

Observations on the Mechanism of Bone Resorption Induced by Multiple Myeloma Marrow Culture Fluids and Partially Purified Osteoclast-activating Factor

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ABSTRACT Supernatant fluids from the cultures of bone marrow cells from 10 of 12 patients with multiple myeloma (MM) caused bone resorption in organ cultures of fetal rat calvaria. In four patients, the marrow cells were cultured with and without indomethacin (1 μ M). The supernatant fluids from indomethacin-treated marrow cultures caused significantly less bone resorption than supernatant fluids of cell cultures without indomethacin. This inhibition of release of bone resorbing factor(s) by myeloma cultures is similar to the previously observed indomethacin-induced inhibition of osteoclast-activating factor (OAF) production by activated human leukocytes. None of the MM supernatants had any effect on cyclic (c)AMP accumulation in resorbing bone *in vitro*.

Four separate preparations of partially purified OAF obtained from phytohemagglutinin-stimulated peripheral human leukocytes were tested for their ability (a) to cause bone resorption in organ cultures of fetal rat and neonatal mouse calvaria and (b) to cause accumulation of cAMP in rat and mouse skeletal tissue *in vitro*. Those dilutions of OAF that caused bone resorption had no effect on accumulation of cAMP in rat or mouse calvaria incubated *in vitro*. In addition, no stimulation of adenylate cyclase activity in membranes prepared from fetal rat calvaria could be found. Bone cell populations isolated by sequential collagenase digestion of fetal rat calvaria also showed no cAMP response to these dilutions of OAF. Parathyroid hormone caused a clear response in all three systems.

Furthermore, no cAMP response to OAF was observed in calvaria in the presence of cholera toxin (1 μ g/ml) and isobutyl-methylxanthine (0.3 mM).

These observations demonstrate that (a) supernatant fluids from MM marrow cultures stimulate bone resorption but do not increase cAMP accumulation *in vitro*; (b) indomethacin interferes with the release of bone resorbing factors by MM bone marrow cultures suggesting that this process requires prostaglandins; and (c) Sephadex G100 or G75 purified OAF does not stimulate adenylate cyclase or increase cAMP accumulation at equivalent bone resorbing concentrations in rat and mouse skeletal tissue.

The resorptive action of MM culture fluids is similar to that of partially purified OAF from activated cultured leukocytes, but different from those of other bone resorbing factors, parathyroid hormone and prostaglandin E₂, which stimulate cAMP production in skeletal tissue.

INTRODUCTION

A potent bone resorbing agent, osteoclast-activating factor (OAF)¹, has been isolated from the supernatant fluid of cultured normal human peripheral blood leukocytes activated by phytohemagglutinin (1). OAF purified from leukocytes is a peptide which may exist in two interchangeable forms (2, 3). Some aspects of the bone resorbing properties of this lymphokine have been characterized (4). OAF has been implicated in the pathogenesis of hypercalcemia and bone

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¹Abbreviations used in this paper: IBMX, isobutyl-methylxanthine; GPP(NH)P, guanylyl imidodiphosphate; OAF, osteoclast-activating factor; PG, prostaglandin; PTH, parathyroid hormone.

TABLE I
Clinical Data in 12 Patients with Multiple Myeloma

Patient	Age	Sex	Hemoglobin	Serum calcium*	Myeloma cells in marrow	M-protein
			g/dl	mg/dl	%	
1	73	F	11.0	13.1	36	1gG/K
2	55	F	7.3	12.4	24	1gA/K
3	56	M	8.3	15.6	66	K†
4	72	M	9.7	13.7	9	1gA/L
5	55	M	7.5	12.0	35	1gG/K
6	51	F	12.0	9.3	10	1gG/L
7	69	M	12.3	9.9	28	1gA/K
8	65	F	9.5	9.4	14	L†
9	77	F	13.1	10.7	15	1gA/L
10	72	M	11.0	9.4	56	1gA/L
11	67	M	10.5	8.9	50	L†
12	85	F	10.1	9.4	60	1gA/L

* Normal serum calcium, 8.5–10.3 mg/dl.

† Light chain disease; K, kappa; L, lambda (light chains).

disease associated with hematologic malignancies, particularly multiple myeloma (5–8). It may also be involved in the mechanism of localized bone loss in such conditions as periodontal disease and rheumatoid arthritis (3, 9).

We wished to determine whether OAF and the factor produced by multiple myeloma cells (presumed to be OAF) would cause accumulation of cyclic (c) AMP in skeletal tissue, an event that occurs in association with the osteoclastic bone resorption stimulated by parathyroid hormone and prostaglandin E₂ (10, 11) but not in association with that mediated by the active metabolites of vitamin D (12).

We have studied the supernatant fluids of short-term cultures of myeloma cells from 12 patients with multiple myeloma, 10 of which contained bone resorbing activity when tested in organ cultures of fetal rat calvaria. Production of this bone resorbing activity was inhibited by culturing the myeloma cells in the presence of indomethacin. Partially purified OAF derived from phytohemagglutinin-activated leukocytes also caused resorption in both rat and mouse calvaria. In contrast to parathyroid hormone and prostaglandins, we could find no effect of either the myeloma cell bone resorbing factor or OAF on cAMP content of neonatal rat or mouse calvaria, or of isolated rat bone cell populations.

METHODS

Myeloma-cell culture. 12 patients with clinically active multiple myeloma were studied, either untreated, or at least 6 wk after their last course of chemotherapy. The diagnosis of multiple myeloma was established by the usual clinical, biochemical and radiological criteria (13). Selected clinical data on the 12 patients whose bone marrow leukocytes

were cultured are given in Table I. Cells were obtained by direct bone-marrow aspiration, washed six times in a balanced salt solution, and cultured at a concentration of $1-2 \times 10^6$ cell/ml (5). Total and differential counts of the washed cells were done on slides stained with Wright's technique prepared with a Shandon cytocentrifuge (Shandon Southern Instruments Inc., Sewickly, Pa.). The cells were cultured in Falcon plastic tissue culture tubes (Falcon Labware, Div. Becton-Dickinson & Co., Oxnard, Calif.) at 37°C in an atmosphere of 5% CO₂ in air. The culture medium was BGJ_B (14) supplemented with 10% heat-inactivated rat serum. Supernatant fluids were harvested after 24 h of culture. Bone marrow from four patients was cultured in the presence and absence of indomethacin (1 μM). The supernatant fluids from all marrow cultures were dialyzed exhaustively at 4°C against 100 times their volume of fresh unsupplemented BGJ_B medium using Spectropor 3 dialysis membrane (Spectrum Medical Industries, Inc., Los Angeles, Calif.) with a nominal molecular weight cutoff of 3,500, in order to remove low molecular weight substances which might affect bone resorption. The supernatant fluids thus obtained were frozen and stored at -20°C for subsequent analysis. Marrow cells obtained from three patients with other hematologic disorders were cultured in the same manner for control purposes.

Bone resorption bioassay. The method used was a modification of the method of Webster et al. (15). 21-d pregnant Wistar rats were decapitated and the fetuses removed. Neonatal mice were treated in a similar fashion. Calvaria were dissected under sterile conditions, care being taken to preserve the periosteum, and placed in sterile ice-cold BGJ_B medium for about 20 min before culture. Two calvaria halves, each obtained from a fetus or neonate of a different litter, were then placed in sterile plastic culture dishes (Falcon 3010) on stainless steel grids that supported the two half calvaria at the gas-liquid interface. The central well of these dishes was filled with 0.75 ml BGJ_B medium supplemented with 15% heat-inactivated rat serum (vol/vol), antibiotics and antifungal agents. The dishes were placed in a humidified incubator at 37°C with 5% CO₂ in air as the gas phase. The medium was changed at 24 h and removed after 72 or 96 h. Supernatant fluids or solutions containing OAF were added

to test dishes in various dilutions. Each experimental group consisted of three or four cultures. The medium removed at stated times was analyzed for calcium using a Fiske calcium titrator (Fiske Associates, Inc., Burlington, Mass.). Bone resorbing activity was expressed either as the change in calcium concentration in meq/liter occurring during the incubation or the net calcium release into the medium at the end of the incubation. Validity of the assay system was always verified using a known concentration of purified bovine parathyroid (PTH) hormone. Statistical differences between treated and control cultures were analyzed by Student's *t* test.

Measurement of skeletal cAMP response. The accumulation of cyclic AMP in skeletal tissue was studied *in vitro* after exposure of calvariae or cultured rat bone cell populations to myeloma marrow culture supernatants or OAF (2). In each experiment, the cAMP response to PTH was tested for control purposes. The cAMP responses were measured as described (16). Briefly, calvaria (parietal and frontal bones) from neonatal Wistar rats or neonatal mice were incubated separately in tubes containing phosphate buffered saline supplemented with 0.1% glucose and 0.1% bovine serum albumin (Pentex; Miles Laboratories, Elkhart, Ind.) for 10 min at 37°C (varying times, 2.5, 5, and 10 min were used for the time-course study). PTH, myeloma marrow culture supernatants, and partially purified OAF were diluted in the incubation buffer and added in the indicated amounts. At the end of the incubation, cAMP was extracted from calvaria with propanol, and assayed by a competitive protein binding method (17). To increase cAMP responsiveness (18) calvaria were preincubated in some experiments in cholera toxin (1 µg/ml) for 30 min, followed by incubation with either isobutyl-methylxanthine (IBMX) (0.3 mM) and cholera toxin, or IBMX alone. With the mouse calvaria only IBMX was used. cAMP responses were also tested in bone cell populations isolated from neonatal rat calvaria by sequential enzyme digestions (19). Digestions were carried out for three consecutive 10-min periods, then two 20-min periods. Cells were harvested at the end of each period of digestion. Populations from the first two digestion periods (designated populations I and II) were combined. Similarly, populations from the fourth and fifth digestion periods (designated populations IV and V) were combined. The cells were plated at uniform concentration (3×10^5 cells/5 ml) in sterile plastic culture dishes (Falcon plastics 3002) and experiments were performed on confluent first subcultures. Cells were extracted and assayed for cAMP in the same manner as for intact calvaria.

Adenylate cyclase was assayed by the method of Salomon et al. (20). Some assays of adenylate cyclase response to purified OAF preparations were carried out in the presence of maximal concentrations of guanylyl imidodiphosphate (GPP[NH]P)(10 µM), to amplify the response.

Prostaglandin assay. Prostaglandins of the E and F series were extracted and assayed as described by Johnston et al. (21). Briefly, myeloma cell incubation fluid was extracted using a sequential ethanol-petroleum ether-chloroform procedure (22). The crude extracts were subsequently purified on silicic acid columns (23), evaporated to dryness, redissolved in assay buffer, and assayed. The radioimmunoassay used a rabbit antiserum to prostaglandin (PG) F_{2α} (21). This antibody cross-reacts 75% with PGF_{1α} and <0.01% with PGA₂, PGB₂, PGE₁, and PGE₂. Consequently, values were expressed as PGF equivalents. To assay PG E₁ and E₂, these were converted to PGF_{1α} and F_{1β} and PGF_{2α} and F_{2β} by sodium borohydride reduction (24). The amount of PGE₁ and PGE₂ present in the sample, expressed as PGE equivalents, was then calculated from the measured PGF levels before and after reduction. Serum contains substances that can inhibit the binding of PG to the antibody. Since these substances are

not removed by the extraction or the column step, an aliquot of control incubation medium containing serum was extracted and processed in the same manner as the unknown samples. An aliquot of this extract was added to every point of the standard curve to correct for the effects of the incubation medium on the assay procedure. The lower detection limit of this assay is 15 pg PGF equivalents. Normally, 1 ml of incubation medium was extracted. PTH was measured by radioimmunoassay using an antiserum (GPI) directed against both C and N-terminal regions of the hormone molecule (25).

Leukocyte culture and OAF production. OAF was obtained from supernates of cultures of normal human leukocytes activated with phytohemagglutinin as described (2). Crude OAF was concentrated by ultrafiltration using Amicon ultrafiltration membranes PM10 and UM2 (Amicon Corp., Scientific Systems Div., Lexington, Mass.). The concentrated activated leukocyte culture supernates, containing crude OAF, were partially purified by column chromatography using Sephadex G100 or G75 (2).

RESULTS

***In vitro* studies of marrow culture supernatant fluids.** Marrow cells from all 12 patients were cultured for 24 h, and the culture supernates were tested for *in vitro* bone resorbing activity in 4 separate experiments. 10/12 Culture fluids contained significant bone resorbing activity (Fig. 1). In each assay, a known amount of PTH was tested as a positive control. Not shown are data from three patients with nonmyelomatous hematologic disorders (Waldenstrom's macroglobulinemia, iron deficiency anemia, and chronic lymphocytic leukemia). Marrow culture supernatant fluids derived from these patients did not show any significant bone resorbing activity.

Characterization of bone resorbing factor(s). Myeloma cell supernatant fluids were assayed for PTH and PGE and F by radioimmunoassay; the concentration of these substances was either unmeasurable, or below the concentrations necessary to produce measurable bone resorption. In previous similar studies, no evidence was found for the presence of PTH, PGE₂, or vitamin D metabolites in active myeloma culture supernatants (5).

Bone marrow from four patients was incubated in the presence of indomethacin 1 µM. The supernates thus obtained caused significantly less bone resorption when compared with bone marrow cultures incubated in the absence of indomethacin (Fig. 1). This must be interpreted as a decrease in production of bone resorbing substance(s) by the myeloma marrow cells, since both indomethacin and PG would have been removed by our dialysis procedure before the bone resorption assay. In separate experiments (data not shown) we have found that indomethacin at 1 µM concentration has no direct effect on the bone resorptive response to OAF or to myeloma culture fluids.

To test whether the bone resorption seen in these experiments was associated with changes in cAMP concentrations in bone, this nucleotide was measured directly after incubation of calvaria with 11 myeloma

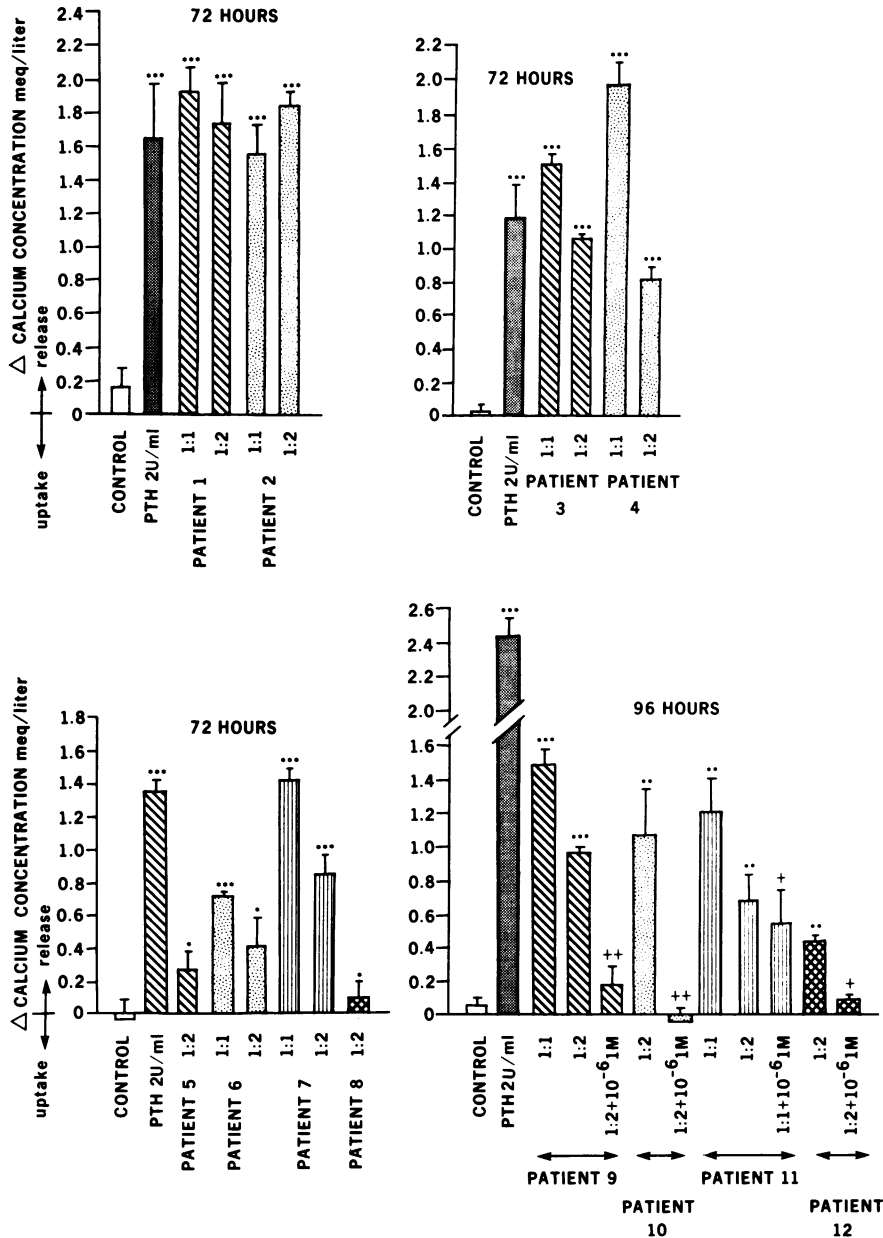


FIGURE 1 Results from 4 separate in vitro bone resorption bioassays performed on bone marrow culture supernates from 12 patients with multiple myeloma. Results are expressed as the change in medium calcium concentration during culture of fetal rat calvaria with myeloma culture fluids for the times shown. Supernates from each patient were tested at the final dilutions indicated. PTH was added for comparison in the doses shown. Vertical bars indicate ± 1 SEM for triplicate cultures. Results were tested for significance of their difference from control values using Student's *t* test (*, NS; **, $P < 0.05$; ***, $P < 0.01$). Significant bone resorption was seen with supernates from 10 of the 12 patients. Two supernates (patient numbers 5 and 8) did not show significant responses. In four patients (lower right panel) the marrow was cultured in the presence and absence of $1 \mu\text{M}$ indomethacin. The indomethacin was removed from the culture media before the bone resorption assay. In all four patients, the resorption response caused by the indomethacin-treated culture fluid was significantly less than the response in the absence of indomethacin (+, $P < 0.05$; ++, $P < 0.01$).

culture supernates. The results (Table II) show no significant changes in bone cAMP concentrations after incubation with any of the myeloma culture fluids, al-

though significant increases in cAMP were always seen in response to PTH.

In vitro studies with purified OAF. Four batches

TABLE II
Effect of Myeloma Marrow Culture Fluid on cAMP
Production in Neonatal Rat Calvaria

Incubation medium	Dilution	cAMP pmol/calvarium
Control	-	9.02±3.2
PTH (2 U/ml)	-	226.2±53.6
Patient 1		
MCS*	1:1	12.0±3.7
MCS	1:2	7.50±0.7
Patient 2		
MCS	1:1	7.10±1.2
MCS	1:2	5.40±1.2
Control	-	26.7±5.6
PTH (2 U/ml)	-	271.6±60.0
Patient 3		
MCS	1:1	27.9±1.5
MCS	1:2	24.5±5.5
Patient 4		
MCS	1:1	22.4±4.2
MCS	1:2	24.1±2.3
Control	-	19.6±2.2
PTH (2 U/ml)	-	262.3±38.0
Patient 6		
MCS	1:1	11.15±0.5
MCS	1:2	20.1±4.2
Patient 7		
MCS	1:1	20.9±6.2
MCS	1:2	11.1±1.1
Patient 8		
MCS	1:1	19.8±6.0
MCS	1:2	23.1±1.1
Control	-	24.34±1.4
PTH (0.5 U/ml)	-	132.6±12.4
Patient 9		
MCS	1:1	25.9±6.9
MCS	1:2	24.29±0.74
Indomethacin (1 μM)	1:2	18.57±1.86
Patient 10		
MCS	1:2	20.49±0.87
Indomethacin (1 μM)	1:2	17.02±4.75
Patient 11		
MCS	1:1	19.97±0.95
MCS	1:2	26.5±1.54
Indomethacin (1 μM)	1:1	27.06±2.95
Patient 12		
MCS	1:2	21.73±2.4
Indomethacin (1 μM)	1:2	24.29±3.2

Values are mean±1 SD for three incubations assayed in duplicate. Culture fluids were tested at the dilutions shown. Four separate experiments were performed, each with its own control values. Whereas PTH caused significant accumulation of cAMP, no significant increases in cAMP concentration above control were seen with active myeloma marrow supernates. Patient 5 was omitted from this study. In patients 9 to 12, assays were also performed on supernates from marrow cultured in the presence of 1 μM indomethacin.

* MCS, myeloma culture supernatant fluid.

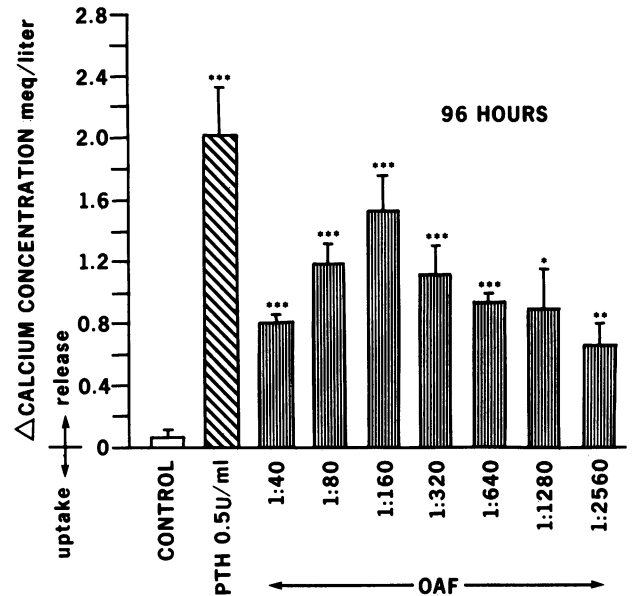


FIGURE 2 In vitro bone resorption bioassay of purified OAF. Results are expressed as in Fig. 1, except that statistical differences from control are expressed as follows: *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$. Significant bone resorption was seen at the dilutions throughout the dose range shown, with a biphasic dose-response pattern. The maximal effect was seen in this experiment at a final dilution of 1:160.

of partially purified OAF were tested with identical results. Results are presented for only two of the batches. The resorptive effect of OAF was dose related; the maximal response was noted at a dilution of 1:160 (Fig. 2). Biphasic dose responses have been reported for OAF (4). No increase in cAMP concentration in the calvaria was seen at this dilution of OAF (Table III).

TABLE III
Effect of OAF (Sephadex G100)* and PTH on cAMP
Levels in Neonatal Rat Calvaria

Incubation medium	cAMP pmol/calvarium
Control	14.2±3.1
PTH (0.5 U/ml)	80.76±17.04
OAF (1:40)	15.12±3.4
OAF (1:80)	15.27±2.7
OAF (1:160)	14.12±2.4
OAF (1:320)	13.27±2.69
OAF (1:640)	16.96±2.33
OAF (1:1280)	16.15±2.49
OAF (1:2560)	13.19±4.33

Values are mean±1 SD for three incubations assayed in duplicate. Whereas PTH caused significant accumulation of cAMP, no significant increase in cAMP concentration above the control value was seen at any dilution of OAF tested. Similar dilutions of OAF caused significant bone resorption, maximal at 1:160 (Fig. 2).

* The same preparation was used as in Fig. 2.

TABLE IV
cAMP Production from Early and Late Bone Cell Populations Stimulated with PTH (1-34) plus OAF (Sephadex G100)

cAMP	
<i>pmol/dish</i>	
Cell population 1 + 2	
Control	29.29±9.8
PTH (1-34) (2 U/ml)	271.41±15.65
OAF (1:160)	30.43±11.39
OAF (1:320)	29.78±12.70
Cell population 4 + 5	
Control	26.45±6.5
PTH (1-34) (2 U/ml)	1,567.61±176.47
OAF (1:160)	25.44±10.88
OAF (1:320)	23.14±5.36

Values are mean±1 SD for three incubations assayed in duplicate. Dilutions of OAF chosen for this experiment were those that caused the highest amount of bone resorption (Fig. 2). Bovine PTH 1-34 (2 U/ml) was also assayed for control purposes. No significant cAMP accumulation resulted from these concentrations of OAF in either early or late eluting bone cell populations.

Recently, Luben et al. (26) noted increases in cAMP production in mouse calvaria and in isolated mouse bone cell populations after stimulation with an OAF purified from human tonsillar tissue. In an attempt to

TABLE V
Effect of OAF (Sephadex G100) and PTH on cAMP Levels in Neonatal Rat Calvaria in the Presence and Absence of IBMX and Cholera Toxin

cAMP	
<i>pmol/calvarium</i>	
Incubation medium	
Control	4.27±1.2
PTH (2 U/ml)	177.42±28.9
OAF (1:40)	4.39±0.99
OAF (1:80)	6.39±1.0
OAF (1:160)	5.19±1.5
OAF (1:320)	4.05±1.6
Control (IBMX + CT)	72.59±12.6
OAF (1:40) IBMX + CT	75.51±6.6
OAF (1:80) IBMX + CT	73.27±23.7
OAF (1:160) IBMX + CT	70.09±10.4
OAF (1:320) IBMX + CT	66.82±12.6

Values are mean±1 SD for three incubations assayed in duplicate. Dilutions of OAF used included those which caused significant bone resorption in vitro. In the presence of IBMX (0.3 mM) and cholera toxin (CT, 1 µg/ml) the control levels of calvarial cAMP are elevated. OAF at several dilutions did not cause any significant elevation above control. In the absence of IBMX and CT, the responses of PTH and OAF are similar to those shown previously. While a marked increase in cAMP was seen with PTH, no significant response was noted with OAF.

clarify the discrepancy between our findings and those of Luben et al., some further studies were undertaken. Isolated rat bone cell populations were tested after stimulation with the same batch of Sephadex G 100 purified OAF at dilutions that caused maximal bone resorption. Again, no significant cAMP accumulation was noted, although the isolated cells retained their characteristic PTH response (Table IV). In an attempt to amplify any small increases in cAMP production undetectable in our previous experiments, calvaria were incubated in the presence of cholera toxin and IBMX. No increases in cAMP accumulation above the expected elevated control levels were noted (Table V). In addition, since the studies of Luben et al. (26) were performed after 5 min of incubation, a cyclic AMP response time-course study was carried out to test the action of Sephadex G75 purified OAF (containing 221 µg/ml protein) at 2.5, 5, and 10 min (Table VI). At 5 and 10 min, there was a slight increase in cAMP production compared with control, although the effect was virtually negligible in comparison with the PTH response (Table VI). A parallel experiment

TABLE VI
cAMP Time-course Study: Effect of OAF (Sephadex G75) and PTH in Rat Calvaria

Incubation medium	Incubation time	cAMP
		<i>pmol/calvarium</i>
Control	2.5 min	22.6±2
PTH (2 U/ml)		43.2±8.3
OAF (1:40)		17.4±6
OAF (1:80)		23.8±2.3
OAF (1:160)		14.4±6.5
OAF (1:320)		19.4±9.6
OAF (1:640)		13.6±1.9
Control	5 min	18.4±4.8
PTH (2 U/ml)		216.0±42.3
OAF (1:40)		19.2±10.3
OAF (1:80)		21.1±6.9
OAF (1:160)		21.9±7.1
OAF (1:320)		22.1±6.6
OAF (1:640)		22.5±6.6
Control	10 min	16.1±2.8
PTH (2 U/ml)		349.5±35.5
OAF (1:40)		21.1±5.2
OAF (1:80)		17.8±5.6
OAF (1:160)		22.3±10.4
OAF (1:320)		22.7±4.9*
OAF (1:640)		22.3±7.7

Values are mean±1 SD for three incubations assayed in duplicate. Incubation times were 2.5, 5, and 10 min. While PTH caused the expected increment in cAMP production with increasing time of incubation, there was no statistically significant increase in cAMP caused by OAF.

* $P < 0.05$.

confirmed the bone resorbing activity of this OAF preparation (data not shown). The overall results of the time-course study provide further evidence that OAF does not stimulate cAMP in a comparable fashion to PTH when both substances cause almost equivalent bone resorption. Finally, adenylate cyclase activity was measured directly in membranes prepared from the neonatal rat calvaria. Although PTH caused marked stimulation of this enzyme, multiple dilutions of OAF did not result in any significant stimulation above basal levels (Fig. 3). Similar experiments (not shown) were performed in the presence of 10 μ M GPP(NH)P. This nucleotide has been shown to increase the sensitivity of renal adenylate cyclase to several PTH analogues (27). In the presence of GPP(NH)P, there was again no stimulation of adenylate cyclase by purified OAF added in multiple dilution.

In a similar series of experiments, neonatal mouse calvaria (and rat calvaria again for comparison) were tested with a different batch of purified OAF. The results are shown in Fig. 4 and Table VII. Although there was a rather high control resorption in the mouse calvaria, dilutions of OAF causing bone resorption significantly greater than control did not cause any elevation of cAMP at 1, 2, 10, and 20 minutes (Table VII). This experiment was repeated in the presence of IBMX with similar findings.

DISCUSSION

This study has demonstrated that the supernatant fluids from multiple myeloma marrow cultures and

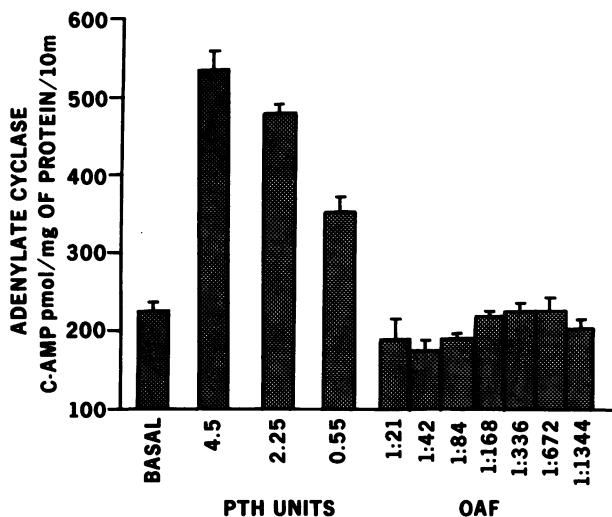


FIGURE 3 Adenylate cyclase activity in membrane preparations from neonatal rat calvaria in response to PTH and purified OAF. While PTH caused the expected increase in enzyme activity, OAF did not cause any such activation at any dose tested. In particular, there was no activation at the dilution which caused maximal bone resorption, 1:160 (Fig. 2).

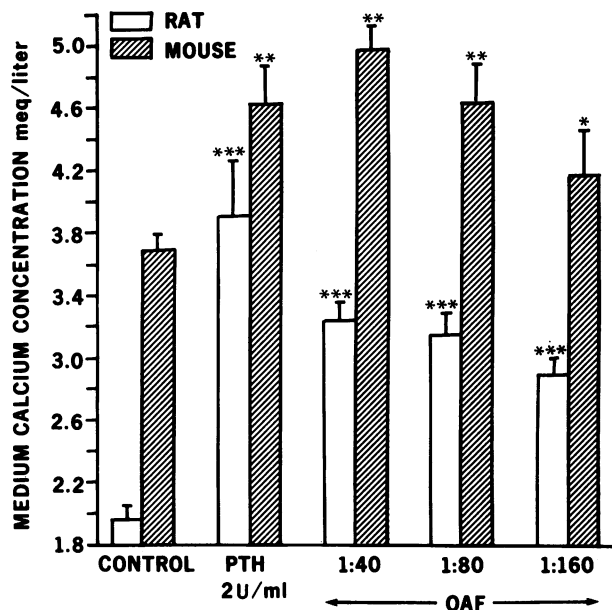


FIGURE 4 In vitro bone resorption assay of purified OAF in rat and mouse calvaria. Results are expressed as the net calcium release into the medium after 96 h incubation. Purified OAF was tested in the final dilutions indicated, and PTH was added for comparison in the dose shown. Vertical bars indicate ± 1 SEM for quadruplicate cultures. Statistical significance was expressed as in Fig. 1. Despite high control bone resorption in mouse calvaria, statistically significant differences from control were obtained with 1:40 and 1:80 dilutions of OAF.

purified preparations of OAF cause active osteoclastic bone resorption unassociated with detectable cAMP accumulation in skeletal tissue. The experiments were performed using multiple dilutions of OAF and myeloma marrow supernates in three different systems: isolated rat skeletal membranes, isolated rat bone cell populations, and intact calvaria from both rats and mice. These data are significant because they demonstrate differences between OAF and two other well-known agents that regulate bone metabolism, PTH and PGE₂. cAMP accumulates concomitantly with bone resorption induced by PTH and PGE (10, 11) and it has been postulated that this nucleotide may directly mediate the resorptive response to these agents, although this has not been conclusively established (28). Whether or not cAMP is directly involved in the chain of intracellular events leading to bone resorption, its accumulation upon stimulation of skeletal tissue by PTH and prostaglandins is an observation that contrasts with the absence of such a response to OAF and must indicate some difference between their respective mechanisms of action. In this context, OAF acts similarly to 1,25(OH)₂ vitamin D, a bone resorbing agent that also appears to act independently of cAMP (12).

TABLE VII
Effect of OAF on cAMP Production in Mouse
Calvaria: Time-course Study

Incubation medium	Incubation time	cAMP <i>pmol/calvarium</i>
Control	1 min	9.3±4.4
PTH (2.5 U/ml)		37.8±0.6
OAF (1:40)		7.9±1.3
OAF (1:80)		10.3±2.5
OAF (1:160)		8.1±0
Control	2 min	10.3±1.9
PTH (2.5 U/ml)		>150
OAF (1:40)		10.0±0.8
OAF (1:80)		8.5±0.9
OAF (1:160)		7.9±1.7
Control	10 min	7.2±1.6
PTH (2.5 U/ml)		>200
OAF (1:40)		7.6±3.1
OAF (1:80)		5.6±1.1
OAF (1:160)		7.3±1.7
Control	20 min	5.6±1.0
PTH (2.5 U/ml)		>200
OAF (1:40)		4.4±0.7
OAF (1:80)		5.2±0.4
OAF (1:160)		5.1±0.4

Values are mean±1 SD for three incubations assayed in duplicate. Incubation times were 1, 2, 10, and 20 min. PTH caused the expected increase in cAMP production, but the exact concentration of cAMP was not measured by serial dilution. There was no increase in cAMP produced by OAF at any time period.

Our findings are at variance with the data reported from another laboratory. Recently, Luben et al., (26) using an OAF purified from human tonsillar lymphocytes, detected cAMP accumulation in association with bone resorption induced by this factor in mouse calvaria *in vitro*. In an attempt to clarify the discrepancy, we employed methods that would amplify small increases in tissue concentration of cAMP that may have been otherwise indistinguishable from control. In the presence of cholera toxin and IBMX in the rat calvaria and IBMX alone in the mouse calvaria, we still could not detect any significant cAMP response to OAF purified from peripheral human leukocytes. Also, there was no adenylate cyclase activation by our OAF preparation in rat skeletal membranes even in the presence of GPP(NH)P.

Luben et al. (26) observed a dissociation between the dose-response curves of OAF-induced cAMP accumulation and bone resorption. They attempted to explain this dissociation on the basis of different OAF responses in two different bone cell types, periosteal derived cells (osteoclast-like) and matrix-derived cells

(osteoblast-like). In our studies, we did not observe cAMP accumulation in response to OAF in either type of bone cell population. However, it would be imprudent to make a definitive statement implying that the mechanism of action of OAF does not involve cAMP mediation. There are well-documented systems in the endocrine literature in which hormones acting via cAMP-dependent protein kinases do not stimulate the accumulation of free cAMP at the doses necessary for biological activity (29). A definitive answer will require studies of OAF-stimulated activation of cAMP-dependent protein kinases. Nevertheless, the evidence presented from several different complimentary lines of investigation shows that skeletal cAMP accumulation does not occur in response to purified OAF or multiple myeloma supernatants. Moreover even if we were unable to measure increases in free cAMP one would perhaps have expected to detect activation of adenylate cyclase if cAMP played an important intracellular role. The data are therefore in clear distinction to the responses observed with PTH and PGE suggesting that a different mechanism of action is operative.

This study has also focussed on the similarities that exist between purified OAF and the active agent in myeloma marrow cell supernates. With neither agent is *in vitro* bone resorption associated with a detectable cAMP accumulation. Another similarity between the factor produced by multiple myeloma cells and OAF is that release of both factors appears to depend on PG. Previous studies have shown that there is an obligate interaction between OAF release by cultured activated leukocytes and PG synthesis (30). This interaction appears to involve macrophage (monocyte)-lymphocyte synergy (31, 32). Indomethacin blocks production of OAF by cultured activated leukocytes and also inhibits the production of the myeloma bone resorbing factor, as shown in this study. It might be inferred from these observations that indomethacin would be useful in the therapy of hypercalcemia in patients with multiple myeloma. We have used indomethacin in maximally tolerated doses prior to any chemotherapy in two hypercalcemic myeloma patients without any effect on the serum calcium concentration. Indomethacin has also been ineffective in the treatment of hypercalcemia in some other malignancies where prostaglandins have been implicated etiologically (33-35). Perhaps indomethacin is without effect *in vivo* because it is not present in sufficient concentration around tumour cells to prevent local PG synthesis.

These experiments add to the weight of evidence that the myeloma factor is similar or identical to OAF. Mundy et al. (5, 6) provided biological and chemical evidence for similarity; our present studies show parallels in the biochemical mode of action and in the mechanism for production. Clearly more studies are

indicated on the mode of action of OAF and its involvement in the osteolysis and hypercalcemia frequently observed is multiple myeloma. Advances may be expected from the purification of the active peptide and the determination of its chemical structure, as well as the development of a radioimmunoassay for OAF. Our experiments by providing evidence for a different biochemical mechanism of action from some other bone resorbing agents may serve to focus further attention on this peptide, which is important in conditions of generalized and localized bone loss.

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