

# Partial Purification of Osteoclast-Activating Factor from Phytohemagglutinin-Stimulated Human Leukocytes

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**ABSTRACT** Osteoclast-activating factor (OAF) is a soluble mediator found in supernates of human peripheral leukocytes which have been cultured with antigens or phytomitogens. OAF is a potent stimulator of osteoclastic resorption of fetal bone in organ culture. The present studies were designed to characterize OAF chemically. Bone resorbing activity from supernates of leukocytes cultured without added plasma was not lost on dialysis using a membrane with a molecular weight cutoff of 3,500, but was lost when heated to 60°C for 30 min. The activity was lost after treatment with trypsin or pronase but not after treatment with ribonuclease or neuraminidase. Papain, which inactivated parathyroid hormone at a concentration of 25 µg/ml, did not inactivate OAF at 250 µg/ml. OAF did not react with an antibody to bovine parathyroid hormone which cross-reacts with human parathyroid hormone. OAF was also distinguished from active metabolites of vitamin D and from prostaglandin by extraction procedures and immunoassay for prostaglandin E<sub>2</sub>.

When the medium from activated leukocytes cultured with autologous plasma was fractionated by gel filtration on Sephadex, bone resorbing activity eluted both with plasma proteins and in lower molecular weight fractions. However, when medium from leukocytes cultured without added plasma was chromatographed, all the OAF activity was eluted in a sharp low molecular weight peak located between chymotrypsinogen (25,000 molecular weight) and ribonuclease A (13,700 molecular weight). This peak contained about 4% of the total protein originally present in the supernate. Its activity was destroyed by overnight incubation at 37°C at pH 6 or 8, but not at pH 7.2. After incubation at 4°C, the activity was lost at pH 3 or 10, but not at pH 4-9.

*Received for publication 1 November 1973 and in revised form 4 January 1974.*

The active fraction from Sephadex G-100 was therefore chromatographed at pH 7.2 on DEAE cellulose and carboxymethyl cellulose. The active material was not adsorbed; however, about sevenfold further purification was achieved by removal of contaminants. The material obtained after sequential Sephadex, DEAE and, carboxymethyl cellulose chromatography stimulated resorption of fetal rat bone in culture at concentrations of 0.75-3 µg protein/ml, indicating that this preparation of OAF was nearly as potent as bovine parathyroid hormone in this system.

## INTRODUCTION

Local resorption of bone is associated with chronic inflammation in such disorders as periodontal disease, rheumatoid arthritis, and chronic osteomyelitis. Cultures of cells associated with chronic inflammation, particularly lymphocytes, can produce soluble factors with a variety of biological activities (1). The production of these factors is generally increased when the cells are stimulated by antigens to which the donor has developed cellular immunity or by nonspecific mitogens, such as phytohemagglutinin (PHA).<sup>1</sup> Recently a new soluble mediator in supernates of activated human peripheral leukocyte cultures was identified by its ability to stimulate resorption of fetal rat bone in organ culture (2). The activity was present in leukocyte cultures stimulated by a solubilized dental plaque antigen or by PHA. The mediator was termed osteoclast-activating factor (OAF), because treated bones showed large numbers of active osteoclasts which appeared to be responsible

<sup>1</sup> *Abbreviations used in this paper:* CM, carboxymethyl; K<sub>D</sub>, partition coefficient; MIF, migration inhibition factor; OAF, osteoclast activating factor; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PHA, phytohemagglutinin; PTH, parathyroid hormone.

TABLE I  
OAF Produced by Human Leukocyte Cultures  
with and without PHA

	[ <sup>3</sup> H]TdR incorporation, cpm/culture	48 h <sup>45</sup> Ca release, treated/control ratio
Leukocyte cultures		
20% plasma - PHA	483 ± 114	1.19 ± 0.11
20% plasma + PHA	14,123 ± 589	1.61 ± 0.17*
No plasma - PHA	230 ± 71	0.96 ± 0.16
No plasma + PHA	18,480 ± 332	2.45 ± 0.20*
Culture medium		
1% PHA	—	0.96 ± 0.08
10% PHA	—	0.89 ± 0.07

Human leukocytes were cultured (approximately 10<sup>6</sup> cells in 1 ml of BGJ medium), with or without 20% autologous plasma, and with or without PHA. At the end of 3 days of culture, the supernatant medium was removed, and the cells were incubated with [<sup>3</sup>H]TdR, 1 μCi/culture, in BGJ for 4.5 h. The incorporation of <sup>3</sup>H into cellular material precipitated with cold trichloroacetic acid (5%) was then determined. Incorporation values are mean ± SEM for six cultures. Supernates from parallel cultures not labeled with [<sup>3</sup>H]TdR were diluted 1:1 with fresh BGJ and assayed for stimulation of bone resorption in organ culture. Fresh BGJ medium with added PHA which had not been used for leukocyte cultures was also assayed. Results are expressed as mean ± SEM treated/control ratios for four pairs of bones.

\* Significantly different from 1.0 (*P* < 0.05).

for the increased resorption. The present studies were designed to characterize chemically the activity in supernates of leukocyte cultures stimulated by PHA and to isolate the active fraction. OAF was found to be a protease-sensitive macromolecule which could be distinguished from stimulators of bone resorption which have been chemically identified previously, such as parathyroid hormone (PTH), 1,25-dihydroxycholecalciferol (1,25-[OH]<sub>2</sub>D<sub>3</sub>), and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).

## METHODS

**Leukocyte cultures.** Human peripheral leukocytes were cultured as described previously (2). White cell-rich plasma and buffy coat were removed from settled heparinized blood. In initial experiments, the cells were spun down and resuspended in medium RPMI 1640 with 20% autologous plasma. In most experiments, the cells were centrifuged, the plasma was removed as completely as possible, and the cell pellet was resuspended at approximately 10<sup>6</sup> cells/ml (60–80% mononuclear cells) in BGJ medium (3), with or without added PHA (1% Gibco crude PHA [Grand Island Biological Co., Grand Island, N. Y.], approximately 30 μg/ml). The medium was usually harvested at 24 and 72 h. Pooled culture medium was sterilized by millipore filtration, concentrated 10- to 20-fold by ultrafiltration using Amicon PM10 membranes (Amicon Corp., Lexington, Mass.), and stored frozen at -20°C. Concentrated media were

diluted with fresh BGJ before assay, or used directly for enzyme studies or chromatography.

**Bone resorption assays.** The method for measuring bone resorption in organ culture has been described previously (3). Rats were injected with 0.4 mCi of <sup>45</sup>CaCl<sub>2</sub> on the 18th day of gestation. On the 19th day, the fetuses were removed and the shafts of the radius and ulna dissected and cultured in BGJ with 1 mg/ml bovine serum albumin or 5% human plasma (both heat inactivated at 60°C for 30 min). After a 24- to 48-h period of preculture in control medium, which served to remove much of the exchangeable <sup>45</sup>Ca, the bones were transferred to test or control media for assay. The test media consisted of appropriate dilutions of leukocyte culture medium or one of the materials described below. Control values for <sup>45</sup>Ca release were obtained by culturing bones in BGJ medium containing 1 mg/ml heat-inactivated bovine serum albumin or 5% heat-inactivated human plasma (for enzyme studies). After 2–4 days in culture, <sup>45</sup>Ca release from bones in the test medium was compared to <sup>45</sup>Ca release in control cultures. When paired radii or ulnae from the same fetus were used, a treated-to-control ratio was calculated. In most experiments, treated bones were not paired with controls from the same fetus. Instead, a group of bones from each litter were used as controls, and the remaining bones were divided among the treatment groups. In these experiments, the percentage release of total <sup>45</sup>Ca was determined by

TABLE II  
Dialysis, Heat Inactivation, and Ultrafiltration of OAF

Experiment	Treatment	48 h <sup>45</sup> Ca release, treated/control ratio
A	None	2.44 ± 0.13*
	Dialyzed	1.17 ± 0.21
B	None	2.26 ± 0.39*
	Dialyzed	2.20 ± 0.31*
	Heated (60°, 30 min, no dialysis)	1.09 ± 0.16
C	None	2.53 ± 0.08*
	PM10 filtrate	1.15 ± 0.23
	PM10 retentate	2.25 ± 0.38*
	XM50 filtrate	1.94 ± 0.14*
	XM50 retentate	1.79 ± 0.07*

Medium from PHA-stimulated leukocyte cultures without added plasma was treated as indicated, then diluted with fresh medium and assayed for bone-resorbing activity. Experiment A: dialysis against BGJ, using Fisher dialyzer tubing (Fisher Scientific Co., Pittsburgh, Pa.), nominal molecular weight cutoff about 12,000. Experiment B: dialysis against BGJ using Spectrapor 3 dialysis membranes (see Methods), nominal molecular weight cutoff about 3,500. Experiment C: samples concentrated to one-fourth the original volume using Amicon filters PM10 or XM50. Retentates were diluted back to the original volume with BGJ for assay, while filtrates were assayed directly. PM10 and XM50 membranes have nominal molecular weight cutoffs of 10,000 and 50,000, respectively.

\* Significantly different from 1.0 (*P* < 0.05).

measuring radioactivity in both bone and medium and was calculated as follows: percent release = (medium dpm / [medium dpm + bone dpm]) × 100.

**Enzyme digestion.** Trypsin (lyophilized, salt-free 1 × crystallized, 150 U/mg protein) was obtained from Worthington Biochemical Corp., Freehold, N. J. Enzymes, obtained from Sigma Chemical Co., St. Louis, Mo., were papain (2 × crystallized, 30 U/mg protein), pronase (protease type VI, 3 U/mg protein), ribonuclease (5 × crystallized type I-A, 75 Kunitz U/mg protein), and neuraminidase (type V, 12 U/μg solid, using bovine submaxillary mucin as substrate). The effects of incubation with enzymes were tested on samples of 10 × concentrated leukocyte supernate and purified bovine parathyroid hormone (kindly provided by Dr. W. Y. W. Au). The samples were exposed to enzymes in BGJ without added albumin or plasma at 37°C for 1 h under an atmosphere of 5% CO<sub>2</sub> in air, pH approximately 7.3–7.4. After incubation, the samples were diluted 10-fold with BGJ containing 5% heat-inactivated human plasma. The final concentrations of enzymes were those which, in preliminary experiments, had been shown to have no effect on control resorption, and additional control cultures with enzyme were used in each experiment to confirm this. Enzymes which did not destroy OAF activity were assayed by using standard substrates after incubation in BGJ for 1 h at 37°C in 5% CO<sub>2</sub>. Bovine submaxillary mucin (Sigma Chemical Co.) was used as substrate for neuraminidase, *Escherichia coli* RNA (Calbiochem, San Diego, Calif.) for ribonuclease, and  $\alpha$ -N-benzoyl-L-arginine ethyl ester (Sigma Chemical Co.) for papain. Under these conditions, neuraminidase retained 15%, ribonuclease 70%, and papain 19% of their originally specified specific activities toward these substrates.

**Extraction procedures.** Concentrated leukocyte culture media were extracted with solvents which are known to remove the potent lipid soluble bone resorbing agents, 1,25-(OH)<sub>2</sub>D<sub>3</sub> (4, 5), and PGE<sub>2</sub> (6, 7) from biological samples. OAF samples, control medium, and medium to which authentic 1,25-(OH)<sub>2</sub>D<sub>3</sub> or PGE<sub>2</sub> had been added were prepared and extracted either with chloroform-methanol (1:1) followed by chloroform (for vitamin D metabolites), or with redistilled ethyl acetate after adjustment of the medium to pH 3.5 with glacial acetic acid (for PGE<sub>2</sub>). Extraction was repeated three times, and the organic phases were pooled and dried under nitrogen, and the residue was dissolved with BGJ for assay.

**Gel filtration chromatography.** All chromatographic steps were carried out at 4°C. 2–3 ml of concentrated leukocyte supernates was applied to columns of Sephadex G-150 or G-100 equilibrated in 0.02 M sodium phosphate buffer with 0.15 M NaCl, pH 7.2, and eluted with buffer. The fractions were pooled and dialyzed against BGJ at a phosphate concentration adjusted to produce a final concentration of 1.0 mM. The dialysis membrane used was boiled Spectrapor 3 (Spectrum Medical Industries, Inc., Los Angeles, Calif.), with a nominal molecular weight cutoff of 3,500. Dialyzed fractions were further diluted with fresh BGJ for assay. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (8). The elution volumes of column fractions were used to calculate the partition coefficient (K<sub>D</sub>), both for OAF activity and for standard calibration proteins (Pharmacia Calibration Kit, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). K<sub>D</sub> was calculated as follows:  $K_D = (V_0 - V_e) / V_1$ , where V<sub>0</sub> was the peak elution volume of the material in question, V<sub>e</sub> was the peak elution volume for blue dextran, and V<sub>1</sub>

TABLE III  
Effect of Enzyme Digestion on Bone Resorbing Activity of OAF and PTH

Experiment	Digestion	Percent release of <sup>45</sup> Ca		
		Control	OAF	PTH
A, medium treated	None	20 ± 1	37 ± 4*	38 ± 3*
	Trypsin 40 μg/ml	19 ± 1	43 ± 4*	20 ± 2
	Trypsin 400 μg/ml	20 ± 1	18 ± 1	19 ± 2
	Neuraminidase 400 μg/ml	19 ± 1	42 ± 4*	37 ± 4*
	Ribonuclease 100 μg/ml	22 ± 2	33 ± 5*	45 ± 4*
	Papain 25 μg/ml	20 ± 1	46 ± 8*	19 ± 1
B, medium treated	None	13 ± 1	33 ± 8*	—
	Papain 250 μg/ml	12 ± 1	30 ± 2*	—
	Pronase 500 μg/ml	11 ± 1	12 ± 1	—
C, Bone treated	None	20 ± 1	53 ± 3*	—
	Trypsin 40 μg/ml 1 day	22 ± 2	46 ± 1*	—

In experiments A and B, media containing OAF, 10 × concentrate, or PTH, 10 μg/ml, and control media were digested with enzymes added at the indicated concentration for 1 h at 37°C, followed by 10-fold dilution with fresh medium before assay. In experiment C, bones were incubated for 1 day, with or without trypsin, washed with fresh medium, and placed in assay medium. Values are mean ± SEM (four to eight bones per point). \* Significant difference from control (P < 0.05).

was the difference between the peak elution volumes of blue dextran and tritiated water under the operating conditions used. K<sub>D</sub> vs. log (molecular weight) gave a linear plot for the calibration proteins used (9).

**Ion exchange chromatography.** Pooled active fractions from Sephadex G-100 chromatography were dialyzed against 0.05 M Tris-HCl, pH 7.2, or against 0.02 M Hepes, pH 7 (Gibco), and chromatographed on columns of DEAE cellulose (Whatman DE 52) or carboxymethyl (CM cellulose (Whatman CM 32), respectively. The columns were washed with starting buffer and then eluted with a linear gradient of 0–0.5 M NaCl in starting buffer. The CM cellulose column was washed with 1.0 M NaCl after elution. The fractions were dialyzed against BGJ for assay as described above.

**Immunoassays.** Leukocyte culture supernates were assayed directly for immunoreactive PTH (10) by Dr. Charles Hawker (Upjohn Co., Kalamazoo, Mich.). Ethyl acetate extracts of acidified supernates were assayed by using a prostaglandin E immunoassay kit (11) (Clinical Assays, Inc., Cambridge, Mass.) by Dr. J. M. Goodson (University of California at San Francisco School of Dentistry).

## RESULTS

Supernates of human peripheral leukocyte cultures activated with PHA contained bone-resorbing activity, while supernates from unstimulated leukocytes were inactive (Table I). PHA alone had no effect on bone resorption. Leukocytes cultured without added serum showed as great a stimulation of thymidine uptake and released as much OAF as did leukocytes cultured with 20% autologous plasma. When OAF-containing media were dialyzed by using cellulose membranes with a nominal molecular weight cutoff of 12,000, much of the activity was lost (Table II), but using Spectrapor 3

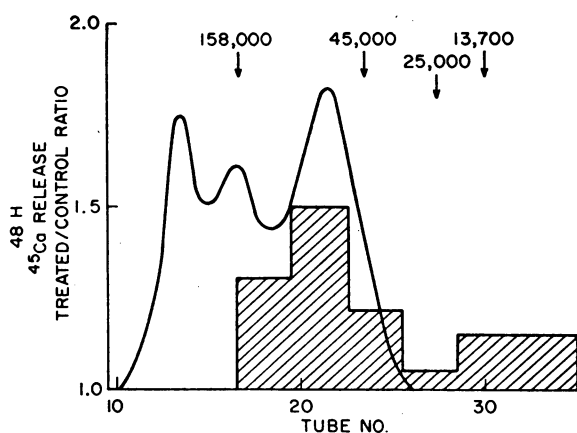


FIGURE 1 Gel filtration of medium from PHA-stimulated leukocyte cultures containing 20% plasma. Concentrated dialyzed medium was placed on a column ( $2.5 \times 35$  cm) of Sephadex G-150 and eluted with 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.2. The solid line represents absorbance at 280 nm; shaded bars represent the stimulation of bone resorption by pooled fractions from the areas indicated. Bone resorption was estimated from the release of  $^{45}\text{Ca}$  by fetal rat bones in tissue culture containing aliquots of the pooled, dialyzed, and concentrated fractions (see Methods) compared with similarly treated fractions of control medium incubated without cells. The arrows at the top of the chromatogram represent the peak elution volumes of aldolase (158,000), ovalbumin (45,000), chymotrypsinogen (25,000), and ribonuclease A (13,700).

with a nominal cutoff of about 3,500, all the activity was retained. Heating to  $60^\circ\text{C}$  for 30 min inactivated OAF in supernates containing no added plasma. Little or no OAF was filterable through an Amicon PM10 filter, but a substantial fraction passed through an XM50 filter. There was no measurable loss of biological activity during ultrafiltration. These results suggested that the molecular weight of OAF was between 10,000 and 50,000, nominal values for 95% rejection of globular proteins by the PM10 and XM50 filters, respectively.

OAF was inactivated (Table III) by treatment with trypsin (400  $\mu\text{g}/\text{ml}$ ) or pronase (500  $\mu\text{g}/\text{ml}$ ), but not by neuraminidase (400  $\mu\text{g}/\text{ml}$ ) ribonuclease (100  $\mu\text{g}/\text{ml}$ ) nor papain (250  $\mu\text{g}/\text{ml}$ ). Bovine PTH was also resistant to neuraminidase and ribonuclease, but was inactivated by trypsin at 40  $\mu\text{g}/\text{ml}$  and papain at 25  $\mu\text{g}/\text{ml}$ . In a separate study, PTH was inactivated by 10  $\mu\text{g}/\text{ml}$  trypsin (data not shown). Pretreatment of bone with trypsin at 40  $\mu\text{g}/\text{ml}$ , the concentration present in bone cultures after dilution of the 400  $\mu\text{g}/\text{ml}$  incubation mixture in the inactivation experiments, did not block the subsequent resorptive response of bone to OAF.

The fact that OAF activity was not lost on dialysis with Spectrapor 3 or on ultrafiltration with PM10 argued against the activity being due to a prostaglandin or vitamin D metabolite. However, since there could

have been retention of such materials by protein binding, active OAF samples were extracted repeatedly with lipid solvents which can remove these materials (Table IV). The bone-resorbing activity of  $1,25\text{-(OH)}_2\text{D}_3$  was extracted from BGJ medium with chloroform-methanol, while the activity of  $\text{PGE}_2$  was extracted with ethyl acetate after acidification (Table IV). Neither extraction procedure yielded biological activity from supernates containing OAF. By immunoassay, the supernate with maximal OAF activity contained 0.5 ng/ml of bovine PTH equivalent. Much of this could be ascribed to nonspecific displacement of labeled hormone, since BGJ alone gave a value of 0.4 ng/ml. There was no detectable  $\text{PGE}_2$  by immunoassay in the ethyl acetate extracts under conditions which should have detected as little as  $2 \times 10^{-9}$  M  $\text{PGE}_2$  in the original medium.

Initial OAF samples obtained from leukocytes cultured in 20% autologous plasma showed widely dispersed activity when chromatographed on Sephadex G-150 (Fig. 1). The greatest biological activity eluted in the albumin fraction, but activity was also found in lower molecular weight fractions. Fractions of supernates from inactive leukocyte cultures showed no bone-resorbing activity when compared with the same fractions from control media, which had been incubated without cells. When OAF was obtained from supernates of leukocytes cultured without added serum, there was no longer any detectable biological activity in the albumin region (Fig. 2), and the activity now eluted as a discrete symmetrical peak on Sephadex G-100 after chymotrypsinogen (25,000 molecular weight) and before ribonuclease A (13,700 molecular weight). No bone-resorbing activity was found in any fraction of medium from unstimulated cultures (1% PHA added after culture). The OAF peak had a  $K_D$  of 0.52. The active fractions from the G-100 column contained only about 4% as much protein as the original supernate (Table V) and showed a similar dose-response curve to crude OAF with no evidence of loss of activity on the column (Fig. 3).

TABLE IV  
Extraction of Bone Resorbing Activity from BGJ Culture Medium by Specific Organic Solvents

Sample	Percent release of $^{45}\text{Ca}$		
	No extraction	Chloroform/methanol	Ethyl acetate
BGJ	$14 \pm 2$	$12 \pm 1$	$15 \pm 2$
$1,25\text{-(OH)}_2\text{D}_3$ (10 ng/ml)	$30 \pm 3^*$	$21 \pm 2^*$	—
$\text{PGE}_2$ ( $10^{-6}$ M)	$33 \pm 8^*$	—	$31 \pm 4^*$
OAF	$38 \pm 4^*$	$15 \pm 2$	$16 \pm 2$

Samples containing  $1,25\text{-(OH)}_2\text{D}_3$  or  $\text{PGE}_2$  were extracted three times using equal volumes of solvent and sample. Pooled extracts were dried under nitrogen at  $4^\circ\text{C}$  and reconstituted to 85% of the volume of the original sample with BGJ medium for assay. Values are mean  $\pm$  SEM ( $n = 4$ ).  
\* Significantly different from control BGJ ( $P < 0.05$ ).

When partially purified OAF from G-100 columns was incubated at 37°C overnight at pH 6 or 8.1, activity was lost (Fig. 4), but activity was not lost at pH 7.4. When incubated at 4°C overnight, the activity was completely lost only at pH 3 or 10, and significant activity was retained over a range of 4.0–8.5 (Fig. 4). However, the maximal recovery appeared to be at neutral pH. Because of these results, further purification was attempted at pH 7.2 by using DEAE and CM cellulose. The activity was recovered in the initial buffer wash and not retarded on either column. There even appeared to be a slight increase in activity in the eluate from the CM cellulose column (Fig. 5). Although OAF was not retained on DEAE or CM cellulose, some contaminants were apparently removed, since the protein content was reduced by about seven-fold without loss of biological activity. Attempts to purify OAF further on CM cellulose at pH 5.3 were unsuccessful. This was ascribed to inactivation, since no biological activity was found either in the column fractions or in the original sample after dialysis against pH 5.3 ammonium acetate buffer.

## DISCUSSION

The studies reported here were designed to compare the chemical characteristics of OAF with those of other stimulators of bone resorption. The original studies (2) of bone-resorbing activity in supernates of activated leukocyte cultures did not distinguish OAF from other agents known to cause bone resorption, such as PTH, active metabolites of vitamin D, or prostaglandins. The fact that OAF is heat labile and nondialyzable suggests that it is macromolecular, like PTH. However, OAF

TABLE V  
Protein Content of Purified OAF Fractions

Active fraction	Protein content
	$\mu\text{g/ml}$
Crude OAF	514
G-100 OAF	21.4
CMC	9.0
DEAE	6.1
DEAE-CMC (sequential)	3.0

Leukocyte culture medium ("crude OAF") was fractionated on Sephadex G-100. Active fractions were pooled and passed through columns of carboxymethyl-cellulose (CMC), DEAE-cellulose, or DEAE-cellulose followed by CMC. The active eluates were concentrated and analyzed for protein, using bovine serum albumin as standard and expressed as protein concentration per milliliter of the original leukocyte culture supernate, which contained maximal bone resorbing activity. These purification procedures caused no measurable loss of biological activity (see Figs. 3 and 5).

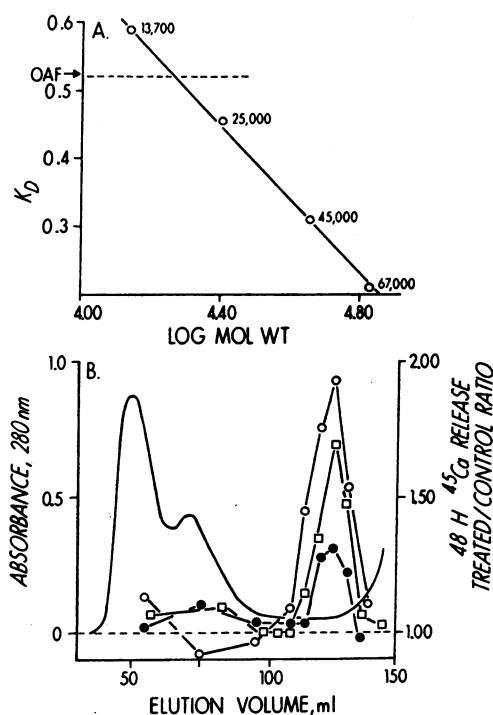


FIGURE 2 Gel filtration of medium from PHA-stimulated leukocyte cultures without added serum. Concentrated medium was placed on a column (2.5 × 45 cm) of Sephadex G-100 and eluted with 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.2. (A). Calibration of the column with standard proteins. Molecular weight values represented are bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), and ribonuclease A (13,700). (B) Chromatography of active OAF samples. Column fractions were pooled, dialyzed against BGJ, and assayed for bone-resorbing activity. The smooth curve is absorbance at 280 nm; the connected points represent activity of media from three separate leukocyte cultures from two normal donors. Control media for the bone resorption assays were BGJ with 1 mg/ml bovine serum albumin.

can be distinguished chemically from PTH by several criteria. OAF is more sensitive to heat and acid than PTH, which can be extracted from parathyroid glands in a biologically active form by using 0.2 N HCl at 100°C (12). OAF is considerably more resistant than PTH to inactivation by either trypsin or papain. In addition, supernates containing OAF activity did not react with an antibody to bovine PTH, which cross-reacts with human PTH at physiological concentrations (10). Since the antibody may not cross-react with the biologically active sequence of PTH, it is conceivable that OAF and PTH have sequences in common in the active part of the molecules. The complete recovery of OAF as a sharp peak from Sephadex without special pretreatment of the gel is also in contrast to the reported behavior of PTH (13).

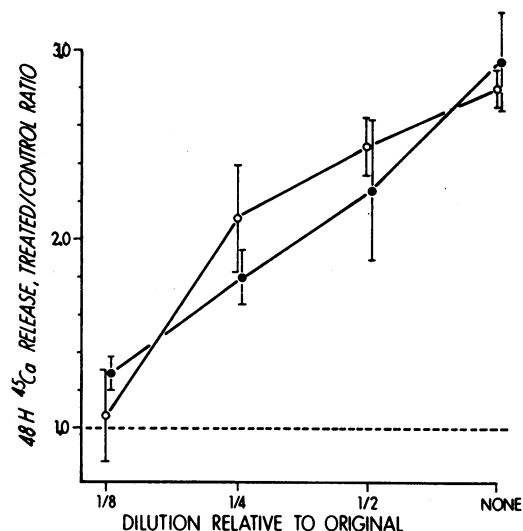


FIGURE 3 Log dose-response curves for crude and purified OAF. Medium from PHA-stimulated leukocyte cultures ("crude OAF") was serially diluted and assayed for bone-resorbing activity (open circles). Pooled active fractions from G-100 chromatography ("purified OAF") were concentrated to the same volume as the original culture medium used for the column run, dialyzed against BGJ medium, then diluted serially for assay (solid circles). Values are mean  $\pm$ SEM for four pairs of bones per point.

OAF was also distinguished by several criteria from the active metabolites of vitamin D or prostaglandins. Retention of full OAF activity during dialysis using a membrane with a 3,500 molecular weight cutoff suggests that the activity was not due to a small molecule. Moreover, no bone-resorbing activity could be recovered by extraction with lipid solvents of active samples containing OAF, although these procedures did result in recovery of biologically active  $1,25(\text{OH})_2\text{D}_3$  and  $\text{PGE}_2$ . Finally, we could not detect  $\text{PGE}_2$  by immunoassay in extracts of OAF-containing media. These results do not rule out the possibility that OAF activity depends on the combination of a protein moiety with some active group resembling prostaglandins or vitamin D metabolites, perhaps as a covalently bonded complex.

Enzyme inactivation studies indicated that OAF activity was dependent on a protein moiety. Failure to inactivate OAF with ribonuclease or neuraminidase indicates that nucleic acids are probably not an important component and that terminal sialic acid residues are either absent or not required for biologic activity. Chromatographic studies also indicated that OAF was a macromolecule, which was not highly charged on the basis of its behavior on ion exchange resins. When OAF was produced in cultures containing added plasma, the elution pattern on Sephadex G-100 was very disperse. This may have been the result of aggregation, binding

to plasma proteins, or distortion of the elution pattern by the large amount of protein which had to be placed on the column to recover significant amounts of activity. Moreover, it is possible that OAF produced by lymphocytes in the presence of added autologous plasma is heterogeneous and that these different active fractions represent different biologically active molecules.

When OAF was produced by normal human leukocytes in the absence of added plasma, a single symmetrical peak of biological activity eluted from Sephadex G-100 with a  $K_D$  of about 0.52. When compared to the elution for globular proteins, this value would correspond to a mass of about 18,000 daltons. However, since we do not know whether OAF is a globular protein, this may not represent the true molecular weight. Sephadex-purified OAF was relatively stable at neutral pH, but rapidly inactivated at both high and low pH, particularly at  $37^\circ\text{C}$ . Since these preparations were not pure, we cannot determine whether this is the result of activation of enzymes present in the fraction or direct inactivation of OAF. Further purification of OAF was attempted by using DEAE cellulose and CM cellulose columns at neutral pH. The biological activity was eluted with the buffer and not adsorbed to the resin. Nevertheless, some purification resulted because of the removal of contaminants, and the final product was biologically active

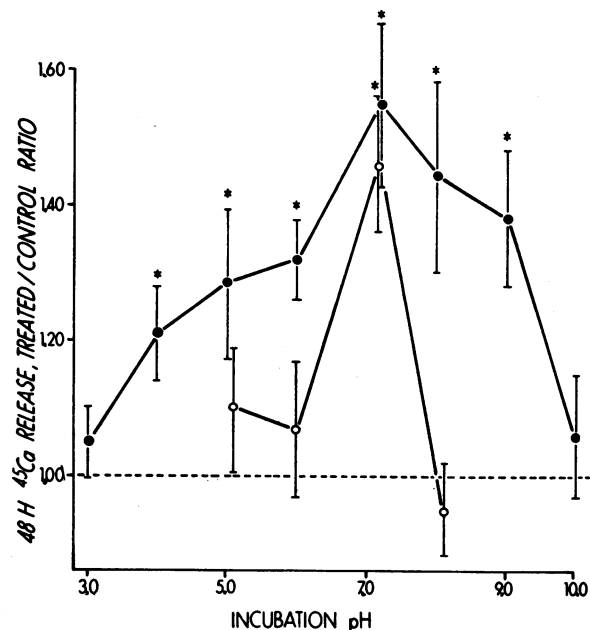


FIGURE 4 Effect of pH on OAF activity. Purified OAF from G-100 chromatography was adjusted (in column buffer) to the indicated pH values, stored overnight at  $4^\circ\text{C}$  or  $37^\circ\text{C}$ , then dialyzed against BGJ medium before assay of bone-resorbing activity. Values are mean  $\pm$ SEM for four to eight pairs of bones per point.  $\bullet$ ,  $4^\circ\text{C}$ ;  $\circ$ ,  $37^\circ\text{C}$ ; \*, significantly different from control ( $P < 0.05$ ).

over a range of 0.75–3  $\mu\text{g}/\text{ml}$  of protein concentration, not much greater than the concentrations of bovine PTH (0.1–1.0  $\mu\text{g}/\text{ml}$ ) which give equivalent stimulation of resorption in this culture system (3). It is likely that this preparation is still quite impure and, therefore, that the concentration of active OAF is much lower than suggested by the protein analysis. On the other hand, we have no evidence that OAF contains only protein. It could conceivably contain substantial amounts of carbohydrate or lipid. However, if it were a highly acidic glycoprotein, then one might have expected it to bind to DEAE cellulose, and if the lipid moiety were large, one might have expected to observe extraction of the active material with lipid solvents.

OAF appears to be chemically distinct from previously characterized lymphokines produced by cultures of activated human leukocytes. Human macrophage migration inhibition factor (MIF) appears to have a molecular weight of about 23,000 and elutes from a Sephadex G-100 column with peak activity in the same fractions as chymotrypsinogen (14). Human mononuclear leukocyte chemotactic factor (15), on the other hand, elutes from Sephadex G-100 coincident with cytochrome C (molecular weight 12,500). The peak activity for OAF is clearly and reproducibly (cf., Fig. 2) between the elution positions of chymotrypsinogen and ribonuclease A (molecular weight 13,700). In addition, both MIF and human mononuclear leukocyte chemotactic factor are heat stable, while OAF is heat labile. Human lymphotoxin (16), which is heat labile, is a considerably larger molecule than OAF, as evaluated by Sephadex G-100 chromatography. We cannot exclude the possibility, however, that OAF represents a variant form of some previously described lymphokine or that OAF has other activities in different biological assay systems than the one used in these studies. Purified guinea pig complement factor C'5a, which has chemotactic activity (17), elutes from Sephadex G-100 at about the same position as OAF. However, preliminary experiments<sup>2</sup> failed to detect any bone-resorbing activity of either guinea pig or human C'5a.

The biological importance of OAF is not yet known. The production of such a potent stimulator of bone resorption by activated leukocytes in vitro suggests that activation of leukocytes in chronic inflammation might also lead to OAF secretion. If so, it could be responsible for some of the changes in bone resorption seen locally in periodontal disease, arthritis, or osteomyelitis. A factor such as OAF could also be important in neoplastic diseases, particularly those that involve proliferation of lymphoid cells which could themselves produce OAF. We have obtained evidence that lymphoid cell lines derived from patients with hematologic neoplasms produce an

<sup>2</sup> L. G. Raisz and R. Snyderman, unpublished results.

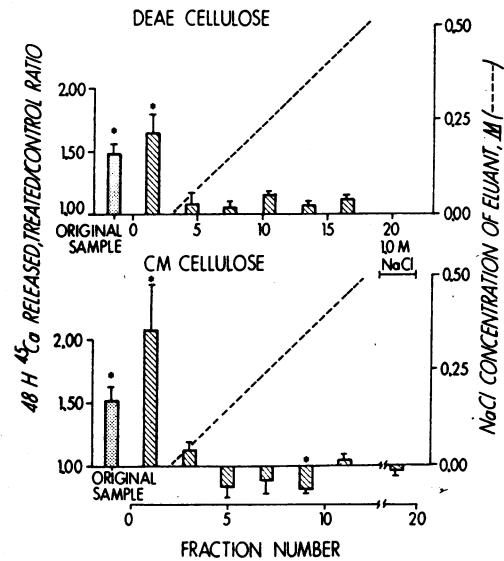


FIGURE 5 Ion exchange purification of OAF. Samples of active fraction OAF from G-100 chromatography were dialyzed against 0.05 M Tris-HCl, pH 7.0, for DEAE cellulose purification, or against 0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, pH 7.0, for CM cellulose purification. For both ion exchangers, the column (0.9  $\times$  10 cm) was washed with starting buffer after applying the same, then eluted with a linear gradient of NaCl, 0–0.5 M in starting buffer. The CM cellulose column was also washed with 1.0 M NaCl after elution. Fractions were dialyzed against BGJ, concentrated to the volume of the original sample, and assayed for bone-resorbing activity. Values are mean  $\pm$  SEM for four pairs of bones per point. \*, significantly different from control ( $P < 0.05$ ).

OAF-like material (18). OAF-like factors could aid the development of bone metastases or promote hypercalcemia in such diseases. Finally, it is possible that in other neoplasms the cellular immunity of the host could result in activation of lymphocytes which produce OAF and affect bone.

#### ACKNOWLEDGMENTS

The authors thank Hollis Simmons and Barbara Beeler for technical assistance.

This work was supported in part by National Institutes of Health grants AM 06205, DE 03725, and 5T01 DE 00003-14, and was aided by grant no. IN-18N from the American Cancer Society.

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