I carrier T cells, and HTLV-I–negative T-cell lines. The excessive expression of ICAM-1 on ATL cells might be specific for ATL because it is induced by HTLV-I tax protein as reported by Fukudome et al and us. ICAM-1 expression on ATL cells was higher in acute-type ATL than in the chronic and lymphoma types of ATL (cases no. 5, 6, and 7) and seems to be related to peripheral ATL cell number. Thus, upregulation of both ICAM-1 and the activated form of LFA-1 on ATL cells appear to be related with the increased aggregation of ATL cells and cell lines. Additionally, MT-1, which did not make cluster formation, did not express ICAM-1 but expressed the activated form of LFA-1. The proliferation of MT-1 was not inhibited by anti-ICAM-1 MoAb but was slightly inhibited by anti-LFA-1 MoAb. It is unknown why ICAM-1 was not expressed on MT-1, but it may be due to the lack of transcriptional factor necessary for ICAM-1 expression on MT-1.

The significance of ICAM-1 expression on ATL cells has raised the following three possibilities. First, ATL cells migrate from circulation into tissue and proliferate in the tissue. However, Uchiyama et al recently reported that E-selectin/
Basal levels of IL-1β (pg/mL/10^6 cells)

<table>
<thead>
<tr>
<th>MoAbs against adhesion molecules</th>
<th>Stimulation Rate (%)</th>
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<tr>
<td>NONE</td>
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<tr>
<td>EDTA</td>
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<tr>
<td>LFA3</td>
<td>0</td>
</tr>
<tr>
<td>VLA4</td>
<td>0</td>
</tr>
<tr>
<td>CD25</td>
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</tr>
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</table>

MoAbs against adhesion molecules

A

Fig 5. Effects of plastic-immobilized MoAbs against adhesion molecules on IL-1β secretion (A) and PTHrP secretion (B) by all cases of freshly isolated ATL cells. The cells (2 x 10^6 cells/mL) were cultured with plastic-immobilized MoAbs against various adhesion molecules containing RPMI 1640 medium with 8% FCS for 48 hours. (A) IL-1β levels in the medium were measured using an ELISA for human IL-1β and the stimulation rate (%) was calculated as follows:

\[
\text{Stimulation Rate} (\%) = \frac{(\text{IL-1β level of added MoAb}) - (\text{Basal level of IL-1β release})}{(\text{Basal level of IL-1β release})} \times 100
\]

(B) PTHrP levels were measured using an RIA for c-terminal region (109-141) of PTHrP and the stimulation rate (%) was calculated as follows:

\[
\text{Stimulation Rate} (\%) = \frac{(\text{PTHrP level of added MoAb}) - (\text{Basal level of PTHrP release})}{(\text{Basal level of PTHrP release})} \times 100
\]

The respective basal levels of IL-1β (pg/mL/2 x 10^6 cells) and PTHrP (pmol/L/2 x 10^6 cells) secretion of the fresh ATL cells are given in the box at the right of each panel.

sialyl Lewis^1^ and VCAM-1/VLA-4 pathway, but not the LFA-1/ICAM-1 pathway, play an important role on ATL cell adhesion to IL-1-activated human umbilical vein endothelial cells as a model of migration of ATL cells.⁴⁴,⁴⁵ Thus, ICAM-1 on ATL cells may not be involved in extravasation. Second, ATL cells can induce the proliferation of resting autologous T cells by the cognate adhesion through ICAM-1 on ATL cells and LFA-1 on T cells.⁴⁶ Therefore, cellular adhesion through ICAM-1 may be related to resting T-cell proliferation. Third, we assessed the mechanism of homotypic adhesion of ATL cells in two phases, ie, an early phase homotypic conjugation at 6 minutes after the culture and a late phase homotypic cluster formation at 48 hours. Our results indicate that anti-LFA-1/ICAM-1 MoAbs dominantly inhibited ATL cell conjugation and cluster formation, whereas all the MoAbs against adhesion molecules comparably but slightly reduced the cluster formation of PHA/PMA T cells. Thus, the significance of consistently high expression of ICAM-1 on the ATL cells involves the homotypic adhesion of ATL cells through LFA-1/ICAM-1 pathway in tissue. Actually, ATL cells frequently proliferate, making nodular formations in tissue. However, our results showed that ATL cell proliferation was more correlated with the degree of active form LFA-1 expression than with ICAM-1 expression, suggesting that LFA-1 activation might be more important than ICAM-1 expression on ATL cell proliferation.

Of note is that immobilized MoAb against LFA-1 or ICAM-1 and cross-linking of LFA-1 or ICAM-1 through Fc-receptors such as CD16, CD32, or CD64, which are highly expressed on fresh ATL cells (data not shown), induced both
the production of IL-1 and PTHrP from the ATL cells and the proliferation of the ATL cells. This finding suggests that such adhesion molecules on ATL cells not only function as glue but also mediate signal transduction. LFA-1/ICAM-1 is involved in tyrosine phosphorylation in T cells or human epidermal carcinoma (KB) cells. Webb et al. also showed that cellular interaction through adhesion molecules such as LFA-3 and CD44 is critically important in the induction of IL-1 and TNF mRNA on monocytes. However, there is little information on the signaling properties of ICAM-1. Although it remains to be discussed which signaling pathway participates, this is the first evidence that the cross-linking of ICAM-1 by direct MoAbs is involved in cytokine production.

ATL cells produce multiple cytokines and hormones, such as IL-1, TNF, IL-2, IL-6, and PTHrP. We have also reported that fresh ATL cells and cell lines produce mRNA and protein of IL-1α and IL-1β in a calcium-dependent manner. However, cytokines are not necessarily required for the proliferation of ATL cells, because the effects of any anticytokine antibodies against such as IL-1 and IL-2 are on spontaneous proliferation of ATL cells were restricted to only special ATL cases. Contrary to cytokines, the inhibition of the homotypic adhesion through LFA-1/ICAM-1 by the soluble MoAbs significantly reduced proliferation. The soluble MoAbs also completely and consistently reduced the mRNA expression and protein release of both IL-1 and PTHrP of all the ATL cells and ATL cell lines tested. On the other hand, cross-linking of LFA-1 or ICAM-1 using immobilized MoAbs markedly amplified the mRNA transcription and protein secretion of both IL-1 and PTHrP and the proliferation of ATL cells. In some cases (cases no. 1, 8, and 9), ATL cells secrete significant IL-10 but little PTHrP, or vice versa, and the inhibition of cytokine secretion from ATL cells by anti-LFA-1/ICAM-1 MoAb did not always correlate with that of the cluster formation and proliferation. Furthermore, MoAb against LFA-3 or CD44 did not reduce cluster formation but did slightly block proliferation of ATL cells. These results suggest that a rich diversity of cellular mechanisms may differently regulate cytokine production and proliferation via different signaling pathway and that cell-cell contact initialized by LFA-1/ICAM-1 may not only transduce signaling by the engagement of ICAM-1 by themselves but also induce redundant signaling through...
the other functioning molecules on the surface of ATL cells such as LFA-3 and CD44. It is noteworthy that IL-1 and PTHrP are representative bone-resorbing factors, suggesting that adhesion-induced secretion of these factors play an important role in the induction of hypercalcemia by bone resorption, which serves an appropriate environment for calcium-dependent proliferation and cytokine production of ATL cells. Thus, homotypic adhesion of ATL cells through LFA-1/ICAM-1 appears to be an initial and critical step for the subsequent events, the signal transduction that adhesion-induced secretion of these factors play an important role in the induction of hypercalcemia by bone resorption, which serves an appropriate environment for calcium-dependent proliferation and cytokine production of ATL cells.

Considering these results, we propose the concept of the combinatorial sequence of multiple phenomena in ATL cell nodular proliferation in multiple tissue and hypercalcemia, ie, high expression of ICAM-1 possibly induced by HTLV-I tax gene, the homotypic and calcium-dependent adhesion through activated LFA-1/ICAM-1 that is the initial and critical step for the subsequent events, the signal transduction from these adhesion molecules, the production of multiple bone-resorbing cytokines such as IL-1 and PTHrP, and proliferation of ATL cells.

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Calcium-dependent homotypic adhesion through leukocyte function-associated antigen-1/intracellular adhesion molecule-1 induces interleukin-1 and parathyroid hormone-related protein production on adult T-cell leukemia cells in vitro

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