Vol. 84, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 14, 1978 Pages 15-22

PURIFICATION OF A LYMPHOKINE: OSTEOCLAST ACTIVATING FACTOR FROM HUMAN TONSIL LYMPHOCYTES

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Received July 21,1978

SUMMARY

Osteoclast activating factor is a lymphokine produced by mitogen-stimulated human lymphocytes. The current studies describe purification to essential homogeneity of the major form of osteoclast activating factor present in supernatants of phytohemagglutinin stimulated lymphocyte cultures. Preliminary chemical and biological characterization of the purified material was carried out. The active factor is a peptide which migrates in polyacrylamide gel electrophoresis as an α-2 fraction in native gels and as a 9,000-dalton species in sodium dodecyl sulfate-urea gels. The purified fraction stimulates bone resorption in vitro at doses between 0.1 and 500 ng/ml, with half-maximal stimulation at approximately 1 ng/ml.

INTRODUCTION

Bone resorbing activity in cultures of human leukocytes was first reported by Horton, et.al. (1), who used buffy coat cells from patients with periodontal disease, activated by dental plaque antigen or by phytohemagglutinin (PHA). The activity, termed osteoclast activating factor (OAF), was subsequently shown to be a peptide-like material secreted by PHA-stimulated lymphocytes from normal individuals, and was partially purified (2). OAF causes stimulation of bone resorption in vitro (1-3) and also inhibits synthesis of collagen by bone fragments in vitro (3-4). Similar activity is produced by primary human myeloma cell cultures (5) and by stable cell lines derived from bone destroying lymphoid tumors such as myelomas and lymphomas (6). Because of the frequency of bone destruction associated with lymphoid tumors and chronic inflammatory diseases, it has been postulated (1-6) that OAF may play a major role in localized pathologic bone destruction. OAF, like other lymphokines, is secreted in very small quantities but with high biological activity (2,3). Therefore, it has been difficult to obtain enough of the material to allow purification by classical procedures. In the current studies, we utilized human tonsils as a readily available source of large numbers of lymphocytes. This allowed us to process large quantities of culture medium from PHA-stimulated lymphocytes, and allowed development of procedures for fractionation of OAF to a high degree of purity. The purification scheme developed yields OAF in a biologically active form, which should facilitate further biological and chemical characterization.

Abbreviations: OAF, osteoclast activating factor; PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate; HBSS, Hanks' balanced salt solution.

0006-291X/78/0841-0015$01.00/0

MATERIALS AND METHODS

Leukocyte cultures: Tonsils were obtained from local hospitals under informed consent procedures approved by the appropriate Human Subjects committees. Tonsils were stored in Hanks' basal salt solution (HBSS) for transport to the laboratory. Upon arrival, they were rinsed several times with fresh HBSS, and were disrupted by mincing, followed by grinding on a stainless steel mesh with several washes using RPMI 1640 medium with 10% fetal calf serum. The material forced through the mesh was centrifuged for two minutes at 25° C at 300 x G to remove particles of connective tissue. The supernatant was then centrifuged at 2000 x G for 10 minutes. The sedimented cells were washed once with fresh medium, and cell counts and viability were determined. Cells were resuspended in RPMI 1640 medium containing 0.5% glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone, and no added serum. Cells were cultured at 1 to 2 x 10^6 cells per milliliter in screw-cap, 2-liter glass flasks containing 600 ml of suspension per flask. Approximately 95% of the cells from each lymphocyte preparation were stimulated with PHA (Sigma type 5 lectin) at a concentration of 2.5 µg/ml. Total viable cell yield from a single tonsil was normally 2 to 4 x 10^9 cells. Cells were incubated at 37° in 5% CO_2: 95% air for 24 hours; approximately 80% of the medium was then removed by decanting and was replaced with fresh medium. Culture was continued for another 48 hours (72 hours total culture). Activation of cells was monitored by comparing [^{3}H] thymidine uptakes between untreated and PHA treated cells. The recovered culture supernatants were pooled and sterilized by filtration. This culture supernatant was concentrated approximately 1000-fold by use of an Amicon ultrafiltration apparatus, using a PM10 filter (nominal 10,000 molecular weight cutoff). This concentration and all subsequent purification steps were carried out at 4° C.

Biological assays of OAF: OAF activity was assayed by the ability of concentrated culture supernatants to cause a net stimulation of the release of calcium from cultured mouse cranial bones (8). Cranial bones were obtained from 2 to 3 day old mice, and were cultured in BGJ_b culture medium (Fitton-Jackson modification; Gibco) containing 4 mg/ml bovine serum albumin. To the treated bones were added appropriate volumes of sample from chromatography or ultrafiltration steps. Control bones received equivalent dilutions of column buffer, or of equivalent fractions from unstimulated lymphocyte cultures. After 72 hours of culture the medium was removed for calcium analysis by atomic absorption spectrometry. Results are expressed as the effect on final medium calcium concentration exerted by the treatment, after compensation for changes in medium calcium by killed bones in parallel cultures, as described previously (7,8).

Gel filtration: Concentrated culture supernatants were fractionated using a 2.5 x 100 cm column of Sephadex G-100 equilibrated and eluted with 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.2. OAF activity eluted from this column as a single major peak between K_D 0.5 and 0.6 (2). This fraction was pooled and concentrated using Amicon UM2 membranes (nominal molecular weight cutoff 1,000).

Ultrafiltration: The concentrated G-100 fractions were made 1.0 M in NaCl, then ultrafiltered across an Amicon PM10 membrane, using 5 mM Tris-HCl in 1.0 M NaCl, pH 7.75. The total ultrafiltrate volume collected was 20 times the initial sample volume. The ultrafiltrate was then concentrated and desalted by ultrafiltration (using a UM2 membrane), with 10 volumes of 5 mM Tris-HCl, pH 7.75. The retentate from this diafiltration contained the OAF activity and was used for subsequent purification steps.

DEAE chromatography: The concentrated sample from ultrafiltration was fractionated using a 0.9 x 25 cm column of DEAE-cellulose (DE52) initially equilibrated with 5 mM Tris-HCl, pH 7.75. The fraction was applied and the column washed with 50 ml of the same buffer. Then a linear gradient of 0 - 0.3 M NaCl was applied over a volume of 300 ml. Fractions were collected and assayed individually for OAF activity. The active fractions were pooled and desalted on UM2 membranes, using ultrafiltration with 5 mM Tris-HCl, pH 7.75, and concentrated to .001 of the original culture volume. This fraction was then applied to a 0.9 x 15 cm column of DEAE-Sephadex A25, and eluted with a gradient of 0 - 0.16 M NaCl. The active fractions were pooled, concentrated and desalted with UM2 membranes.

Polyacrylamide gel electrophoresis (PAGE): Samples were electrophoresed in slab gels 0.75 mm thick using a discontinuous buffer system: separation gel,7% acrylamide (1/40 methylene bisacrylamide) in 0.375 M Tris-HCl buffer, pH 8.5; stacking gel, 3% acrylamide (1/40 methylene bisacrylamide) in 0.05 M Tris-phosphate, pH 6.4; electrode buffer, 0.025 M Tris-glycine, pH 8.3. These conditions were chosen after preliminary trial and error experiments to determine optimum conditions for both separation and recovery of biological activity from gels. Samples were separated either at room temperature or 4°C at 150V for 3 - 5 hours. Gels were then stained with Coomassie blue (9). Alternatively, gels were cut into segments and eluted overnight at 4°C with culture medium containing 10% fetal calf serum. Control samples were prepared by eluting corresponding gel segments from lanes which had been run with sample buffer only. Aliquots of experimental or control gel eluates were used for bioassay of OAF activity as described above. Sample elution from the gels using these procedures did not interfere with normal bone calcium release, or with stimulation of calcium release induced by parathyroid hormone or OAF (data not shown).

SDS-PAGE: Samples were analyzed on 0.75 mm thick slab gels (15% acrylamide, 1/20 methylene bisacrylamide) containing 7 M urea and 0.1% sodium dodecyl sulfate (SDS) using a discontinuous buffer system (9). Samples were treated at 100°C for 3 minutes with 1% SDS and 0.1% 2-mercaptoethanol before layering on gels. Electrophoresis was carried out at 100 V for 5-8 hours, and gels then were stained with Coomassie blue.

End group analysis: Pooled and concentrated fractions from the second step of ion exchange chromatography were desalted by UM2 ultrafiltration, lyophilized and analyzed for amino-terminal residues after dansylation followed by acid hydrolysis (10).

RESULTS AND DISCUSSION

Quantitation of OAF activity: As shown previously (2,3), the stimulation of calcium release from mouse cranial bones by OAF was markedly dose-dependent (Figure 1). Stimulation increased sharply over approximately a 30-fold range of concentration, reached a maximum and then decreased gradually. The reasons for the decrease in activity at supramaximal doses are not clear; however, this would appear to be an inherent property of OAF in this system, since it was observed to approximately the same extent in samples at all stages of purification. For the present study, its primary significance was that each OAF sample had to be assayed at multiple dilutions in order to arrive at a reliable estimate of the actual concentration. For comparison of samples in different experiments, and especially in order to monitor recovery, it was necessary to define a unit of OAF activity. Therefore, one "bone-resorbing unit" of activity was defined as the quantity of OAF activity necessary to produce half-maximal stimulation of calcium release from mouse cranial bones, measured on the ascending slope of the dose-response curve (Figure 1).

Ultrafiltration/diafiltration: OAF activity was remarkably stable to ultrafiltration, whether the primary recovery was in the retentate or ultrafiltrate. There was essentially no detectable loss of OAF activity in any of the several ultrafiltration steps of the purification procedure. As previously shown by Mundy and Raisz (11), near physiologic osmolarities (e.g., 0.15 M NaCl) OAF penetrated Amicon PM10 membranes only slightly (Table 1, Step 1). However, when the samples were made 1 M in NaCl, considerably more penetration of the PM10 membrane was observed (Table 1, Step 3). This change in

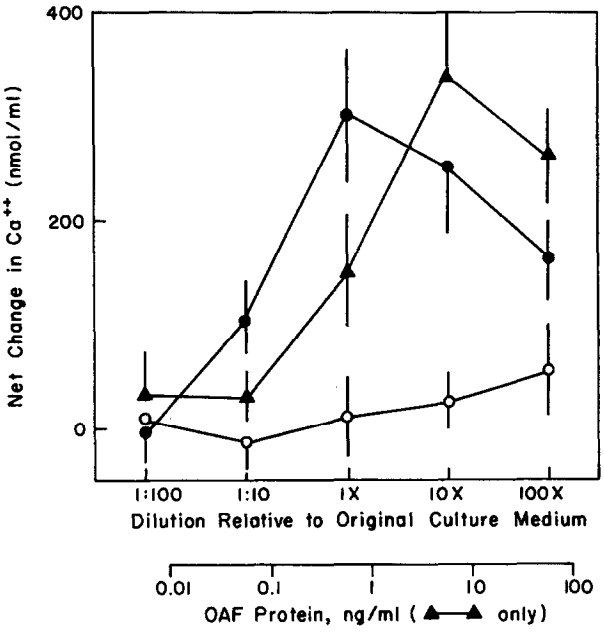

Figure 1. Log dose-response curves for resorption of cultured bone by culture medium from PHA-stimulated (●——●) or unstimulated (○——○) lymphocytes, or by purified OAF (▲——▲) from the second stage of ion-exchange chromatography (see Figure 2). All samples were concentrated approximately 1000 X relative to initial volume of lymphocyte culture. Appropriate volumes of these concentrates were added to mouse cranial bones in culture. Calcium release is expressed relative to bones cultured without additions; all cultures were for 72 hours. Values are mean ± S.E.M. for four to six bones. The dose for purified OAF was not corrected for losses of activity during purification; therefore, the position of this curve to the right of that for crude culture medium reflects approximately 10% recovery of OAF activity. The actual protein concentration of the purified OAF sample is reflected by the lower scale on the abscissa.

penetration of the PM10 membrane was most likely due to a change in the permeability of the membrane at high ionic strengths, since similar increases in penetration were found for other proteins when they were ultrafiltered in 1 M NaCl (unpublished data). The removal of large molecular weight proteins from OAF culture medium by PM10 ultrafiltration in 1 M NaCl resulted in approximately 100-fold purification. The ultrafiltered activity was subsequently re-concentrated and desalted using UM2 membranes, which OAF activity did not penetrate under any conditions.

Ion exchange chromatography: Concentrated, desalted ultrafiltrates containing OAF also contained residual higher molecular weight proteins, principally albumin. These were present due to trailing of peaks in the original gel filtration, along with minimal but significant penetration of the PM10 membrane by albumin at high ionic strength. These contaminants were removed by two steps of ion exchange chromatography, on DEAE cellulose and subsequently on DEAE Sephadex A25 (Figure 2). The former column was

TABLE I

SUMMARY OF OAF PURIFICATION STEPS AND RECOVERY

	STEP	TOTAL ACTIVITY* (UNITS**)	SPECIFIC ACTIVITY (UNITS/ μg PROTEIN)
0.	Starting Material	100	0.02
1.	Concentration (PM10 Membrane)	95	0.03
2.	Gel Filtration (Sephadex G-100)	69	0.62
3.	Ultrafiltration at 1 M NaCl (PM10 Membrane)	68	52
4.	Concentration/Desalting (UM2 Membrane)	65	79
5.	DEAE-cellulose chromatography***	32	248
6.	DEAE-Sephadex A25 chromatography***	12	890

* Activity recovered at end of step, per 100 bone resorbing units in original sample.

** One bone resorbing unit = half-maximal stimulation of calcium release from cultured bones in serial dilution bioassay. See text for details.

*** Ion-exchange steps were followed by concentration and desalting using UM2 membranes. This produced no significant losses or changes in specific activity.

used because of a relatively higher capacity, while the latter was used because it produced sharper fractionation peaks than DEAE-cellulose. In both cases, OAF activity eluted at approximately 0.10 M NaCl (Figure 2). The second step of DEAE ion exchange chromatography produced a sharp peak of coinciding OAF activity and protein concentration (Figure 2B). Correlation of the bone resorption dose-response curve (Figure 1) of this fraction with the measured protein concentration indicated that one "bone-resorbing unit" (half maximal stimulation of resorption) corresponded to approximately 1 ng purified OAF per ml of bioassay culture medium.

Assessment of homogeneity: The OAF peak from DEAE-Sephadex A-25 chromatography was analyzed by both native gel and SDS-urea gel electrophoresis. There was a single protein band in both types of gel which ran in an α-2 region of the native gel (Figure 3A). OAF biological activity was recovered from the identical region of the gel as the protein band. Relative to baseline elevations in parallel blank gels, it was estimated that the single major band in OAF-containing native gels accounted for over 97% of the Coomassie blue-staining material in the sample. In SDS-urea gels, the OAF band ran as a 9,000-dalton species, based on comparison with low molecular weight standards (Figure

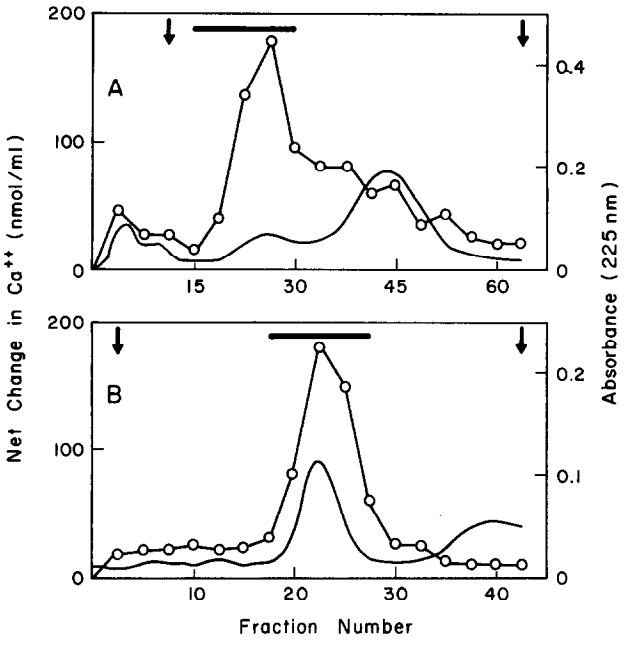

Figure 2. Chromatography of OAF on DEAE-cellulose (Figure 2A) and subsequently on DEAE-Sephadex A25 (Figure 2B). See text for details of conditions. Biological activity was measured by adding aliquots of individual samples to mouse bones in culture (O———O). Calcium release is expressed relative to bones cultured with aliquots of column buffer from parallel columns (run without sample). Arrows mark the beginning and end of linear NaCl gradients: 0 - 0.3 M NaCl for DEAE-cellulose and 0 - 0.16 M NaCl for DEAE-Sephadex A25. The solid bars represent fractions pooled for subsequent purification or analysis.

3B). The discrepancy between OAF's behavior on SDS-urea gels (MW = 9,000) and on Sephadex G-100 (MW = 17,000; ref. 2) suggests that the active species may aggregate under nondissociating conditions. Mundy and Raisz (11) have also reported evidence for aggregation of OAF, and similar evidence has been presented for other lymphokines. We have seen no evidence for species of OAF smaller than the 9,000-dalton band on SDS-urea gels, although the system reliably resolves peptides as small as bacitracin (MW = 1,400). Therefore, the 9,000-dalton species would appear to be the "monomer" from which larger aggregates could form.

Amino terminal analysis of OAF was carried out by a dansylation procedure (10), and revealed that the OAF fraction exhibited a single α-amino dansyl residue, corresponding to glutamic acid or glutamine. This is additional evidence, although not absolute proof, that the peptide band shown in Figure 3 is highly uniform and possibly homogeneous.

Purification of OAF from human lymphocytes should facilitate studies of the chemical structure of this material, as well as studies of the role of OAF in bone diseases.

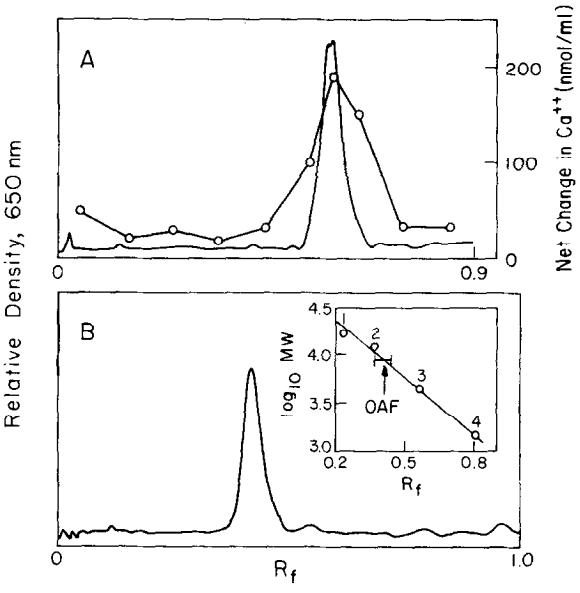

Figure 3. Densitometric scans of Coomassie blue-stained polyacrylamide gels. Sample used was the pooled active fraction from DEAE-Sephadex A25 chromatography (see Figure 2B). Figure 3A: Native gel, 7% acrylamide, pH 8.5. Sections of a parallel (unstained) gel were eluted with culture medium as described in the text, then assayed for bone resorption (O———O). Calcium release is expressed relative to bones cultured with aliquots eluted from an identical gel run without sample. Figure 3B: Gel with 15% acrylamide, 0.1% SDS and 7 M urea. Inset: Migration of molecular weight standards in this gel system. Standards used were: (1) myoglobin, 17,200 MW; (2) cytochrome c, 12,300 MW; (3) adrenocorticotropin, 4,600 MW; (4) bacitracin, 1,400 MW. OAF migrated at Rf = 0.52 ± 0.04 in six separate runs, indicating a molecular weight of approximately 9,000 in this system.

Heretofore, such studies have suffered from uncertainty as to the identity and uniqueness of the material under consideration. The same has been true with studies of other lymphokines, because most of these factors are also secreted in low quantities with high biological activity, making their purification very difficult. This report represents one of the first highly homogeneous preparations of a lymphokine. Hopefully, similar techniques of mass culture and biochemical purification will improve the production of highly purified fractions of other lymphokines. This would allow more precise biochemical characterization of the structures and mechanisms of action of lymphokines, leading to a better understanding of their functions in living organisms.

Acknowledgements: This study was supported by USPHS grants DE04766 and RR09070, and by American Cancer Society grant BC-263. The technical assistance of M. A. Mohler, P. D. Zayac and N. P. Browning is gratefully acknowledged.

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