MONOCYTES REGULATE OSTEOCLAST-ACTIVATING FACTOR PRODUCTION BY RELEASING PROSTAGLANDINS*

By TOSHIYUKI YONEDA‡ and GREGORY R. MUNDY§

From the Division of Endocrinology and Metabolism, Department of Medicine, University of Connecticut School of Medicine, Farmington, Connecticut 06032

Two local factors which may be responsible for bone resorption in a variety of chronic inflammatory and neoplastic diseases are the lymphokine osteoclast-activating factor (OAF)[1] and prostaglandins (PGs) (1, 2). OAF is a potent mediator of bone resorption which is produced by phytohemagglutinin (PHA)-activated leukocytes and by certain neoplastic lymphoid cells (2, 3). PGs, which are also potent mediators of bone resorption (4), are frequently found in increased concentrations in chronic inflammatory tissue (5). The interactions of these two factors may be very important in the localized bone destruction which occurs in diseases such as rheumatoid arthritis and periodontal disease and also around metastatic tumor deposits in bone.

Most reports of the effects of PGs on the production of lymphokines have suggested that PGs are inhibitory (6–9). However, recently we showed that prostaglandin synthesis was necessary for OAF production by activated leukocytes (10). OAF production was inhibited by the presence of structurally unrelated inhibitors of prostaglandin synthesis such as indomethacin, flufenamic acid, and R020-5720. OAF production was restored when PGs of the E series were added to activated leukocyte cultures together with indomethacin (10). Although these data showed that PGs were necessary for the production of OAF by stimulated leukocytes, they did not show which cells in the leukocyte population were responsible for OAF production and which were responsible for the synthesis of PGs.

The presence of both lymphocytes and monocytes is required in the activated leukocyte population for OAF release to occur (11, 12). The experiments reported here were performed to determine the nature of the interaction between the monocyte and the lymphocyte in OAF production and the role of PGs in this interaction. They show that enriched activated lymphocytes produce OAF, but only in the presence of PGs of the E series which are released by adherent monocytes.

Materials and Methods

Leukocyte Culture. Our leukocyte culture technique has been described previously (13). Normal human peripheral blood leukocytes were obtained from plateletpheresis from normal

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‡ On leave from second department of oral- and maxillo-facial-surgery, Osaka University Dental School, Osaka, Japan.

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[1] *Abbreviations used in this paper:* act. lc, PHA-activated lymphocytes; culture media; ^{3}H-AA, ^{3}H-amino acid; [^{3}H]TdR, [^{3}H]thymidine; MCM, monocyte-conditioned medium; OAF, osteoclast-activating factor; PGs, prostaglandins; PHA, phytohemagglutinin.

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donors at the Connecticut Red Cross, Farmington, Conn. The leukocyte buffy coat was removed 60 min after the addition of 5 ml of 6% dextran to the plasma-leukocyte-erythrocyte mixture for acceleration of erythrocyte sedimentation. The leukocytes were suspended in BGJ medium (14) (Grand Island Biological Co., Grand Island, N.Y.), centrifuged three times at 150 *g* for 5 min to remove platelets, and resuspended in BGJ medium without added serum at a concentration of 2–2.5 × 10^6 cells/ml in Falcon plastic tubes (12 × 75 mm) or tissue culture dishes (100 × 20 mm) (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.). The cells were stimulated with 1% phytohemagglutinin-M (PHA-M) (Grand Island Biological Co.) and cultured for 24–72 h at 37°C in an atmosphere of 5% CO_2 in air. At the end of culture, the supernates were collected by careful aspiration and frozen at −20°C until the OAF assay. The remaining leukocytes were pulsed with [^{3}H]thymidine (New England Nuclear, Boston, Mass.) or ^{3}H-amino acids (New England Nuclear) for 4–6 h and the incorporation of ^{3}H into the trichloroacetic acid-insoluble cell fraction was measured.

Preparation of Adherent Monocytes. Leukocytes were collected in BGJ medium supplemented with 4 mg/ml of bovine serum albumin (BGJ, BSA_4, Reheis Chemical Co., Div. of Armour Pharmaceutical Co., Chicago, Ill.) in a similar manner to that described above. After 24–48 h, when adherent cells were observed under phase-contrast microscopy, nonadherent cells were removed by vigorous pipetting. The adherent cells were evaluated for viability by trypan blue exclusion, cytoplasmic spreading by Wright's staining, phagocytotic ability by uptake of latex beads (15) (0.8 microns, Sigma Chemical Co., St. Louis, Mo.), and for nonspecific esterase activity (16). More than 95% of the adherent cells were viable, >85% of the viable cells were capable of phagocytosis, and >92% of the cells showed nonspecific esterase activity and cytoplasmic spreading. These adherent cells were designated enriched monocytes. Using this method, 1.5–2.0 × 10^5/ml of monocytes were recovered from each leukocyte culture. The enriched monocytes were detached from the bottom of dishes by incubating with trypsin-EDTA (1X, Grand Island Biological Co.) for 10–15 min, suspended in the medium, and counted on a hemocytometer. Enriched monocyte culture was carried out in BGJ, BSA_4 for 48–72 h. At the end of this period, the monocyte culture media was harvested and the monocytes were detached and recombined with the nonadherent enriched lymphocytes. This mixed population of cells was then cultured in BGJ medium in a similar manner to the leukocyte cultures to assess DNA and protein synthesis and the production of OAF by the cells.

Preparation of Nonadherent Lymphocytes. Nonadherent cells obtained after 24–48 h of culture on plastic surfaces were washed three times, resuspended in BGJ, BSA_4, replated on new dishes, and cultured for a further 24 h. After that, the cells which had still not adhered to the dishes were again replated on new dishes and cultured for a further 24 h. The final resulting nonadherent cells were designated enriched nonadherent lymphocytes. They were cultured in BGJ in a similar manner to that of the leukocytes (see above).

Drugs. Indomethacin (Sigma Chemical Co.), D-1,6-chloro-α-methyl-carbazole-z-acetic acid (R020-5720, Hoffman-LaRoche Inc., Nutley, N. J.), flufenamic acid (Aldrich Chemical Co. Inc., Milwaukee, Wis.), and prostaglandin E_1, E_2, $F_{1\alpha}$, and $F_{2\alpha}$ (Sigma Chemical Co.) were all dissolved in 95% ethyl alcohol and added to the cultures to the desired concentration 2 h before activation of cells with PHA. An identical concentration of ethyl alcohol was added to some control cultures. There was no detectable difference in control cultures to which alcohol had been added compared with those to which it was not added.

Bioassay for Bone Resorbing Activity As a Result of OAF. Bone resorbing activity, as a result of OAF in the leukocyte culture supernates, was assessed by methods which have been previously described (13, 17). Pregnant mice were injected subcutaneously with 0.05 mCi of ^{45}Ca on the 16th d of gestation. On the next day, the fetuses were removed from the mothers and mineralized shafts on their radii and ulnae were dissected free from surrounding soft tissue and the cartilagenous ends. These bones were cultured in BGJ medium for 24 h to allow for exchange of loosely complexed ^{45}Ca with stable calcium in the medium. The bones were then cultured either in the test media containing the cell culture supernates to be assayed or in the control media. The cell culture supernates were diluted 1:1 or 1:3 with fresh BGJ medium before assay for bone resorbing activity. Bone resorbing activity of OAF was expressed as the percent of total radioactivity released from the bone into the culture medium during the culture period or as the ratios of ^{45}Ca release from the test bones compared with the bones cultured in control media. The results were expressed as means and standard errors for four pairs of bone

TABLE I

Effects of Prostaglandin Synthetase Inhibitors on [^{3}H]Thymidine ([^{3}H]TdR) and ^{3}H-Amino Acid (^{3}H-AA) Incorporation and OAF and PG Production by Leukocyte Cultures

PHA	Inhibitors	[^{3}H]TdR incorporation*	^{3}H-AA incorporation*	OAF production‡ (treated/control ratios of ^{45}Ca release)	PGE
1%	*10 μM*	*cpm*	*cpm*		*ng/ml*
−	—	1,868 ± 168	452 ± 77	0.80 ± 0.07	2.00
−	Indomethacin	2,432 ± 261	806 ± 91	1.07 ± 0.05	ND
−	R020-5720	2,310 ± 241	517 ± 57	1.05 ± 0.08	0.07
−	Flufenamic acid	1,562 ± 279	466 ± 52	1.08 ± 0.13	ND
+	—	27,767 ± 1,536§	2,625 ± 211§	1.97 ± 0.26§	12.80
+	Indomethacin	22,889 ± 1,835§	2,319 ± 103§	1.03 ± 0.02‖	0.34
+	R020-5720	27,081 ± 1,516§	2,512 ± 367§	0.98 ± 0.16‖	0.80
+	Flufenamic acid	26,245 ± 1,551§	2,510 ± 285§	1.07 ± 0.15‖	0.52

ND, not detectable. The leukocytes were cultured for 48 h in BGJ medium without serum supplementation.

* Values expressed as means ± SEM for three leukocyte cultures.

‡ Values expressed as means ± SEM for four pairs of bone cultures.

§ Significantly greater than corresponding control, $P < 0.05$.

‖ Significantly different from stimulated cultures without inhibitor, $P < 0.05$.

cultures. Statistical differences were analyzed using Student's *t* test.

Diafiltration of Media. In some experiments, prostaglandins were removed from cell culture supernates by diafiltration across Amicon UM-2 membranes (Amicon Corp. Scientific Sys. Div., Lexington, Mass.) using continuous replacement with 10 vol of BGJ media. These membranes have a nominal molecular weight cutoff of 1,000 daltons. All steps were carried out at 4°C.

Prostaglandin Measurement. The amount of total PGE and total PGF in culture supernates was measured according to the method of Zurier and Sayadoff (18). In brief, after addition of ^{3}H-PGE_1 and-$PGF_{2\alpha}$ (New England Nuclear) for calculation of recovery, the culture supernates were extracted twice with 2:1 ethylacetate: cyclohexane, and the extracts were dried under nitrogen. The residues were vortexed in 0.4 ml of 10% methanol in 60:40 benzene-ethylacetate and chromatographically separated on silicic acid column. PGE and PGF in the samples were sequentially eluted with 3% and 30% methanol in 60:40 benzene:ethyl acetate, respectively. The fractions were then dried and reconstituted with 0.2 M phosphate buffer (pH 7.0) containing 1% rabbit serum. Radioimmunoassays for PGE and PGF content were carried out in the manner previously described (19). Measurements were corrected for final recovery of added tritiated PG, which averaged 40%.

Results

Effects of PGs Synthesis Inhibitors on [^{3}H]Thymidine and ^{3}H-Amino Acid Incorporation, OAF Production, and PGs Synthesis by Leukocytes. PHA-stimulated leukocytes incorporated 10- to 15-fold more ^{3}H-amino acids than corresponding unstimulated leukocytes (Table I). This increased incorporation was not affected by the addition of indomethacin (20, 21), R020-5720 (22), and flufenamic acid (21), known inhibitors of PGs synthesis (Table I). In the same cultures, however, these inhibitors completely blocked OAF production by the stimulated cells (Table I), as we have reported previously (10). In parallel with this, PGE content of the stimulated leukocyte media was also decreased to undetectable amounts by the addition of these prostaglandin synthetase inhibitors (Table I). Unstimulated leukocytes cultured with the drugs showed no

TABLE II

Effects of Varying Concentrations of Indomethacin on [^{3}H]TdR and ^{3}H-AA Incorporation and OAF Production by PHA-Stimulated Leukocytes Cultured for 48 h

Concentration	[^{3}H]TdR incorporation*	^{3}H-AA incorporation*	OAF production‡ (treated/control ratios of ^{45}Ca release)
	cpm	*cpm*	
0	2,229 ± 272	918 ± 90	1.39 ± 0.03
10 μM	3,724 ± 142	1,372 ± 111	1.04 ± 0.02§
1 μM	9,895 ± 124	1,258 ± 106	1.05 ± 0.03§
0.1 μM	7,653 ± 437	1,499 ± 136	1.11 ± 0.03§
10 nM	3,415 ± 174	677 ± 88	1.17 ± 0.03§
1 nM	2,083 ± 413	671 ± 38	1.17 ± 0.03§

For experimental details see Materials and Methods.

* Values expressed as means ± SEM for three leukocyte cultures.

‡ Values expressed as means ± SEM for four pairs of bone cultures.

§ Significantly less than corresponding sample obtained from leukocyte cultured without indomethacin.

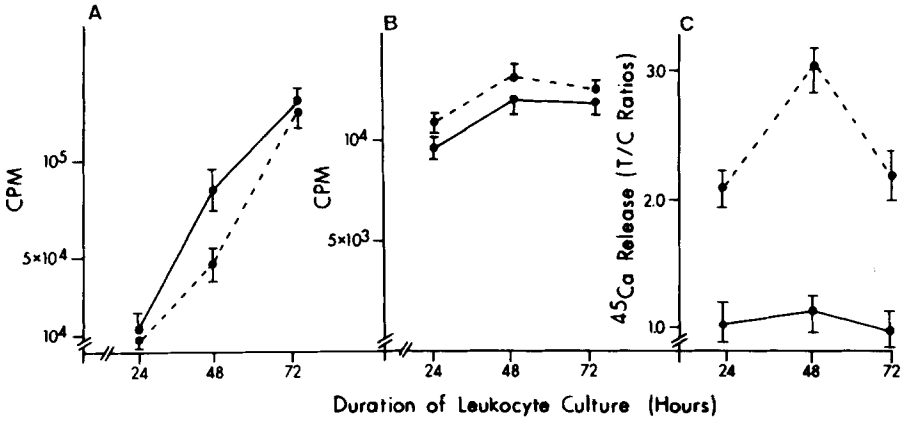

FIG. 1. Time-course of leukocyte activation and OAF production in the presence or absence of 10 μM indomethacin. Three leukocyte cultures, with or without indomethacin, were assessed at each time point of 24, 48, and 72 h. ●- - -●, leukocytes + PHA; ●——●, leukocytes + PHA + Indomethacin. A, [^{3}H]TdR incorporation; B, ^{3}H-AA incorporation; and C, OAF production.

significant changes except for decreased PGE synthesis (Table I). Indomethacin, a potent inhibitor of PGs synthesis, blocked OAF production at concentrations of 1 nM to 10 μM (Table II). Fig. 1 shows a time-course of leukocyte activation and OAF production in the presence or absence of indomethacin (10 μM). Indomethacin did not affect leukocyte activation as assessed by the incorporation of radioactive isotopes into DNA and protein by the stimulated cells, whereas OAF production was completely inhibited by indomethacin through the entire culture period (Fig. 1). Flufenamic acid and R020-5720 demonstrated the same time- and concentration-dependent inhibition on OAF production as indomethacin (data not shown).

Effect of PGs Synthesis Inhibitors on Biological Activity of OAF. To exclude the possibility that these drugs were inhibiting the biological activity of OAF rather than inhibiting OAF production, bones were cultured in OAF-containing media with the inhibitors and bone resorbing activity was measured. There was no inhibition of bone resorption

TABLE III

Effect of PGs Synthesis Inhibitors on Bone Resorbing Activity As a Result of OAF

PGs synthesis inhibitors added to OAF-containing media	72-h ^{45}Ca release (treated/control)* ratios
10 μM	
None	1.50 ± 0.08
Indomethacin	1.52 ± 0.11
R020-5720	1.76 ± 0.14
Flufenamic acid	1.66 ± 0.06

OAF was produced by stimulated leukocytes cultured for 48 h. The inhibitors of PGs synthesis were added directly to the OAF-containing media and the samples were assayed for bone resorbing activity.

* Values are means ± SEM for four pairs of bone cultures.

TABLE IV

Effects of Indomethacin, R020-5720 and Flufenamic Acid on OAF Production When Added to Leukocyte Cultures 24 h after the Stimulation of the Cells with PHA

PGs synthesis inhibitors	OAF production* (treated/control ratios of ^{45}Ca released)
10 μM	
None	2.68 ± 0.17
Indomethacin	2.97 ± 0.47
R020-5720	2.76 ± 0.13
Flufenamic acid	1.88 ± 0.08

* Values are means ± SEM for four pairs of bone cultures.

by 10 μM indomethacin, R020-5720, or flufenamic acid (Table III). Furthermore, when the inhibitors were added to leukocyte cultures 24 h after the stimulation of the cells with PHA, inhibition of OAF production or diminution of OAF effects on bone were not observed with indomethacin or R020-5720 (Table IV). Slightly less OAF was produced in the presence of flufenamic acid (Table IV).

Requirement of the Presence of Monocytes for OAF Production by PHA-Stimulated Lymphocytes. PHA-stimulated nonadherent enriched lymphocytes showed an increase in [^{3}H]thymidine and ^{3}H-amino acid incorporation as compared with corresponding unstimulated lymphocytes, whereas OAF was not produced by these cells at all (Table V). However, as suggested previously (11, 12), this failure of OAF production was restored by reconstitution of stimulated nonadherent enriched lymphocytes (2.0×10^6 cells/ml) with enriched monocytes (1.2×10^5 cells/ml) (Table V). Monocytes (Table V) and unstimulated lymphocytes (data not shown) incorporated little [^{3}H]thymidine or ^{3}H-amino acids and produced no OAF.

OAF Production by PHA-Activated Lymphocytes Cultured with Conditioned Media of Monocytes. The data shown in Table V indicate that activated enriched lymphocytes require the presence of monocytes and/or soluble factors derived from monocytes to produce OAF. To examine this further, nonadherent PHA-stimulated lymphocytes (1.5×10^6 cells/ml) were cultured with monocyte-conditioned medium (MCM) for

TABLE V

Requirement of the Presence of Monocytes for OAF Production by PHA-Activated Lymphocytes (act. lc)

act. lc	Monocytes	[^{3}H]TdR incorporation*	^{3}H-AA incorporation*	OAF production‡ (treated/control ratios of ^{45}Ca release)
		cpm	*cpm*	
+		4,092 ± 339	1,671 ± 60	0.86 ± 0.18
+	+	4,362 ± 91	1,332 ± 44	1.80 ± 0.33§
	+	56 ± 2	69 ± 4	0.84 ± 0.15
Unseparated PHA-activated leukocytes		6,633 ± 226	2,769 ± 250	2.37 ± 0.34§

Adherent and nonadherent cells were separated described in Materials and Methods. The adherent monocytes were cultured at a 2 × 10^5-cell/ml concentration. The nonadherent lymphocytes were cultured at a 1.5 × 10^6-cell/ml concentration. Act. lc, PHA-activated lympocytes.

* Values are means ± SEM for three leukocyte cultures.

‡ Values are means ± SEM for four pairs of bone cultures.

§ Significantly >1.0, $P < 0.05$.

48 h (Table VI). MCM was obtained from the 48-h culture of enriched monocytes (2.0 × 10^5 cells/ml) treated with or without indomethacin (10 μM) and diluted 1:1 with fresh BGJ. Activated enriched lymphocytes that were depleted of monocytes did not produce OAF by themselves, but released OAF when they were cultured with MCM (Table VI). On the other hand, when activated enriched lymphocytes were cultured with conditioned medium from monocytes treated with indomethacin, they failed to produce OAF (Table VI). Neither MCM nor conditioned medium from monocytes treated with indomethacin contained OAF. There was no significant change in the incorporation of [^{3}H]thymidine or ^{3}H-amino acids into the cells cultured with or without MCM (Table VI). The concentration of PGE in the MCM was measured using a radioimmunoassay. The conditioned media from monocytes cultured with indomethacin contained less PGE (0.08 ng/ml) than the media from monocytes cultured without indomethacin (1.6 ng/ml).

OAF Production by PHA-Activated Lymphocytes Cultured in Media Containing Exogenous PGE_1 or PGE_2. The previous results show that soluble factors released by monocytes are required for activated lymphocytes to synthesize OAF and it seemed likely that these soluble factors may be PG. To determine if PGs could obviate the necessity for the presence of monocytes for OAF production by lymphocytes, PGE_1 and PGE_2 (0.1 μM) were added directly to activated enriched lymphocyte cultures (Table VII). In this experiment, the supernates of stimulated lymphocyte cultures were assayed for OAF content and immunoreactive PGE concentrations before and after diafiltration across an Amicon UM 2 membrane, a procedure which was required to remove the exogenously added PGE_1 or PGE_2 before assay for OAF activity. To test the efficacy of diafiltration, the same concentrations of PGE_1 and PGE_2 were added to control media, diafiltered, and assayed for bone resorbing activity and immunoreactive PGE in a similar manner. The concentration of PGE in the diafiltered lymphocyte supernates and in the control media was <0.1 ng/ml, which was <1/400 that of the nondiafiltered supernates and control media to which PGE_1 and PGE_2 were added. This indicates that most PGE in the supernates and the control media was removed

TABLE VI

Effect of MCM on Production of OAF and Incorporation of [^{3}H]TdR and ^{3}H-AA by PHA Lymphocytes

Conditions of leukocyte culture			[^{3}H]TdR incorporation*	^{3}H-AA incorporation*	OAF production‡ (treated/control ratios of ^{45}Ca release)
act. lc	MCM	MCM + Indo			
			cpm	*cpm*	
+			4,089 ± 60	4,635 ± 227	1.02 ± 0.13
+	+		3,268 ± 501	4,814 ± 24	2.52 ± 0.45§
+		+	3,512 ± 566	4,924 ± 387	1.09 ± 0.09
	+				0.97 ± 0.03
		+			1.01 ± 0.03

The methods of preparation of lymphocyte and monocyte cultures are indicated in Methods. Indomethacin (10 μM) was added to the monocytes and the media harvested of 3 d of culture. The duration of the lymphocyte culture was 48 h. The bones were cultured for 72 h.

MCM + Indo, media from monocytes cultured with indomethacin (10 μM).

* Values are means ± SEM for three lymphocyte cultures.

‡ Values are means ± SEM for four pairs of bone cultures.

§ Significantly greater than corresponding control, $P < 0.01$.

TABLE VII

Elaboration of OAF by act. lc. with PGE_1 or PGE_2 Added Exogenously

act. lc	PGE_1	PGE_2	OAF production* ^{45}Ca release (treated/control ratio)	
			Before diafiltration	After diafiltration
	0.1 μM	*0.1 μM*		
+			—	0.93 ± 0.07
+	+		2.81 ± 0.29‡	1.86 ± 0.24‡
+		+	2.83 ± 0.30‡	1.36 ± 0.03‡
	+		1.34 ± 0.03‡	0.92 ± 0.12
		+	1.31 ± 0.03‡	0.95 ± 0.13

Experimental details are indicated in Methods.

* Values are means ± SEM for four pairs of bone cultures.

‡ Significantly >1.0, $P < 0.05$.

during the diafiltration process. No bone resorbing activity was present in these solutions after diafiltration across the Amicon UM 2 membrane (Table VII), indicating that bone resorbing activity present in the lymphocyte culture supernates after this procedure could not be ascribed to PGs. In contrast, the supernates of activated enriched lymphocytes depleted of monocytes cultured with exogenously added PGE_1 or PGE_2 (0.1 μM) showed significant bone resorbing activity after diafiltration (Table VII). When activated lymphocytes were cultured with 1 nM PGE_1 (approximately the amount detected in the MCM), slight but significant recovery of OAF production was observed (treated/control ratio of ^{45}Ca release 1.19 ± 0.02).

Failure of PGE to Augment OAF Activity. Although the results shown in Table VII indicate that OAF was produced by activated lymphocytes cultured in the presence of PGE_1 or PGE_2, and that these exogenously added prostaglandins could substitute for the presence of monocytes in the leukocyte population as far as OAF production was concerned, it might still be possible that PGE augmented OAF activity rather than OAF production. Removal of PGs during diafiltration indicates this explanation

TABLE VIII

The Failure of PGE_1 to Augment the Biological Activity of OAF

OAF-containing media	PGE_1		OAF activity* (treated/control ratios of ^{45}Ca release)
	0.1 μM	1 nM	
+			1.72 ± 0.06
+	+		1.77 ± 0.07
+		+	1.74 ± 0.1
	+		1.20 ± 0.05
		+	1.04 ± 0.08

OAF was produced by stimulated leukocytes cultured for 48 h. PGE_1 was added directly to the OAF-containing media and the samples were assayed for bone resorbing activity.

* Values are means ± SEM for four pairs of bone cultures.

is unlikely. To exclude this possibility further, we added PGE_1 in varying concentrations to submaximal doses of OAF (Table VIII). We found no augmentation of OAF activity.

Discussion

In the present study, we have found that: (*a*) OAF was not produced by stimulated lymphocytes unless monocytes coexisted with them in the leukocyte population (Table V); (*b*) OAF was produced by stimulated lymphocytes cultured with MCM, but not with conditioned medium from monocytes treated with indomethacin (Table VI); (*c*) PGE concentrations were much higher in MCM (1.6 ng/ml) than in conditioned media from monocytes treated with indomethacin (0.08 ng/ml); (*d*) OAF was produced by stimulated enriched lymphocytes cultured with exogenously added PGE_1 or PGE_2 (Table VII); and (*e*) PGE_1 was unable to enhance OAF activity (Table VIII). These findings lead us to conclude that PG of the E series synthesized and released by monocytes are required for activated lymphocytes to produce OAF.

The fact that nonadherent enriched lymphocytes depleted of monocytes were capable of producing OAF in the presence of MCM or exogenously added PGE_1 (Tables VI and VII) indicates that the lymphocyte is the cell which is responsible for OAF production. This confirms earlier data which showed that OAF production was markedly diminished when PHA-stimulated leukocytes were irradiated (11). Because lymphocytes are much more sensitive to irradiation than monocytes, these authors suggest that reduction in OAF as lymphocytes were depleted indicates that lymphocytes were responsible for OAF production. However, this evidence is indirect and there can be other interpretations of the data. Now we have shown that lymphocytes depleted of monocytes are capable of producing OAF, this is direct evidence that the lymphocyte is the cell source of OAF production.

The amounts of OAF which was produced by PHA-activated nonadherent enriched lymphocytes cultured with exogenously added PGE_1 was not significantly different from that produced by activated lymphocytes cultured with MCM or unseparated leukocytes. Thus, it is likely that PGE by itself is sufficient to replace the requirement of the presence of monocytes for OAF production by activated lymphocytes. However, we cannot exclude the possibility that some other monocyte-derived factor, as well as PGE, is necessary for OAF production.

Most reports of the effects of PGs on the secretion of inflammatory mediators have suggested that PGs are inhibitory. For example, the release of histamine by human and monkey mast cells and β-glucuronidase and other lysosomal hydrolases by human neutrophils is blocked by PGE (23). Similarly, the secretion of lymphokines such as macrophage migration inhibition factor (7) and leukocyte inhibitory factor (9) are also suppressed by PGE. Moreover, Gordon et al. (8) have shown that PGE_1 inhibits migration inhibition factor secretion by cultured guinea pig peritoneal exudate cells which consist of 60–80% macrophages and had been already found to produce PGE_1 when cultured in vitro (24). Therefore, Morley (6) has proposed a hypothesis that lymphokines activate macrophages to produce PGE, which in turn inhibit further lymphokine production by a negative feed-back mechanism. This inhibitory effect of PGE on lymphokine production has been considered to be a self-protective mechanism from the progressive development of unregulated immune responses (23). In contrast, we have shown here that the presence of PGE is required for the production of another lymphokine, OAF. Also, the evidence reported here indicates that PGE resorbs bone not only directly but also indirectly by enhancing OAF production upon the appropriate stimulation.

As PGE has been known to stimulate intracellular cyclic AMP accumulation, the enhancing effects of PGE on OAF production may be mediated by cyclic AMP. However, Koopman et al. (7), Bourne et al. (23), and Lomnitzer et al. (9) have shown that isoproterenol and PGE (both stimulators of adenylate cyclase), theophylline, and other methylxanthine derivatives (phosphodiesterase inhibitors), and dibutyryl cyclic AMP (an analogue of the endogenous nucleotide) all inhibit histamine release and lymphokine production together with elevation of intracellular cyclic AMP. It seems reasonable to suggest that cyclic AMP may be a negative modulator of inflammation and immunity in these circumstances. However, the situation may be different with OAF production. As PGs are necessary for OAF production, it is possible that in this case intracellular cyclic AMP enhances rather than inhibits the production of the lymphokine. Studies are now in progress to investigate the relationship between cyclic AMP and OAF production.

Of particular interest is the role of monocytes in OAF production. In the present study, monocytes have behaved as helper cells by releasing PGs to allow OAF production by activated lymphocytes. This is another mechanism by which monocytes can influence the process of bone resorption. Other work from our laboratory has demonstrated that monocytes directly resorb both devitalized bones by a mechanism which is not mediated by the action of osteoclasts (25) and live bones through an osteoclast-mediated mechanism which is prostaglandin-dependent (26). There is also other evidence which suggests that monocytes and macrophages synthesize and release PGs (27–29), collagenase (30), and hydrolytic enzymes (31), which are all capable of resorbing bone or degrading bone matrix. It is also possible that circulating mononuclear cells may transform or differentiate into osteoclasts (32). Thus, it seems likely that monocytes possess multifunctional capacities in the bone resorption process and may reveal each function separately or synergistically depending on the environmental conditions.

Although the PGs synthesis inhibitors blocked OAF production completely, they showed no consistent effect on the incorporation of [^{3}H]thymidine and ^{3}H-amino acids by the cells (Table I, Fig. 1). Phillips et al. (33) have also demonstrated that

[^{3}H]thymidine, [^{3}H]uridine, and [^{14}C]leucine incorporation by PHA-stimulated lymphocytes were not affected by indomethacin, phenylbutazone, and aspirin. Therefore, it is clear that PGs are not essential for the stimulation of lymphocytes (10, 33). Thus, the pathway or mechanism of OAF production in the lymphocyte may be different from that of cell transformation.

In the experiment shown in Table II, [^{3}H]thymidine and ^{3}H-amino acid incorporation by the stimulated cells increased after the addition of indomethacin, in spite of decreased OAF production. Similar results have been described by Goodwin et al (34). They demonstrated the presence of prostaglandin-producing suppressor cells which adhered to glass-wool in patients with Hodgkin's disease (34) and normal persons (35). Our leukocytes were obtained from normal human peripheral blood obtained from plateletpheresis donations. We observed an increase in incorporation of radioactive materials into lymphocytes after adding indomethacin in only one experiment of many. This finding in one experiment and discrepancy from others' results is unexplained, but may be a result of differences in the source of leukocytes or by differences in cell separation techniques.

The incorporation of [^{3}H]thymidine and ^{3}H-amino acids into PHA-stimulated, nonadherent enriched lymphocytes was significantly less than that of stimulated unseparated leukocytes (Table V). Reconstitution of the lymphocytes with enriched monocytes did not increase the incorporation to that of unseparated leukocytes (Table V). There are a number of possible explanations. We cannot be sure that the proportions of T lymphocytes, B lymphocytes, and monocytes are the same in the reconstituted population as in the unseparated cells. There may also have been some impairment of mitogen sensitivity or vitality of some of the cells during the process of leukocyte separation. Using different separation techniques others have found that reconstituted cells incorporated more [^{3}H]thymidine than unseparated cells (11).

Our findings show that the presence of prostaglandins are necessary for OAF to be produced by activated lymphocytes. The usual source of PGs is likely to be the monocyte. However, it is also likely that PGs produced by neoplastic cells (36, 37) or by chronic inflammatory cells (5, 38, 39) also modulate OAF production by lymphocytes. Thus, PGs produced by neoplastic or inflammatory cells may influence bone resorption directly by activating osteoclasts and indirectly through their influences on OAF production.

Summary

Osteoclast-activating factor (OAF), a powerful stimulator of osteoclastic bone resorption, is released by peripheral blood mononuclear cells on exposure to phytohemagglutinin (PHA) or a specific antigen to which the leukocytes have been previously exposed. Both lymphocytes and monocytes are required in the leukocyte population for OAF release to occur. In this study we examined the relationship between the lymphocyte and monocyte in OAF production. Biological activity, as a result of OAF, was assessed by a bioassay based on the release of previously incorporated ^{45}Ca from fetal rodent long bones in organ culture. We found that an enriched lymphocyte population depleted of monocytes by serial adherence does not release OAF after stimulation with PHA, although the cells are activated as assessed by [^{3}H]thymidine and ^{3}H-amino acid incorporation. When conditioned media harvested from adherent cells which did not contain OAF was added to the enriched lympho-

cytes, OAF release occured. Media harvested from adherent cells which were cultured with indomethacin (10 μM), an inhibitor of prostaglandin synthesis, did not permit OAF release by activated lymphocytes. When PGE_1 and PGE_2 (0.1 μM) were added exogenously to the enriched lymphocyte population, OAF release occurred after stimulation with PHA. These results indicate that, (*a*) the activated lymphocyte is the cell of origin of OAF, (*b*) prostaglandins produced by monocytes are necessary for OAF production by activated lymphocytes, and (*c*) monocyte prostaglandins can influence bone resorption indirectly by regulating OAF production as well as directly by osteoclast activation. The interactions of OAF and prostaglandins at bone resorbing sites may be important in inflammatory and neoplastic diseases associated with bone destruction.

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References

1. Raisz, L. G., C. L. Trummel, G. R. Mundy, and R. A. Luben. 1975. Immunologic factors influencing bone resorption, role of osteoclast activating factor from human lymphocytes and complement mediated prostaglandin synthesis. *In* Calcium-Regulating Hormones, R. V. Talmage, M. Owen, and J. A. Parsons, editors. Excerpta Medica, Amsterdam. 149.
2. Horton, J. E., L. G. Raisz, H. A. Simmons, J. J. Oppenheim, and S. E. Mergenhagen. 1972. Bone resorbing activity in supernatant fluid from cultured human peripheral blood leukocytes. *Science (Wash. D. C.).* **177:**793.
3. Mundy, G. R., L. G. Raisz, R. A. Cooper, G. P. Schechter, and S. E. Salmon. 1974. Evidence for the secretion of an osteoclast stimulating factor in myeloma. *New Engl. J. Med.* **291:**1041.
4. Klein, D. C., and L. G. Raisz. 1970. Prostaglandins: stimulation of bone resorption in tissue culture. *Endocrinology.* **86:**1436.
5. Robinson, D. R., A. H. Tashjian, and L. Levine. 1975. Prostaglandin-stimulated bone resorption by rheumatoid synovia. A possible mechanism for bone destruction in rheumatoid arthritis. *J. Clin. Invest.* **56:**1181.
6. Morley, J. 1974. Prostaglandins and lymphokines in arthritis. Prostaglandins. **8:**315.
7. Koopman, W. J., M. H. Gillis, and J. R. David. 1973. Prevention of MIF activity by agents known to increase cellular cyclic AMP. *J. Immunol.* **110:**1609.
8. Gordon, D., M. A. Bray, and J. Morley. 1976. Control of lymphokine secretion by prostaglandins *Nature (Lond.).* **262:**401.
9. Lomnitzer, R., A. R. Rabson, and H. J. Koornhof. 1976. The effects of cyclic AMP on leukocyte inhibitory factor production and on the inhibition of leukocyte migration. *Clin. Exp. Immunol.* **24:**42.
10. Yoneda, T., and G. R. Mundy. 1979. Prostaglandins are necessary for Osteoclast-Activating Factor production by activated peripheral blood leukocytes *J. Exp. Med.* **149:**279.
11. Horton, J. E., J. J. Oppenheim, S. E. Mergenhagen, and L. G. Raisz. 1974. Macrophage-lymphocyte synergy in the production of Osteoclast Activating Factor. *J. Immunol.* **113:** 1278.
12. Chen, P., C. Trummel, J. Horton, J. J. Baker, and J. J. Oppenheim. 1976. Production of osteoclast-activating factor by normal human peripheral blood rosetting and nonrosetting lymphocytes. *Eur. J. Immunol.* **6:**732.
13. Trummel, C. L., G. R. Mundy, and L. G. Raisz. 1975. Release of osteoclast activating

factor by normal human peripheral blood leukcoytes. *J. Lab. Clin. Med.* **85:**1001.
14. Biggers, J. D., B. L. Gwatkin, and S. Heyner. 1961. Growth of embryonic avian and mammalian tibiae on relatively simple chemically defined medium. *Exp. Cell Res.* **25:**41.
15. Ackerman, S. K., and S. D. Douglas. 1978. Purification of human monocytes on microexudate-coated surfaces. *J. Immunol.* **120:**1372.
16. Koski, I. R., D. G. Poplack, and R. M. Blease. 1976. A non-specific esterase stain for the identification of monocytes and macrophages. *In* In Vitro Methods in Cell-Mediated and Tumor Immunity. B. R. Bloom and J. R. David, editors. Academic Press, Inc., New York. 359.
17. Raisz, L. G. 1965. Bone resorption in tissue culture. Factors influencing the response to parathyroid hormone. *J. Clin. Invest.* **44:**103.
18. Zurier, R. B., and D. M. Sayadoff. 1975. Release of prostaglandins from human polymorphonuclear leukocytes. *Inflammation.* **1:**93.
19. Jaffe, B. M., H. R. Behrman, and C. W. Parker. 1973. Radioimmunoassay measurement of prostaglandins E, A and F in human plasma. *J. Clin. Invest.* **52:**398.
20. Vane, J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol.* **231:**232.
21. Flower, R. D. 1974. Drugs which inhibit prostaglandin biosynthesis. *Pharmacol. Rev.* **26:**33.
22. Gant, Z. N., H. Baruth, L. O. Randall, C. Ashley, and J. R. Paulsrud. 1975. Stereoisomeric relationships among anti-inflammatory activity, inhibition of platelet aggregation and inhibition of prostaglandin synthetase. *Prostaglandins.* **10:**59.
23. Bourne, H. R., L. M. Lichtenstein, K. L. Melmon, C. S. Henney, Y. Weinstein, and G. M. Shearer. 1974. Modulation of inflammation and immunity by cyclic AMP. Receptors for vasoactive hormones and mediators of inflammation regulate many leukocyte functions. *Science* (*Wash. D. C.*). **184:**19.
24. Bray, M. A., D. Gordon, and J. Morley. 1974. Role of prostaglandins in cellular immunity. *Br. J. Pharmacol.* **52:**253P.
25. Mundy, G. R., A. J. Altman, M. D. Gondek, and J. G. Bandelin. 1977. Direct resorption of bone by human monocytes. *Science* (*Wash. D. C.*). **196:**1109.
26. Dominguez, J. H., and G. R. Mundy. 1978. Monocyte-mediated osteoclastic bone resorption. *Clin. Res.* **26:**630A.
27. Humes, J. L., R. J. Bonney, L. Pelus, M. E. Dahlgren, S. J. Sadowski, F. A. Kuehl, Jr., and P. Davies. 1977. Macrophages synthesize and release prostaglandins in response to inflammatory stimuli *Nature* (*Lond.*). **269:**149.
28. Kurland, J. I., and R. Bockman. 1977. Prostaglandin E production by human blood monocytes and mouse peritoneal macrophages. *J. Exp. Med.* **147:**952.
29. Bray, M. A., and D. Gordon. 1978. Prostaglandin production by macrophages and the effect of anti-inflammatory drugs. *Br. J. Pharmacol.* **63:**635.
30. Wahl, L. M., C. E. Olsen, A. L. Sandberg, and S. M. Mergenhagen. 1977. Prostaglandin regulation of macrophage collagenase production. *Proc. Natl. Acad. Sci. U.S.A.* **74:**4955.
31. Unanue, E. R. 1976. Secretory function of mononuclear phagocytes. A review. *Am. J. Pathol.* **83:**396.
32. Walker, D. G. 1975. Control of bone resorption by hematopoietic tissue. The induction and reversal of congenital osteopetrosis in mice through use of bone marrow and splenic transplants. *J. Exp. Med.* **142:**651.
33. Phillips, C. A., E. Z. Girit, and J. E. Kay. 1978. Changes in intracellular prostaglandin content during activation of lymphocytes by phytohemagglutinin. *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett.* **94:**115.
34. Goodwin, J. S., R. P. Messner, A. D. Bankhurst, G. T. Peake, J. H. Saiki, and R. C. Williams, Jr. 1977. Prostaglandin-producing suppressor cells in Hodgkin's disease. *New Engl. J. Med.* **297:**963.

35. Goodwin, J. S., A. D. Bankhurst, and R. P. Messner. 1977. Suppression of human T-cell mitogenesis by prostaglandin. Existence of a prostaglandin-producing suppressor cell. *J. Exp. Med.* **146:**1719.
36. Tashjian, A. H., E. F. Voelkel, L. Levine, and P. Goldhaber. 1972. Evidence that the bone resorption-stimulating factor produced by mouse fibrosarcoma cells is prostaglandin E_2. *J. Exp. Med.* **136:**1329.
37. Voelkel, E. F., A. H. Tashjian, Jr., R. Franklin, E. Wasserman, and L. Levine. 1975. Hypercalcemia and tumor-prostaglandins: the VX_2 carcinoma model in the rabbit. *Metab. Clin. Exp.* **24:**973.
38. Dayer, J-M., S. M. Krane, R. G. G. Russell, and D. R. Robinson. 1976. Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells. *Proc. Natl. Acad. Sci. U.S.A.* **73:**945.
39. Bonta, I. L., and M. J. Parnham. 1978. Prostaglandins and chronic inflammation. *Biochem. Pharmacol.* **27:**1611.