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# Calcium Dependency of the Production of Interleukin 1 and the Expression of Interleukin 1 Receptors of Human Adult T-Cell Leukemia Cells *in Vitro*[1]

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## ABSTRACT

**The effect of calcium on the production of interleukin 1 (IL 1) and the expression of IL 1 receptors (R) of adult T-cell leukemia (ATL) cells was studied *in vitro*. ATL cells freshly obtained from patients and ATL cell lines produced limited amounts of IL 1 by culturing in a low-calcium concentration of medium (<0.01 mM). However, the production of IL 1 was enhanced by the addition of calcium chloride to the medium in a concentration-dependent manner and reached the maximum at the higher calcium concentration (3–4 mM) than at the standard calcium concentration of medium (1.26 mM). The production of IL 1 from ATL cells was further enhanced by calcium ionophore. Furthermore, the expression of IL 1R on ATL cells was augmented in proportion to the extracellular calcium concentration and calcium ionophore. In accordance with the change of the extracellular calcium concentration, the intracellular calcium concentration of ATL cells detected by Fura 2 was changed. However, this calcium dependency was not observed in the human T-cell leukemia virus I-negative acute T-cell leukemia cells. These results suggest that calcium plays a critical role in the regulation of the production of IL 1 and the expression of IL 1R on ATL cells.**

## INTRODUCTION

ATL[3] is a unique T-cell malignancy which is caused by the human T-cell leukemia virus type I. The complication of hypercalcemia is experienced by about 70% of ATL patients, is one of the most difficult problems to treat since the acute and critical elevation of the number of peripheral WBCs and the abrupt swelling of lymph nodes are often parallel with the increase in the serum calcium concentration, and often results in death (1–3). We have reported in a series of studies related to ATL that ATL cell lines and ATL cells freshly obtained from patients produce interleukin 1 (IL 1) which not only stimulates the growth of ATL cells by an autocrine mechanism but also possesses a bone-resorbing activity that is one of the most important causes of hypercalcemia in ATL patients (4–7). It has been reported that calcium plays an important role in the regulation of the growth and the functions of many kinds of cells (8, 9). Furthermore, several reports suggest a potential role of calcium in the activation and secretion events associated with the production of IL 1 from monocytes and monocytic cell lines (10, 11). These investigations suggest a possibility that calcium also regulates the production of IL 1 from ATL cells *in vitro*. In order to assess this possibility, we have analyzed the effect of calcium and calcium ionophore on the production of IL 1 and the expression of IL 1R of ATL cells *in vitro*.

## MATERIALS AND METHODS

**Subjects.** Peripheral blood lymphocytes from 10 patients with ATL admitted to our hospital were tested in this study. ATL was diagnosed according to the following criteria: (*a*) the presence of a highly convoluted nucleus, (*b*) the expression of CD2 antigen and CD4 antigen, (*c*) the patient has anti-ATL-associated antigen antibody in the serum. All patients had >80% convoluted nucleus-positive cells and >95% had CD4-positive cells in their peripheral blood.

**Preparation of ATL Cells.** Leukemic cells were isolated from heparinized peripheral blood from ATL patients by centrifugation over a lymphocyte separation medium (Litton Bionetics, Kensington, MD) and were washed with 10 mM PBS, pH 7.4. The obtained cells were incubated in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) containing 10% FCS (Grand Island Biological Co., Grand Island, NY) using plastic culture dishes (Falcon 3002; Falcon Plastics, Oxnard, CA) at 37°C for 2 h in 5% $CO_2$ and 95% air. After the incubation, nonadherent cells were removed by gentle washing with PBS and were used as ATL cells. The ATL cell line, MT2, established by Dr. I. Miyoshi (Kochi Medical College, Nangoku, Japan) (12), was maintained *in vitro* by culturing in RPMI 1640 medium containing 10% FCS.

**Preparation of Calcium-free Medium.** For this study, we prepared a calcium-free medium based on the reports by Corradin *et al.* (13). This contained 100 mg glucose, 800 mg sodium sulfate, 40 mg potassium chloride, 4.8 mg sodium monohydrogen phosphate, 6 mg potassium dihydrogen phosphate, 4.9 mg magnesium sulfate, 4.7 mg magnesium chloride, 0.6 mg phenol red (Nakarai Chemical Co., Kyoto, Japan), 2 ml 50-fold concentrated essential amino acids, 2 ml 100-fold concentrated nonessential amino acids, 2.5 ml 100 mM sodium pyruvate, 2 ml 100 mM glutamine, 0.5 ml *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 2 ml penicillin/streptomycin (5000 units/5000 μg/ml), 1.3 ml 7.5% sodium bicarbonate (Flow Laboratories, North Ryde, New South Wales, Australia), 2.5 ml nucleic acid precursor (1 mg/ml; Dojin Laboratories, Kumamoto, Japan), 0.45 ml 2N sodium hydroxide, and 0.05 ml 0.1 M 2-mercaptoethanol (Nakarai Chemical Co.) in 100 ml distilled water. The medium was used after sterilization through a Millipore filter (0.30-μm pore size, Japan Millipore Ltd., Tokyo, Japan). The calcium concentration of this medium is <0.01 mM.

**Preparation of IL 1-like Factors from ATL Cells.** ATL cells ($1 \times 10^6$/ml) were incubated in a 5-ml medium containing various concentrations of calcium chloride and 1% FCS using plastic culture dishes at 37°C for 48 h in 5% $CO_2$ and 95% air. The culture supernatant was collected by centrifugation, dialyzed against 100-fold volumes of RPMI 1640 medium, sterilized by Millipore filtration, and used as factors derived from ATL cells.

**Assay of IL 1-like Activity.** Thymocytes ($1.5 \times 10^6$) of 4-week-old A.TH mice (bred in our laboratory) were cultured in 0.2 ml of Eagle's-Hanks' amino acid medium described by Corradin *et al.* (13) containing 10% FCS with serially diluted IL 1 samples in flat-bottomed microtiter culture plates (Falcon 3072) at 37°C for 3 days in 5% $CO_2$ and 95% air (14). The cells were pulsed with 0.5 μCi [$^3$H]thymidine for the last 24 h and were harvested with the aid of an automated cell harvestor. The results were expressed as units of IL 1 produced by $10^6$ ATL cells using human recombinant IL 1α (kindly donated by Dr. M. Yamada, Dainippon Pharmaceutical Co., Osaka, Japan) as the standard.

**Measurement of Intracellular Calcium Concentration.** MT2 cells ($10^6$/ml) were cultured in the medium containing various concentrations of calcium chloride with 1% FCS at 37°C for 24 h. The recovered cells were loaded with 1 μM Fura 2 AM (Dojin Laboratories) at 37°C for 20 min and washed with PBS, and the fluorescence of Fura 2-loaded cells was measured in an Hitachi fluorescence spectrophotometer using an excitation wavelength of 350 nm and emission at 500 nm. The intracellular calcium concentration was expressed as nM using the following formula (15):

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[3] The abbreviations used are: ATL, adult T-cell leukemia; IL 1, interleukin 1; IL 1R, IL 1 receptor; PBS, phosphate-buffered saline; FCS, fetal calf serum.

$$(Ca^{2+})_i = \frac{F - F_{min}}{F_{max} - F} \times 135 \text{ nM}$$

where $F$ is the experimental value; $F_{max}$ was given by the presence of 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO); $F_{min}$ was given by the presence of 1 mM $MnCl_2$ (Nakarai Chemical Co.).

**Radiolabeling of IL 1α.** IL 1R on ATL cells was assayed as previously reported (5, 16). Recombinant human IL 1 α was labeled by the method of Dower *et al.* (17). Briefly, 1 μg of IL 1α in 50 μl 0.1 M borate buffer, pH 8.5, was added to 1 mCi of $^{125}$I-labeled Bolton Hunter reagent (2200 Ci/mM; New England Nuclear, Boston, MA) that had been evaporated by a gentle stream of dry nitrogen. The reaction was allowed to proceed on ice for 1 h and was terminated by the addition of 5 μl of 1 M glycine ethyl ester. Thirty μl of 2% gelatin in PBS (0.05 M phosphate, 0.15 M buffered saline), pH 7.4, was added as a carrier, and labeled IL 1α was separated by chromatography on a 1-ml column of Biogel P-10 (Bio-Rad Laboratories, Richmond, CA). Aliquots (100 μl) were collected, and fractions containing protein-bound radioactivity were pooled. The specific activity of $^{125}$I-labeled IL 1α was $7.6 \times 10^6$ cpm/μg protein. $^{125}$I-labeled IL 1α migrated as a single $M_r$ 17,000 band on sodium dodecyl sulfate-polyacrylamide gels. Although the radiolabeled IL 1α lost about 80% of its activity when assayed by murine thymocyte proliferative responses, it retained binding activity to rabbit anti-IL 1α antibody.

**Binding of $^{125}$I-labeled IL 1α to ATL Cells.** After *in vitro* culture under the various conditions, the ATL cells were washed three times with PBS containing 1 mg/ml bovine serum albumin (Sigma Chemical Co.) and the cell number was adjusted to $3 \times 10^6$ cells/0.2 ml PBS. $^{125}$I-labeled IL 1α (0.5 nM) was added to the cell suspensions in Eppendorf tubes and incubated at 4°C for 1 h. Then, the cell suspensions were layered over 200 μl of a mixture of 20% olive oil and 80% di-*N*-butyl phthalate by the method of Uchiyama *et al.* (18) and were centrifuged at 10,000 × *g* at 4°C for 1 min to separate free and bound IL 1. The nonspecific binding of $^{125}$I-labeled IL 1α was determined by incubated cells with labeled IL 1 in the presence of a 100-fold excess of cold IL 1. The specificity of IL 1R was assessed by competition experiments using unlabeled IL 1α, IL 1β (kindly donated by Dr. Y. Hirai, Otsuka Pharmaceutical Co., Tokushima, Japan), recombinant human interleukin 2 (kindly donated from Takeda Pharmaceutical Co., Osaka, Japan), and recombinant human interferon α and γ (kindly donated from Kyowahakko Kogyo Co., Tokyo, Japan) as reported previously (5, 16). The results were expressed as the mean cpm (specific binding), in which nonspecific binding was subtracted, and SE of triplicate determinations.

**Statistical Analysis.** Statistical analysis of the difference between the control and the experimental group was performed using Student's *t* test.

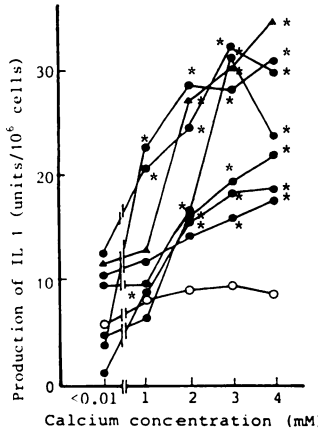


Fig. 1. Effect of calcium on the production of IL 1 by ATL cells. ATL cells (●) freshly obtained from patients, MT2 cells (▲), and the human T-cell leukemia virus type I-negative acute T-cell leukemia cells (○) ($1 \times 10^6$/ml) were incubated in a 5-ml medium containing various concentrations of calcium chloride at 37°C for 48 h and the culture supernatant was collected, dialyzed, and used as IL 1-like factors. The IL 1 activity of the culture supernatant was assayed by the stimulation of murine thymocyte proliferative responses. The results are expressed as units of IL 1 produced by $10^6$ ATL cells using human recombinant IL 1α as the standard. *, significantly different group ($P < 0.05$) from the control group (cultured in the low-calcium medium) of each ATL cells.

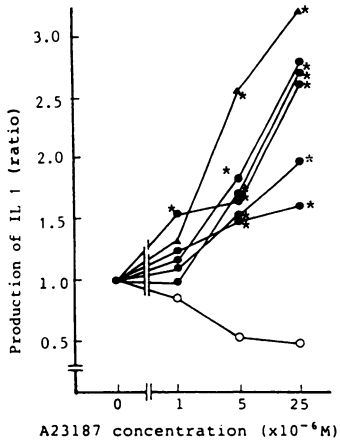


Fig. 2. Effect of calcium ionophore on the production of IL 1 by ATL cells. ATL cells (●), MT2 cells (▲), and the human T-cell leukemia virus type I-negative acute T-cell leukemia cells (○) ($1 \times 10^6$/ml) were cultured in a 5-ml medium containing 1 mM calcium chloride in the absence or the presence of various concentrations of A23187 for 48 h. The IL 1 activity of the culture supernatant was assayed by the stimulation of murine thymocyte proliferative responses. The results are expressed as ratios of the amount of IL 1 produced in each condition to the amount of IL 1 produced without A23187. *, significantly different group ($P < 0.05$) from the control group (without A23187) of ATL cells.

## RESULTS

**Production of IL 1 by ATL Cells Is Dependent on Extracellular Calcium Concentration.** Initially, we assessed the effect of the concentration of calcium in the culture medium on the *in vitro* production of IL 1 by ATL cells. ATL cells were incubated in a medium containing various concentrations of calcium chloride and the IL 1-activity of the culture supernatant was assayed by the stimulation of murine thymocyte proliferative responses. As shown in Fig. 1, ATL cells produced only limited amounts of IL 1 in a low-calcium medium. However, the addition of calcium chloride to this medium enhanced the production of IL 1 by ATL cells in a concentration-dependent manner. The maximum IL 1-production was induced at 3 or 4 mM calcium chloride, which was higher than that of the standard medium condition, 1.26 mM. The IL 1-activity produced by ATL cells was absorbed by anti-IL 1α antibody but not by anti-IL 1β antibody as reported previously (4, 7). On the other hand, the production of IL 1 by the human T-cell leukemia virus type I-negative acute T-cell leukemia cells was not enhanced by the addition of calcium chloride. These results suggest that the production of IL 1 by ATL cells is dependent on the calcium level in the culture medium.

**Effect of Calcium Ionophore on the Production of IL 1 by ATL Cells.** We next assessed the effect of calcium ionophore, A23187, which augments the influx of the extracellular calcium into cells, on the production of IL 1 by ATL cells *in vitro*. A23187 was added to the culture of the standard calcium medium at the final concentration of $1–25 \times 10^{-6}$ M. A23187 was removed from the culture supernatant by dialysis. As shown in Fig. 2, A23187 augmented the production of IL 1 by ATL cells in a concentration dependent manner. The viability of cells at $25 \times 10^{-6}$ M remained 95% as assessed by a trypan blue dye exclusion test at 48 h of the culture. On the other hand, A23187 had no effect or reduced the production of IL 1 by the human T-cell leukemia virus type I-negative acute T-cell leukemia cells.

These results further suggest that calcium plays an important role in the production of IL 1 by ATL cells.

**Intracellular Calcium Concentration of ATL Cells Cultured under Various Conditions.** Next, we studied whether the culture conditions, changing the extracellular calcium concentration, resulted in the change of the intracellular calcium concentration. MT2 cells were cultured in a medium containing various concentrations of calcium chloride at 37°C for 24 h and the intracellular calcium concentration was measured by Fura 2. As shown in Table 1, the intracellular calcium concentration of MT2 cells increased with the increase in the extracellular calcium concentration. Furthermore, it was further increased by calcium ionophore. These results are consistent with our previous report in which we measured the intracellular calcium concentration by Quin 2 (19). These results suggest that the manipulation of the extracellular calcium concentration results in the change of the intracellular calcium concentration and leads to the change of the activity to produce IL 1 in ATL cells.

**Effect of Calcium on the Expression of IL 1R on ATL Cells.** The initial step of the IL 1 action on cells is the binding of IL 1 on the specific IL 1R expressed on the cells (16, 17). We have reported that ATL cells express a larger number of IL 1R than normal T-cells (5). Then, we finally studied the effect of calcium on the expression of IL 1R on ATL cells. ATL cells were incubated in a medium containing various concentrations of calcium chloride at 37°C for 48 h and $^{125}$I-labeled IL 1$\alpha$ binding to the cells was assayed. As shown in Fig. 3, the expression of IL 1R on ATL cells was increased in proportion to the concentration of calcium chloride in the medium. On the other hand, the expression of IL 1R on the human T-cell leukemia virus type I-negative acute T-cell leukemia cells was not enhanced but, rather, reduced in the higher calcium concentration. Furthermore, as shown in Fig. 4, the expression of IL 1R on ATL cells was enhanced by calcium ionophore. These results suggest that the expression of IL 1R on ATL cells is also regulated by calcium.

Table 1 *Intracellular calcium concentration of MT2 cells cultured in vitro*

MT2 cells ($1 \times 10^6$/ml) were cultured in a medium containing various concentrations of $CaCl_2$ and calcium ionophore as indicated at 37°C for 24 h. The recovered cells were loaded with 1 $\mu$M Fura 2 AM at 37°C for 20 min and washed and the fluorescence of Fura 2-loaded cells was measured in a fluorescence spectrophotometer.

| Group | Concentration of $CaCl_2$ (mM) | Agents added | Intracellular calcium concentration (nM) |
|---|---|---|---|
| 1 | 0.01 | –[a] | 118.6 ± 8.9 |
| 2 | 1 | – | 136.0 ± 15.1 |
| 3 | 2 | – | 219.1 ± 2.5[b] |
| 4 | 3 | – | 261.1 ± 8.5[b] |
| 5 | 4 | – | 318.9 ± 45.7[b] |
| 6 | 1 | 5 $\mu$M calcium ionophore | 163.2 ± 18.0 |
| 7 | 1 | 25 $\mu$M calcium ionophore | 285.7 ± 31.7[c] |

[a] None added.
[b] Significantly different ($P < 0.05$) from group 1.
[c] Significantly different ($P < 0.05$) from group 2.

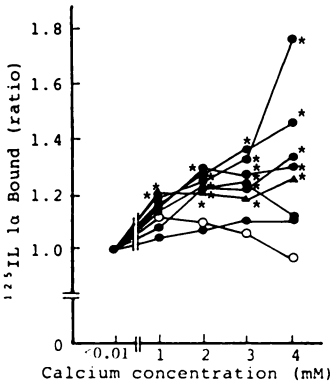


Fig. 3. Effect of calcium on the expression of IL 1R on ATL cells. ATL cells (●), MT2 cells (▲), and the human T-cell leukemia virus type I-negative acute T-cell leukemia cells (○) ($1 \times 10^6$/ml) were incubated in a 5-ml medium containing various concentrations of calcium chloride for 48 h. The obtained cells were treated with $^{125}$I-labeled IL 1$\alpha$ at 4°C for 1 h and the cell-bound radioactivities were counted. The results are expressed as the ratios of the specific binding (cpm) of $^{125}$I-labeled IL 1$\alpha$ on the cells cultured in each condition to the cpm of the cells cultured in a low-calcium medium. *, significantly different group ($P < 0.05$) from the control group (without calcium chloride) of ATL cells.

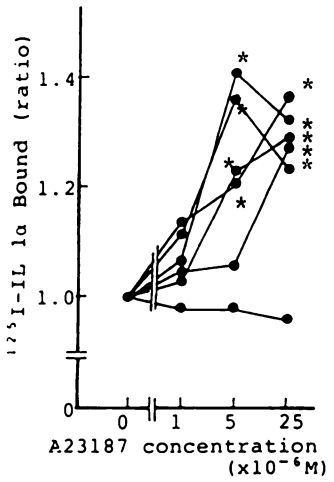


Fig. 4. Effect of calcium ionophore on the expression of IL 1R on ATL cells. ATL cells ($1 \times 10^6$/ml) were cultured in a 5-ml medium containing 1 mM calcium chloride in the absence or the presence of various concentrations of A23187 for 48 h. The obtained cells were treated with $^{125}$I-labeled IL 1$\alpha$ and the cell-bound radioactivities were counted. The results are expressed as the ratios of the specific binding (cpm) of $^{125}$I-labeled IL 1$\alpha$ to ATL cells cultured in each condition to the cpm of ATL cells cultured without A23187. *, significantly different group ($P < 0.05$) from the control group (without A23187) of ATL cells.

## DISCUSSION

It is well known that calcium has a regulatory role in the growth and the functions, especially the secretory events of a number of proteins, of several kinds of cells including lymphocytes (8, 9, 20). In a low-calcium medium, lymphocytes cannot be stimulated to proliferate by mitogenic lectins; on the other hand, excessive concentrations of calcium act on lymphocytes with toxic effects (21). It is well known that IL 1 is a regulatory molecule for the induction of many types of immune and inflammatory responses (22, 23). Recently, IL 1 has been shown to function as a growth factor for several kinds of tumor cells such as Epstein-Barr virus-transformed B-cell lines and acute myelogenous leukemias (24, 25). It is further reported that IL 1 plays an autocrine role in the proliferation of T-cell clones (26). We have reported that IL 1 augments the growth of ATL cells by an autocrine mechanism (5). Furthermore, we have reported that the *in vitro* growth of ATL cells is dependent upon the extracellular calcium level (19). In this investigation, we found that the production of IL 1 by ATL cells was limited in a low-calcium medium but was enhanced by the increase in the extracellular calcium level to the excessively high concentration. The intracellular calcium concentration of ATL cells was also increased by the increase in the extracellular calcium concentration. Furthermore, the production of IL 1 by ATL cells was augmented by a calcium ionophore which stimulates the influx of extracellular calcium into the cells. These results suggest that the production of IL 1 by ATL cells is dependent upon the extra- and intracellular calcium concentration. On the other hand, the production of IL 1 by the human T-cell leuke-

mia virus type I-negative acute T-cell leukemia cells was not markedly affected by changing the extracellular calcium concentration. The production of IL 1 by these cells was not augmented but, rather, decreased by the addition of calcium ionophore. With regard to the calcium dependency of the production of IL 1, contrary results have been reported (10, 11, 27). In our investigation, the production of IL 1 by ATL cells depends on the calcium concentration, but the human T-cell leukemia virus type I-negative acute T-cell leukemia cells do not. Therefore, the calcium dependency of the IL 1 production depends upon the cell types and in ATL cells calcium plays a central role in the production of IL 1.

In the previous paper, we reported that IL 1 stimulated the growth of ATL cells by an autocrine mechanism (5). The initial step of the IL 1 action in these responses is the binding of IL 1 on the specific IL 1R expressed on the cells (17). In fact, the number of IL 1R on ATL cells is 10 times more than on normal T-cells (5). The expression of IL 1R on ATL cells was also dependent upon the extracellular calcium concentration and further augmented by a calcium ionophore. However, it is difficult to conclude that calcium primarily and/or directly augments the expression of IL 1R on ATL cells. IL 1 produced by ATL cells may function as a stimulatory signal for the expression of IL 1R. It has been reported that the expression of IL 1R is induced with the down- and upregulation mechanisms by IL 1 (28, 29). We have found that the expression of IL 1R on some ATL cells can be upregulated by exogeneous IL 1 (data not shown). Thus, a possibility is considered that the expression of IL 1R on ATL cells might be secondarily induced as a result of the enhanced IL 1 production by the increase of calcium level. With regard to the calcium dependency of the IL 1R-expression on ATL cells, further molecular analysis is required.

Calcium influx into cells augments some enzyme systems such as the protein kinase C-dependent pathway and calmodulin-dependent kinase pathway and induces second signal transduction mechanisms and the synthesis of machineries required for the replications. In this investigation, we did not study extensively these points. However, it may be the next step to study the several signal transduction systems induced by the calcium influx into ATL cells.

The important question raised by these investigations may be the *in vivo* relevance of these phenomena. It has been reported that IL 1 is one of the most important factors which cause hypercalcemia by a marked increase in osteoclastic bone resorption systems *in vitro* and *in vivo* (6, 7, 30–33). We have reported that ATL cells freshly obtained from patients and ATL cell lines produce IL 1 which stimulates not only the growth of ATL cells but also bone resorption systems and causes the hypercalcemia in ATL patients. In this study, we found that a maximum production of IL 1 by ATL cells was induced at an excessively higher concentration of the extracellular calcium level than at the standard calcium level and that the IL 1 production was calcium dependent. Furthermore, we have reported that the growth of ATL cells is also dependent upon the extracellular calcium level (19). We often observed in clinical studies that the patients with ATL have accompanying hypercalcemia [calcium often >15 mg/ml; normal calcium level approximately 8 mg/ml (19)]. Furthermore, the number of ATL cells in the peripheral blood of patients parallels the serum calcium level. Accordingly, in ATL patients, the increased serum calcium level may stimulate the production of IL 1 by ATL cells and the growth of ATL cells and the enhanced production of IL 1 inversely results in the hypercalcemia by stimulating osteoclastic bone resorption systems. Thus, IL 1 and hypercalcemia bring about a vicious circle in the growth of ATL cells *in vivo*.

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