[CANCER RESEARCH 48, 4284–4287, August 1, 1988]

Production of Bone-resorbing Activity Corresponding to Interleukin-1α by Adult T-Cell Leukemia Cells in Humans[1]

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ABSTRACT

The physicochemical properties and relationship of bone-resorbing activity and interleukin 1 (IL-1) produced by adult T-cell leukemia (ATL) cells and cell line were studied *in vitro*. The culture supernatant of ATL cell line, MT2, and peripheral blood lymphocytes freshly obtained from ATL patients had both IL-1 activity detected by the stimulation of murine thymocyte-proliferative responses and bone-resorbing activity detected by the stimulation of ^{45}Ca release from prelabeled murine fetal bones. By Sephacryl S-200 column chromatography, both activities were eluted as a single peak at approximately M_r 15,000. By the chromatofocusing technique, the isoelectric point values of both activities were estimated as pH 4.8 and 5.2. Furthermore, both activities were absorbed with rabbit anti-IL-1α antiserum, but not with anti-IL-1β antiserum. These results suggest that ATL cells and cell line produce bone-resorbing activity which corresponds to IL-1α and that this IL-1α is one of the most important causes of hypercalcemia in ATL patients.

INTRODUCTION

Patients with ATL,[3] caused by an infection of human T-cell leukemia-lymphoma virus I often suffer from hypercalcemia which is one of the most difficult problems to treat and often results in death (1, 2). However, the pathogenesis of hypercalcemia in ATL patients is not fully understood. Recently, it has been reported that colony-stimulating factor, transforming growth factor, tumor necrosis factor, and interleukin 1β have BRA *in vitro* (3–8). Thus, hypercalcemia in ATL patients might be induced with some factors produced by leukemic cells. In the previous paper, we reported that ATL cells and cell lines produced BRA and IL-1α (9, 10). These reports suggest a possibility that BRA produced by ATL cells is mediated by IL-1α. In order to clarify this possibility, we have analyzed the physicochemical properties of BRA produced by ATL cells and cell lines. In this paper, evidence will be presented to show that ATL cells and cell lines produce BRA which corresponds to IL-1α and that this IL-1 is one of the most important causes of hypercalcemia in ATL patients.

MATERIALS AND METHODS

Subjects. Eight patients with ATL admitted in our hospital were used for this study. The diagnosis for ATL was performed by the following criteria: (*a*) neoplastic cells have a highly convoluted nucleus; (*b*) the cells have T-cell markers which form rosettes with sheep erythrocytes and are CD4 positive; and (*c*) the patient has anti-ATL-associated antigen antibodies in his/her serum.

Preparation of Peripheral Blood Lymphocytes. PBL were collected from heparinized peripheral blood by centrifugation over a lymphocyte separation medium (Litton Bionetics, Kensington, MD), washed with 10 mM PBS (pH 7.2), and suspended in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) containing 10% FCS (GIBCO), penicillin (50 units/ml; Flow Laboratories, North Ryde, New South Wales, Australia), and streptomycin (50 μg/ml; Flow).

ATL Cell Line. The ATL cell line used in this experiment was MT2 established by Dr. I. Miyoshi (Kochi Medical College, Nangoku, Japan) (11). It was maintained *in vitro* by culturing in RPMI 1640 containing 10% FCS, penicillin, and streptomycin at 37°C in 5% CO_2 and 95% air.

Preparation of Culture Supernatants. PBL (5×10^6/ml) from normal control or ATL patients and MT2 cells (1×10^6/ml) were cultured in RPMI 1640 containing 5% FCS for 2 days. The culture supernatants were concentrated 10 times using an ultrafiltration unit with YM-5 Diaflo membranes (Amicon Co., Lexington, MA), were dialyzed against RPMI 1640, and the activities of IL-1 and BRA were measured.

Assay of IL-1 Activity. IL-1 activity was determined by the stimulation of murine thymocyte proliferative responses according to the method of Mizel *et al.* (12). Thymocytes (1.5×10^6) from A.TH mice were cultured with serially diluted IL-1 sources in Eagle's Hanks' amino acid culture medium (Flow) described by Corradin *et al.* (13) containing 10% FCS in flat-bottomed microtiter culture plates for 3 days. The cells were pulsed with 1 μCi of tritiated thymidine (Amersham, Buckinghamshire, UK) for the last 24 h, were harvested with the aid of an automated cell harvester and the [^{3}H]thymidine incorporated into thymocytes was counted (14). The results were expressed as units of IL-1 produced by 1×10^7 ATL cells using ultrapure IL-1 (Genzyme Co., Boston, MA) as the standard.

Assay of BRA. BRA was assayed by culturing shafts of the radius and ulna of ICR mouse fetuses, which had been prelabeled with ^{45}Ca *in vivo*, in BGJ medium (GIBCO) according to the method of Raisz (15). After a 24-h period of preculture, the bones were transferred to the new medium containing the culture supernatant of ATL cells (20%) and 0.2% bovine serum albumin. After 5-day culture, the released ^{45}Ca was counted. Percentage of ^{45}Ca release was calculated as

$$(\text{medium cpm}/\text{medium cpm} + \text{bone cpm}) \times 100$$

The results were expressed as the means and standard deviations in quadruplicate cultures. The minimum prostaglandin E_1 concentration to elicit a significant BRA was 10^{-8} M in this system.

Sephacryl S-200 Column Chromatography of MT2 Culture Supernatant. The culture supernatant of MT2 cells (100 ml) was concentrated with an ultrafiltration device using YM-5 Diaflo membrane (Amicon), was applied on a Sephacryl S-200 column (3.5 × 60 cm; Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with PBS, was eluted with PBS at the rate of 10 ml/h, and aliquots of 5 ml were collected. The column size was determined by chromatography of marker proteins: blue dextran (void volume), human immunoglobulin G (M_r 150,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 44,000), soybean trypsin inhibitor (M_r 22,000), and cytochrome *c* (M_r 15,000). The protein concentration of each fraction was measured by the absorbance at 280 nm. IL-1 activity and BRA of each fraction were assayed by the method described above.

Isoelectric Chromatofocusing Technique. The active fractions of the Sephacryl S-200 column chromatography (Fig. 1, *Fraction 58-65*) were pooled, concentrated, and dialyzed against 0.025 M imidazole hydro-

Received 12/11/87; revised 4/4/88; accepted 4/25/88.

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[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and for Cancer Research from the Ministry of Health and Welfare, Japan (60-29).

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[3] The abbreviations used are: ATL, adult T-cell leukemia; BRA, bone-resorbing activity; FCS, fetal calf serum; IL-1, interleukin 1; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; BGJ, Biggers, Gwatkin, and Jackson.

chloride buffer, pH 7.4. The sample was applied on a column of PBE 94 gel (10 ml; Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.025 M imidazole hydrochloride buffer, pH 7.4, was eluted with polybuffer 74-hydrochloride, pH 4.0, and aliquots of 5 ml were collected. The pH value of each fraction was measured by a pH meter. IL-1 activity and BRA of each fraction were assayed by the method described above.

Source of IL-1α, IL-1β, and Their Antisera. Recombinant human IL-1α and β were kindly supplied by Dr. M. Yamada (Dainippon Pharmaceutical Co., Osaka, Japan) and Dr. Y. Hirai (Ohtsuka Pharmaceutical Co., Tokushima, Japan), respectively (16, 17). Anti-IL-1α antisera and anti-IL-1β antisera were prepared by immunizing rabbits with IL-1α or IL-1β as reported previously (9).

RESULTS

Production of BRA by ATL Cells and Cell Line. ATL cell line MT2, normal PBL, and PBL (more than 80% of lymphocytes were CD4-positive ATL cells) obtained from ATL patients were cultured *in vitro* and the IL-1 activity and BRA produced in the culture supernatant were measured. As shown in Table 1, the culture supernatant of MT2 cells and PBL from ATL patients (seven out of eight) possessed IL-1 activity detected by murine thymocyte-proliferative responses and BRA detected by ^{45}Ca release from prelabeled murine fetal bones. On the other hand, PBL from 10 normal volunteers studied did not produce significant amounts of both activities. BRA was parallel with IL-1 activity. Furthermore, a significant correlation was observed between the levels of IL-1 and bone-resorbing activities in the culture supernatant of ATL PBL and the serum calcium level of ATL patients ($r = 0.727$ and 0.726, respectively).

Physicochemical Properties of BRA. To study the physicochemical properties of IL-1 activity and BRA, we fractionated the culture supernatant of MT2 cells with a Sephacryl S-200 column. As shown in Fig. 1, IL-1 activity was eluted as a single peak at the molecular weight of approximately 15,000. No fractions contained detectable amounts of interleukin 2 activity as assayed by the growth of interleukin 2-dependent murine cytotoxic T-cell line (18). BRA was also eluted as a single peak by the same profile as IL-1 activity. The elution patterns and the peaks of IL-1 activity and BRA of the culture supernatants

Table 1 *Interleukin-1 and bone resorbing activities from ATL cells and cell line*

MT 2 cells (1×10^6/ml) and PBL (5×10^6/ml) from one normal control and eight ATL patients were cultured in RPMI 1640 medium containing 5% FCS for 2 days. The culture supernatants were concentrated 10 times using an ultrafiltration unit with YM-5 diaflo membrane and dialyzed against RPMI and the activities of IL-1 and bone resorbing were determined.

Culture supernatant from	IL-1 activity[a] (units)	BRA[b] ^{45}Ca release (%)	Serum calcium level (mg/ml)
Medium alone		15.6 ± 1.0	
Normal PBL[c]	<10	16.0 ± 2.1	8.6
ATL PBL 1	340	43.5 ± 5.7	14.6
ATL PBL 2	324	42.1 ± 6.3	15.5
ATL PBL 3	246	42.3 ± 7.1	12.4
ATL PBL 4	86	34.8 ± 5.9	10.9
ATL PBL 5	62	28.4 ± 2.5	14.4
ATL PBL 6	45	27.5 ± 3.6	10.1
ATL PBL 7	32	21.3 ± 3.9	9.0
ATL PBL 8	11	16.3 ± 1.8	8.7
MT2	360	43.7 ± 4.7	

[a] IL-1 activity was determined by the stimulation of murine thymocyte-proliferative responses. The results are expressed as units of IL-1 produced by 1×10^7 ATL cells using ultrapure IL-1 as the standard.

[b] BRA was assayed by culturing with shafts of the radius and ulna of ICR mouse fetuses, prelabeled with ^{45}Ca *in vivo*, in BGJ medium. The results are expressed as the means and standard deviations in quadruplicate cultures.

[c] Total of 10 normal controls were studied. Since all cases of normal controls produced less than 10 units of IL-1 activity and 16.0% of BRA, only the result of one case was shown.

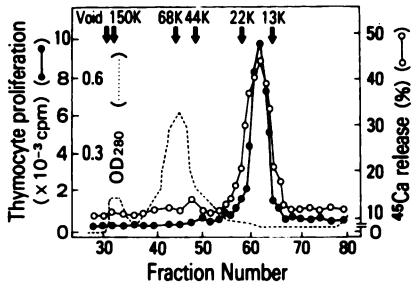

Fig. 1. Fractionation of IL-1 and bone-resorbing activities of MT2 culture supernatant by Sephacryl S-200 column chromatography. The culture supernatant (100 ml) was concentrated, was applied to a Sephacryl S-200 column (3.5 × 60 cm) equilibrated with 10 mM PBS, pH 7.4, was eluted with PBS at a flow rate of 10 ml/h and aliquots of 5 ml were collected. A_{280} was monitored (– – – –). The elution position of the molecular weight standards [blue dextran (*void volume*), human immunoglobulin G (*150K*), bovine serum albumin (*68K*), ovalbumin (*44K*), soybean trypsin inhibitor (*22K*), and cytochrome *c* (*13K*)] are shown. The thymocyte-stimulating activity (●) and the bone-resorbing activity (○) of each fraction (final concentration of 20%) were measured by the method described in Table 1, *legend*.

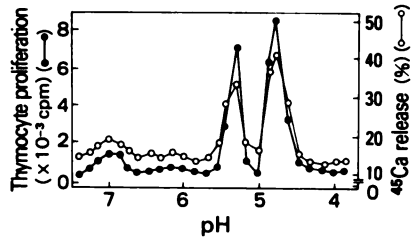

Fig. 2. Isoelectric chromatofocusing of IL-1 and bone-resorbing activities of MT2 culture supernatant. The active fractions of the Sephacryl S-200 column chromatography (Fig. 1, *58-65*) were pooled, concentrated, and dialyzed against 0.025 M imidazole hydrochloride buffer, pH 7.4, were applied on a column of PBE 94 gel (10 ml), were eluted with polybuffer 74-hydrochloride, pH 4.0, and 5-ml aliquots were collected. The thymocyte-stimulating activity (●) and the bone-resorbing activity (○) of each fraction (final concentration of 20%) were assayed by the method described in Table 1, *legend*.

of PBL from seven ATL patients were identical to that of MT2 (data not shown). The active fractions of the Sephacryl S-200 column chromatography were pooled, were fractionated by a chromatofocusing column, and the isoelectric point values of IL-1 activity and BRA were determined. As shown in Fig. 2, both activities were fractionated as two peaks at pH 4.8 and 5.2. Thus, both activities were acidic.

BRA Produced by ATL Cells Corresponds to IL-1α. In the previous paper, we reported that ATL cell lines produced IL-1α (9). Then, we studied the effects of rabbit anti-human recombinant IL-1α or β antiserum on IL-1 activity and BRA. As shown in Table 2, recombinant human IL-1α and β showed both IL-1 and BR activities. Anti-IL-1α antibody inhibited both IL-1 and BR activities of IL-1α, but not of IL-1β, and anti-IL-1β antibody inhibited both IL-1 and BR activities of IL-1β, but not of IL-1α. The activities of IL-1 and BR of the culture supernatant of MT2 cells and PBL from ATL patients was also inhibited by anti-IL-1α antibody, but not by anti-IL-1β antibody. These results suggest that IL-1 and bone-resorbing activities from ATL cells were mediated by the same or closely related molecules which corresponded to IL-1α.

BRA of IL-1α, IL-1β, and ATL-derived IL-1. Recently, Gowen *et al.* (3) and Dewhirst *et al.* (4) reported that IL-1β produced by human peripheral mononuclear cells had BRA. Thus, we compared BRA of human recombinant IL-1α and ATL-derived IL-1 and IL-1β. As shown in Fig. 3, on the unit basis of IL-1 activity, IL-1α, IL-1β, and ATL-derived IL-1 showed almost the same dose-response curves as BRA.

Table 2 *Effect of anti-IL-1α or β antibody on IL-1 activity and BRA from ATL cells and cell line*

The IL-1 activity and BRA of recombinant IL-1α, β, and IL-1-like factors from MT2 cells and ATL cells from ATL Case 1 were measured in the absence or presence of rabbit anti-recombinant human IL-1α or β antiserum (1%). The results are expressed as the means ± SD of [^{3}H]thymidine cpm of triplicate cultures and of percentage of ^{45}Ca release in quadruplicate cultures.

Factors (10 U/ml)	Anti-IL-1 antibody (1%)	IL-1 activity [^{3}H]Thymidine incorporation (cpm)	IL-1 activity % inhibition	BRA ^{45}Ca release (%)	BRA % inhibition
Recombinant IL-1α		8,494 ± 743		40.2 ± 3.1	
	Anti-IL-1α	1,325 ± 23	84.5	14.7 ± 1.8	63.4
	Anti-IL-1β	7,964 ± 604	6.3	37.4 ± 5.6	7.0
Recombinant IL-1β		7,988 ± 827		38.1 ± 4.9	
	Anti-IL-1α	7,233 ± 104	9.5	35.6 ± 2.0	6.6
	Anti-IL-1β	1,086 ± 55	86.4	16.3 ± 2.4	57.2
MT2		8,033 ± 781		39.3 ± 4.7	
	Anti-IL-1α	1,368 ± 60	82.9	15.4 ± 0.9	60.8
	Anti-IL-1β	6,832 ± 549	15.0	35.8 ± 2.4	8.9
ATL Case 1		7,709 ± 408		36.2 ± 2.8	
	Anti-IL-1α	1,665 ± 87	78.4	14.0 ± 0.7	61.3
	Anti-IL-1β	6,182 ± 241	19.8	34.7 ± 1.9	4.1

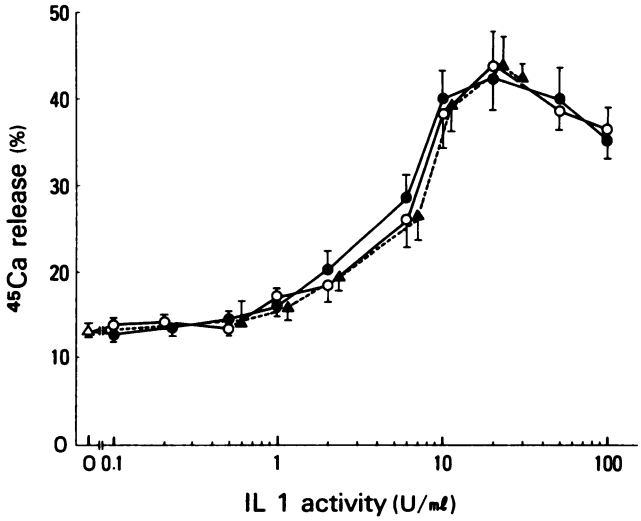

Fig. 3. Bone-resorbing activities of recombinant human IL-1α, IL-1β, and IL-1-like factor from MT2. The bone-resorbing activity of recombinant IL-1α (●), recombinant IL-1β (○), and IL-1-like factor from MT2 (▲) was assayed by the method described in Table 1, *legend*. IL-1 activities are expressed as units of thymocyte-stimulating activity by comparing with ultrapure human IL-1 as the standard. The results are expressed as the means and standard deviations of percentage of ^{45}Ca release in quadruplicate cultures.

DISCUSSION

In this paper, we have shown that ATL cells obtained from ATL patients and ATL cell line produced BRA. Their molecular weights were estimated to be about 15,000 by Sephacryl S-200 column chromatography and their isoelectric point values were determined to be pH 4.8 and 5.2 by a chromatofocusing technique. BRA from ATL cells were absorbed with rabbit anti-IL-1α antibody but not with anti-IL-1β antibody. These results suggest that BRA produced by ATL cells corresponds to IL-1α. We always observed two peaks of IL-1 and bone-resorbing activities by the chromatofocusing technique. The activities of two peaks were absorbed with anti-IL-1α antibody, suggesting that both peaks correspond to IL-1α (data not shown). The difference between the IL-1α pI values might be caused either with the microheterogeneity of amino acid sequences or with the difference of glycosylation. In order to clarify these possibilities, further studies about the purification and the determination of amino acid sequences are progressing at present. Recently, Dewhirst *et al.* purified the osteoclast-activating factor from the culture supernatant of human PBL and reported that it corresponded to IL-1β (4). Gowen *et al.* also reported that recombinant murine IL-1β had BRA (5). As shown in this paper, recombinant human IL-1α and IL-1 from ATL cell line also has a comparable BRA as IL-1β. Recent evidence suggests that not only IL-1 but also colony-stimulating factors, transforming growth factors, and tumor necrosis factors have BRA (5–8). Dodd *et al.* reported that the culture supernatant of ATL cell line stimulated the maturation of human monocytic cell line U937 (19). Since osteoclasts are considered to be cells of monocyte-macrophage lineages (20) and to play an important role in the bone turnover, they suggested that the factors from ATL cells stimulated osteoclasts to resorb bones and resulted in hypercalcemia, although they did not study the physicochemical properties of the factors from ATL cell lines. Our results suggest that one of these factors is IL-1α. In the previous paper, we reported that ATL cell lines produced IL-1α (9). In this paper, we also found that ATL cells freshly obtained from ATL patients produced IL-1α. The production of IL-1α by PBL from ATL patients was not derived from the monocytes contaminated in the PBL, because more than 90% of ATL PBL is CD4-positive T-cells. PBL from ATL patients produced IL-1 activity without any stimulations, while normal monocytes produced IL-1 activity only when they were stimulated with silica or phorbol myristate acetate. Furthermore, IL-1 produced by monocytes is mainly IL-1β, while PBL from ATL patients produce IL-1α. Thus, ATL cells themselves in the PBL seem to produce IL-1α. There is a significant correlation between the level of IL-1 and bone-resorbing activities produced by ATL cells and the serum calcium level in ATL patients (Table 1). All of these results suggest that IL-1α produced by ATL cells is one of the most important causes of hypercalcemia in ATL patients. The hypercalcemia in ATL patients is a consequence of an increasing turnover of bones by BRA from ATL cells. The question is whether hypercalcemia has some effects on ATL cells. In the previous paper, we reported that the *in vitro* growth of ATL cells and cell lines depended on the concentration of calcium ion in the culture medium and that the maximum growth of ATL cells is induced at a higher concentration of calcium ion than the normal serum calcium level (21). Thus, we consider that ATL cells produce IL-1 which stimulates the activity of osteoclasts causing the release of calcium from bones and subsequently the higher concentration of serum calcium works stimulatory on the growth of ATL cells. Namely, the network of IL-1-BRA-calcium may regulate the abnormal growth of ATL cells *in vivo*.

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Cancer Res 1988;48:4284-4287.