

# Identification and Characterization of Osteoclast-like Cells and Their Progenitors in Cultures of Feline Marrow Mononuclear Cells

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**ABSTRACT** The predominant cell responsible for bone resorption, the multinucleated osteoclast, has been difficult to study because of inaccessibility. When feline marrow-derived mononuclear cells are established in long-term culture, multinucleated cells form within 48 h, reaching maximum numbers at 16 d. We have observed that these cultured cells have many of the features of osteoclasts. Morphologically, they are multinucleated, contain large numbers of branched mitochondria, have a peripheral cytoplasm lacking organelles (a clear zone), and have extensive cell-surface processes. In addition to these ultrastructural features, the cells contain a tartrate-resistant acid phosphatase, the activity of which is increased by parathyroid hormone (PTH) and inhibited by calcitonin. PTH, prostaglandin E<sub>2</sub>, and 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub> increased multinucleated cell formation, while calcitonin inhibited the stimulatory effects of PTH. Time-lapse cinemicrographic and autoradiographic studies indicated that the multinucleated cells formed by fusion of the mononuclear progenitors. The multinucleated cells were phagocytic and stained with nonspecific esterase, consistent with their being derived from immature monocytes. Further, cell populations enriched for multinucleated cells release <sup>45</sup>Ca from devitalized bone. Density-gradient centrifugation on Percoll was used to enrich and characterize the mononuclear progenitors of these multinucleated cells. The progenitor cells were found predominantly in Percoll density layers of 1.065 to 1.08 g/ml and were enriched up to 30-fold as compared to unfractionated cells. The bone marrow mononuclear cells that formed the multinucleated cells were initially nonadherent to plastic, stained heavily with nonspecific esterase, and appeared to be immature monocytes histologically. These data indicate that the multinucleated osteoclast-like cells in our cultures are derived from nonadherent monocytic progenitor cells that are responsive to osteotropic hormones. The ability to grow and characterize these cells *in vitro* should facilitate studies to elucidate the role these cells play in normal and pathologic states of bone resorption.

Studies using a variety of model systems including quail-chick chimeras, parabiotic rats, and bone marrow transplantation have shown that osteoclast precursors are present in hemopoietic tissues, and have suggested that osteoclasts result from fusion of mononuclear phagocytes (1–6). Several *in vitro* studies have shown that mononuclear phagocytes such as peripheral blood monocytes can form multinucleated giant cells, but these multinucleated cells differ ultrastructurally from osteoclasts (7, 8).

Although some investigators have described cells with os-

teoclast-like features in cultures isolated from rodent calvaria (9, 10), these cells do not resemble osteoclasts morphologically. Technical difficulties have hampered attempts to obtain a homogeneous, readily identifiable osteoclast population from adult mammals and have been a major limitation in understanding the pathophysiology of age-related bone loss and other metabolic bone diseases.

Recently, Testa et al. (11) described a technique in which they established feline bone marrow cells in long-term culture. These investigators observed multinucleated cells with some

of the morphological characteristics of osteoclasts in their cultures. These cells were multinucleated, survived for several months *in vitro*, ultrastructurally had a clear zone with adjacent ruffled borders, and showed increased numbers of nuclei per cell with continued culture. However, the evidence that these cells were osteoclasts was incomplete, since these cells were not tested for their capacity to resorb bone or respond to osteotropic hormones. In the current investigation, similar feline marrow-derived multinucleated cells have been shown to have other characteristics of osteoclasts in addition to ultrastructural appearance. These include the capacity to resorb bone and the presence of tartrate-resistant acid phosphatase which was increased by parathyroid hormone (PTH)<sup>1</sup> and inhibited by calcitonin. The multinucleated cells were phagocytic and contained nonspecific esterase, consistent with their being derived from immature monocytes. Their mononuclear precursors were nonadherent to plastic, appeared like immature monocytes morphologically, and fused to form multinucleated cells in response to PTH, prostaglandins E<sub>2</sub>, and 1,25(OH)<sub>2</sub> vitamin D.

## MATERIALS AND METHODS

### Cell Culture

Cat marrow cells were cultured as described by Testa et al. (11). Briefly, 12-16-wk-old female cats were killed by intracardiac injection of sodium methohexital. The femurs were disarticulated, the ends removed, and the bone marrow cells were flushed out with a syringe fitted with an 18 gauge needle containing alpha-minimal essential medium (alpha-MEM) (Gibco Laboratories, Grand Island, NY). A single cell suspension was prepared by repeated pipetting with a 10-ml pipette. In selected experiments, unfractionated bone marrow cells were incubated for 2 h in alpha-MEM plus 30% horse serum at 37°C in plastic tissue culture flasks, and the nonadherent cells collected. The nonadherent and adherent cells were then cultured separately in alpha-MEM with 30% horse serum. All cultures were observed daily and the number of multinucleated cells in 20 microscopic fields at a final magnification of 250 was counted using an inverted phase-contrast microscope. The cells were cultured at a final density of 10<sup>6</sup> cells/ml in alpha-MEM containing 30% (vol/vol) horse serum (Flow Laboratories, McLean, VA) and 500 U/ml penicillin and 50 µg/ml streptomycin (Gibco Laboratories) in either 25-cm<sup>2</sup> flasks (Falcon Labware, Oxnard, CA), 6-well (35-mm diameter) or a 24-well (16-mm diameter) plates (Linbro, Flow Laboratories, McLean, VA), and incubated at 37°C in a humid 5% CO<sub>2</sub>-air atmosphere. The cultures were fed weekly by removing half of the medium and replacing it with fresh medium. Cells removed with the spent medium were not replaced.

### Histology and Ultrastructural Studies

Cultures were examined by phase-contrast microscopy at × 40 and × 200. For histologic studies on bone marrow cultures, the culture medium was removed and the adherent cells fixed *in situ* with 5% glutaraldehyde for 5 min. The cells were washed with water, allowed to air dry, and then stained with Wright's Giemsa stain or hematoxylin. In selected experiments, marrow cultures were allowed to air dry before fixation and then stained with either periodic-acid Schiff stain, alpha-naphthyl-ASD-butyrates (nonspecific esterase) or naphthol ASD chloroacetate (granulocytic esterase) by standard techniques (12). Marrow cultures were then examined by light microscopy. Other cultures were stained for acid phosphatase activity as described below. Cytospin slides were prepared by placing 0.1 ml of unfractionated bone marrow cells or cells from each layer of the gradient (2 × 10<sup>5</sup> cells/ml) in a cytocentrifuge (Shandon Southern Instruments, St. Sewickley, PA) followed by centrifugation at 1,000 rpm for 10 min to transfer the cells to glass slides. Slides were air dried, fixed with methanol, and stained with Wright's Giemsa stain, and a differential cell count done. 200 cells were counted in each specimen.

For ultrastructural studies, cells grown on plastic plates were fixed in modified Karnovsky's solution (13) containing 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, 320 mosmol for 90 min at 4°C. After washing and additional fixation in 1% osmium tetroxide (60

min at 4°C), the cells were washed again, then stained en bloc with aqueous uranyl acetate prior to embedding in Embed 812 (Polysciences, Inc., Niles, IL) araldite. Ultrathin sections were counterstained in lead acetate and uranyl acetate before viewing in a Philips 301 electron microscope at an operating voltage of 60 kV. For studies with bone particles present in the cultures (see below), cultures were decalcified with EDTA (250 mM, pH 6.8) for 1 h after fixation and then processed as described above.

**EFFECTS OF OSTEOTROPIC HORMONES ON MULTINUCLEATED CELL FORMATION:** PTH (200 ng/ml), calcitonin (100 ng/ml), prostaglandin E<sub>2</sub> (10<sup>-5</sup> to 10<sup>-7</sup> M) (Sigma Chemical Co., St. Louis, MO) or 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub> (10<sup>-6</sup> to 10<sup>-8</sup> M) (Roche Laboratories, Nutley, NJ) were added to bone marrow cultures 2 wk after the cultures were started. The number of multinucleated cells formed was then scored 24 h later.

### Acid Phosphatase Determinations

The effect of osteotropic hormones on acid phosphatase activity was determined after cells had been grown for 2 wk in six-well plates (Linbro). Bovine PTH (200 ng/ml), calcitonin (100 ng/ml), or PTH and calcitonin were added to the cells and the cultures continued an additional 24 h. PTH (1-84) was obtained from the National Pituitary Agency (sp. act. 1,300 IU/mg protein). Salmon calcitonin (Calcimar T.M.) was obtained from the Armour Pharmaceutical Co. (Phoenix, AZ). The medium was aspirated and the cells washed twice with phosphate-buffered saline. The cells in each well were then fixed for 30 seconds in 1 ml citrate-buffered acetone (0.15 M sodium citrate, pH 5.4, in 60% (vol/vol) acetone). The cells were then rinsed in deionized water and stained (1 ml per well) for acid phosphatase activity for 1 h at 37°C in the dark using a histochemical kit (Sigma Chemical Co.). The acid phosphatase staining reaction involves the enzymatic hydrolysis at acid pH of a naphthol AS-BI phosphoric acid substrate to yield a naphthol AS-BI which instantly couples with fast garnet GBC in the staining mixture to form an insoluble maroon dye at the sites of enzyme activity. Because the acid phosphatase activity of the osteoclast is insensitive to inhibition by tartrate (14), we added sodium tartrate (27 mM) to some of the culture wells simultaneously with the acid phosphatase staining mixture. The cells were then rinsed in deionized water, and counter stained with acid hematoxylin (pH 3.3) for 5 min, rinsed with deionized water for 3 min and allowed to air dry. All concentrations of the hormone were tested in triplicate. Cells were scored for acid phosphatase activity (staining density) by counting 20 microscopic fields at × 1,000 in each culture. Scoring was done without knowledge of treatment group. At least 20 multinucleated cells were counted at random within each well.

**TIME-LAPSE CINEMICROGRAPHY:** After 1 wk of culture, 200 ng/ml PTH was added to the marrow cultures and the cultures placed at 37°C in an incubation chamber fitted to an inverted microscope (Nikon Instruments, Garden City, NY) equipped with a Bolex H-16 cine camera. Exposures were taken every 30 s for 18 h. Time-lapse studies were done on four separate experiments.

**AUTORADIOGRAPHY:** Cells were cultured for 1 wk as described above. 5 µCi/ml of [<sup>3</sup>H]thymidine (30 Ci/mmol [ICH, Irvine, CA]) was added to each of the cultures, and the cultures incubated for 24 h. The cultures were then washed extensively with alpha-MEM 30% horse serum. 200 ng/ml PTH was then added to the cultures and the cultures were then incubated for 18 h at 37°C. The cultures were then processed for autoradiography as described by Roodman et al. (15).

**ENRICHMENT OF OSTEOCLAST PROGENITORS:** Percoll density gradients were prepared by diluting Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) with 1.5 M sodium chloride (9:1). This stock Percoll was further diluted with 0.15 M sodium chloride to give a density range of 1.05-1.10 g/ml. The density of each layer was calculated from the refractive index. Ten 1-ml fractions were layered in conical 15-ml tubes, and 3 × 10<sup>8</sup> feline marrow cells suspended in 3 ml alpha-MEM were layered over the top of the gradient. The discontinuous gradients were centrifuged at 400 g for 30 min at 12°C and the cells at the interfaces collected. The cells were washed twice with alpha-MEM and a nucleated cell count done. Cytospin slides for histology were prepared from each fraction as described above. The cells in each fraction were then cultured separately. Unfractionated bone marrow cells (controls) or cells from each Percoll density layer were cultured in alpha-MEM 30% (vol/vol) horse serum at a density of 10<sup>6</sup> cells/ml in 25-cm<sup>2</sup> flasks. Cultures were examined for periodic-acid Schiff staining, nonspecific esterase or granulocytic esterase staining as described above.

### Bone Resorption

Fractionated and nonfractionated bone marrow cells were cultured in 24 well plates (Linbro) at 10<sup>6</sup> cells/ml per well or were fractionated on Percoll density gradients as described above. After 2 wk in culture, supernatant medium was removed and diluted by 50% with fresh medium. PTH (200 ng/ml),

<sup>1</sup> *Abbreviations used in this paper:* PTH, parathyroid hormone; alpha-MEM, alpha-minimum essential medium.

calcitonin (100 ng/ml), or PTH and calcitonin were added, and the medium added back to the cells. Devitalized bone particles ( $\leq 25 \mu\text{M}$  diameter) prelabeled with  $^{45}\text{Ca}$  (16) (kindly provided by Drs. S. L. Teitelbaum and Z. Bar-Shavit) and sterilized by ultraviolet irradiation overnight were added to each culture well, (0.11 mg/well). The cells were incubated for 48 h at  $37^\circ\text{C}$  in a humid atmosphere of 5%  $\text{CO}_2$ -air. To determine the amount of  $^{45}\text{Ca}$  released from the bone particles into the medium, 0.2-ml aliquots of medium were removed and counted in a liquid scintillation counter. Trichloroacetic acid (100% wt/vol) was then added to the cultures (0.1 ml/well) and the cultures incubated for 24 h to solubilize the remaining bone particles. The medium was then aspirated from the wells and counted in a scintillation counter. Results are expressed as percent release of total  $^{45}\text{Ca}$  into the culture medium during the culture period.

## RESULTS

Multinucleated cells (Fig. 1) began to appear in significant numbers in unfractionated feline marrow cultures after 2 d (Table I). Maximum numbers of multinucleated cells were found after 16 d of culture and remained stable until day 24 of culture. Such cultures have been maintained for more than 6 wk. Ultrastructurally, the nuclei of these cells were uniform with prominent nucleoli and a minimum of heterochromatin which was displayed as a thin band at the nuclear periphery. Abundant cytoplasmic organelles were present and included lipid droplets, free ribosomes (both singly and as polyribosomes), rough endoplasmic reticulum, and branched pleomorphic mitochondria (Fig. 2). In most cells, areas of peripheral cytoplasm (clear zones) that lacked organelles, other than free ribosomes were identified and circumferential bundles of microfilaments were present at the cell surface in these areas (Fig. 2). Contractile apparatuses, both microtubules and microfilaments, also were distributed throughout the cytoplasm of these cells. Other cell surface specializations included extensive cytoplasmic membrane foldings (Fig. 3B). Associated with these folded cell edges were focal areas of thickened cell membranes which resembled coated pits and which occasionally appeared as internalized or intracellular structures (Fig. 3C). Also associated with these areas of the cells were numerous vacuoles, some of which contained amorphous granular material (Fig. 3A). At least two distinct types of monocytoïd cells were regularly observed in the 14-d-old feline marrow cultures. One type (Fig. 4) contained lipid droplets, a subplasmalemmal band of microfilaments and a single nonlobulated nucleus with a prominent nucleolus and a peripheral rim of condensed chromatin; these features are consistent with the ultrastructural characteristics of the multinucleated cells. The

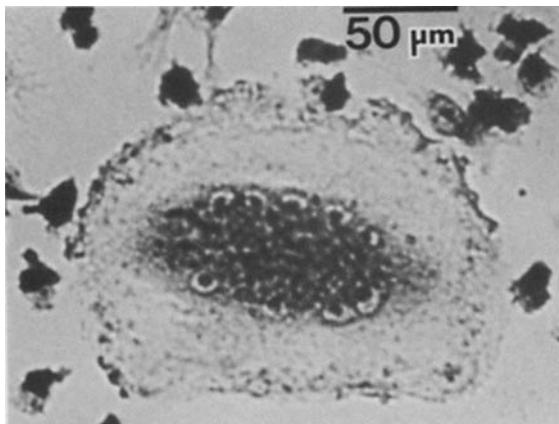


FIGURE 1 Light microscopic appearance of multinucleated cells. Cells were processed and stained as described in Materials and Methods. The cells have a granular cytoplasm, with a clear peripheral cytoplasm and prominent nucleoli. Bar,  $50 \mu\text{m}$ .  $\times 300$ .

TABLE I  
Time Course of Multinucleated Cell Formation from Unfractionated Feline Bone Marrow Cultures

Days in culture	Multinucleated cells in 20 fields	
	Expt. 1	Expt. 2
0	0	0
1	0	0
2	2	2
3	16	5
4	19	15
5	24	18
6	14	17
7	16	22
9	16	11
16	38	38
24	36	—

Unfractionated cat bone marrow was cultured in alpha-MEM with 30% horse serum as described in Materials and Methods. Results represent the mean of duplicate determinations in two independent determinations. Cells were counted at  $\times 250$  (20 random fields), and the number of cells containing three or more nuclei was scored.

other monocytoïd cells were typical monocytes with amorphous granules, small but uniform mitochondria, and a bilobed nucleus (Fig. 5).

The effects of osteotropic hormones on multinucleated cell formation was then examined. Addition of PTH (200 ng/ml) (Table II) increased the number of multinucleated cells twofold as compared to untreated control cultures, while calcitonin (100 ng/ml) alone had no significant effect on multinucleated cell formation. However, addition of calcitonin blocked the stimulatory effects of PTH on multinucleated cell formation in these cultures. Addition of prostaglandin  $\text{E}_2$  and  $1,25\text{-}(\text{OH})_2$  vitamin  $\text{D}_3$ , substances which stimulate osteoclast formation *in vivo*, also significantly increased the number of multinucleated cells (Table III).

PTH and calcitonin also affected acid phosphatase activity of the multinucleated cells (Table IV). PTH stimulated acid phosphatase activity approximately twofold, and this increased enzyme activity was not inhibited in the presence of 27 mM sodium tartrate. Tartrate appeared to cause a slight but significant increase in PTH stimulated acid phosphatase activity. The reason for this is unclear. Calcitonin alone had no effect on acid phosphatase activity, but when added together with PTH, inhibited the stimulatory effect of PTH.

The multinucleated cells appear to arise by fusion of mononuclear precursors rather than endomitosis. Time-lapse cinemicrographic studies showed mononuclear cells fusing with multinucleated cells. The fusion process occurred within 12 to 16 h. Further support that the multinucleated cells arise by fusion was seen in the autoradiographic studies. The nuclei in the majority of multinucleated cells were not labeled when the cultures were incubated with  $^3\text{H}$ thymidine for 24 h, washed, and then cultured with PTH for an additional 18 h (Fig. 6). We found that the multinucleated cells that contained labeled nuclei also contained predominantly one labeled nucleus, with the other nuclei in the cell being unlabeled.

Fractionation of feline marrow cells on discontinuous Percoll density gradients enriched multinucleated cell progenitors (Table V). The progenitor cells were found predominantly in layers of density 1.07–1.08 gm/ml and with Wright's staining were immature monocyte-macrophages histologically. The

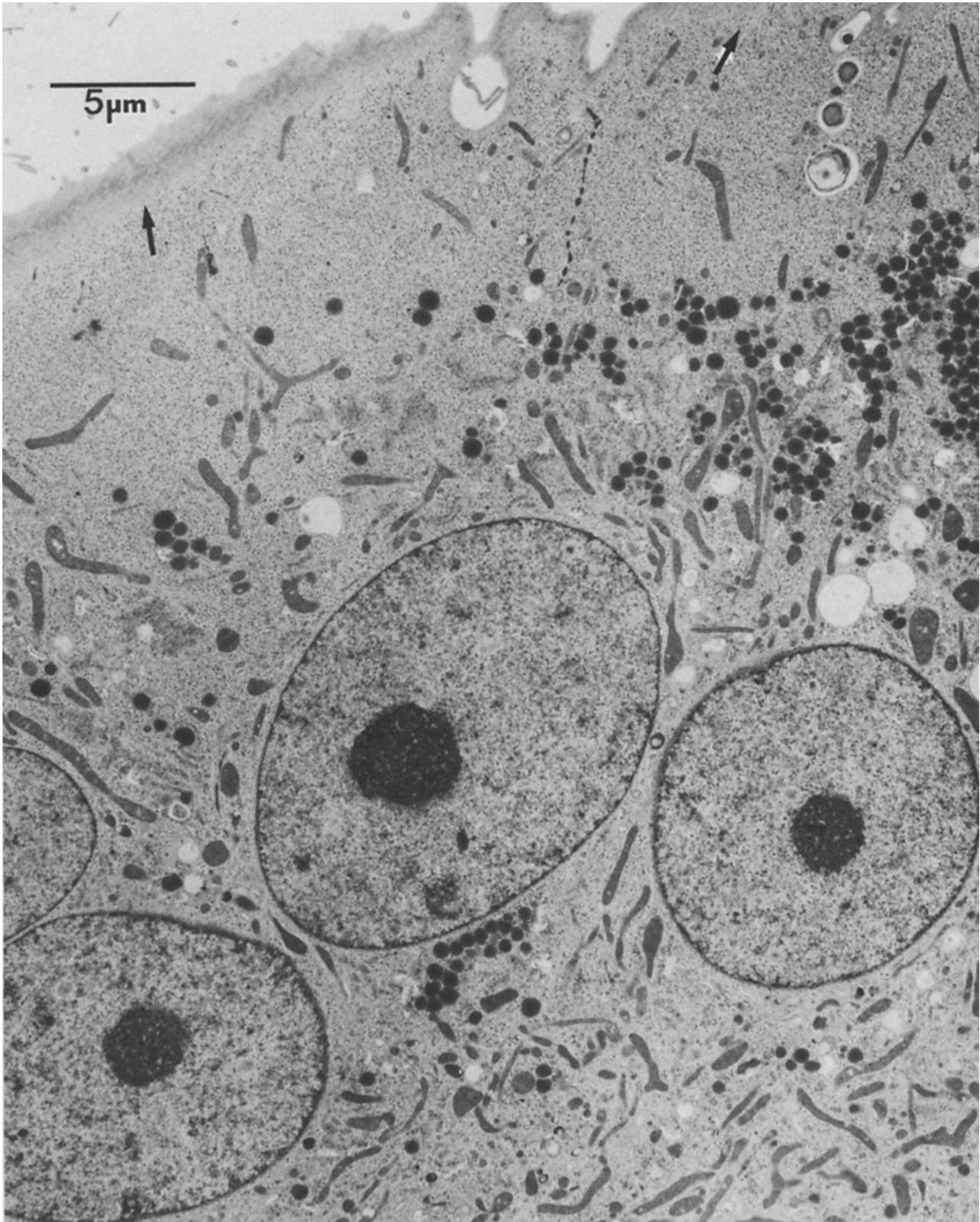


FIGURE 2 Typical ultrastructural morphology of a cultured multinucleated cell derived from cat bone marrow mononucleated cells. Cat marrow cells were cultured for 14 d and then processed for routine electron microscopy (see Materials and Methods). This multinucleated cell contains abundant cytoplasmic organelles including lipid droplets, rough endoplasmic reticulum and branched mitochondria. The peripheral cytoplasm (arrow) lacks organelles other than free ribosomes. Bar, 5  $\mu\text{m}$ .  $\times 4,900$ .

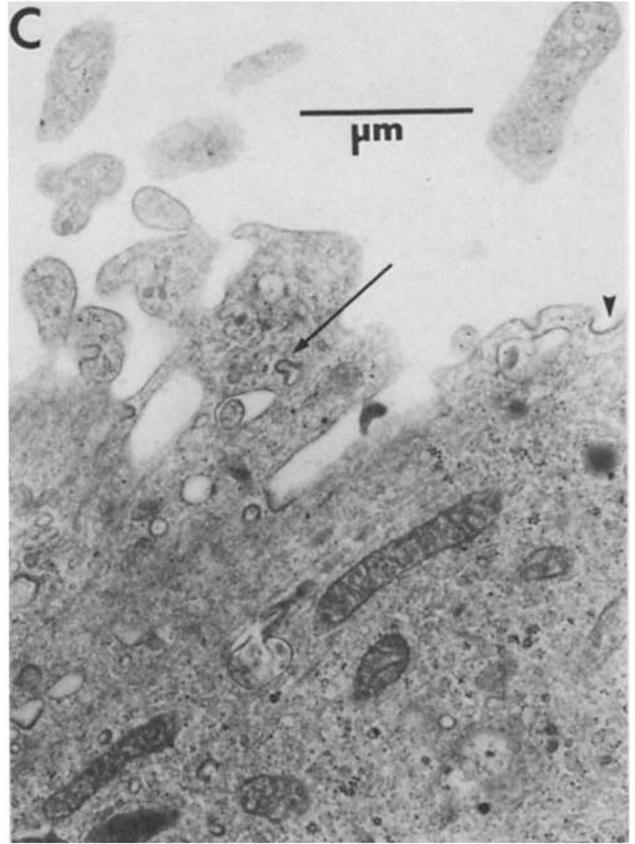
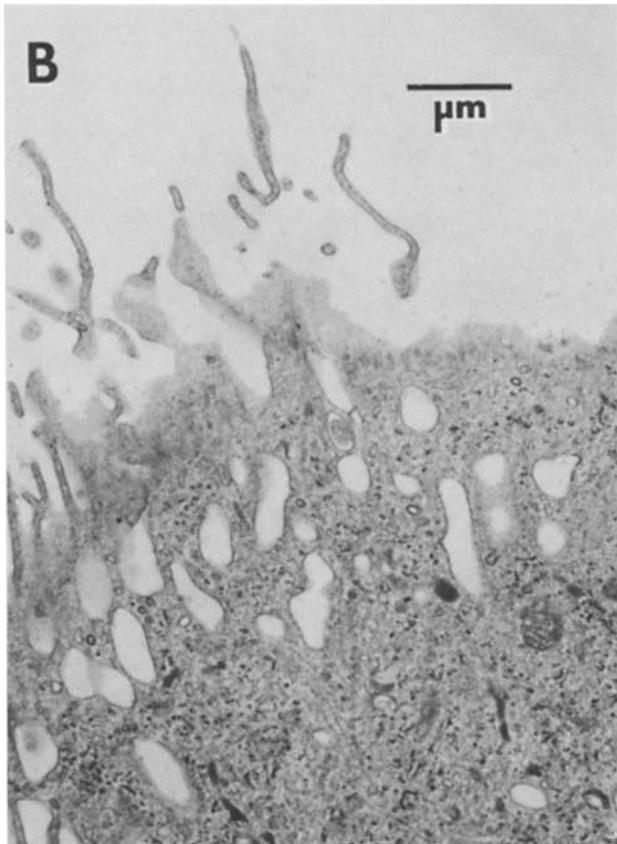
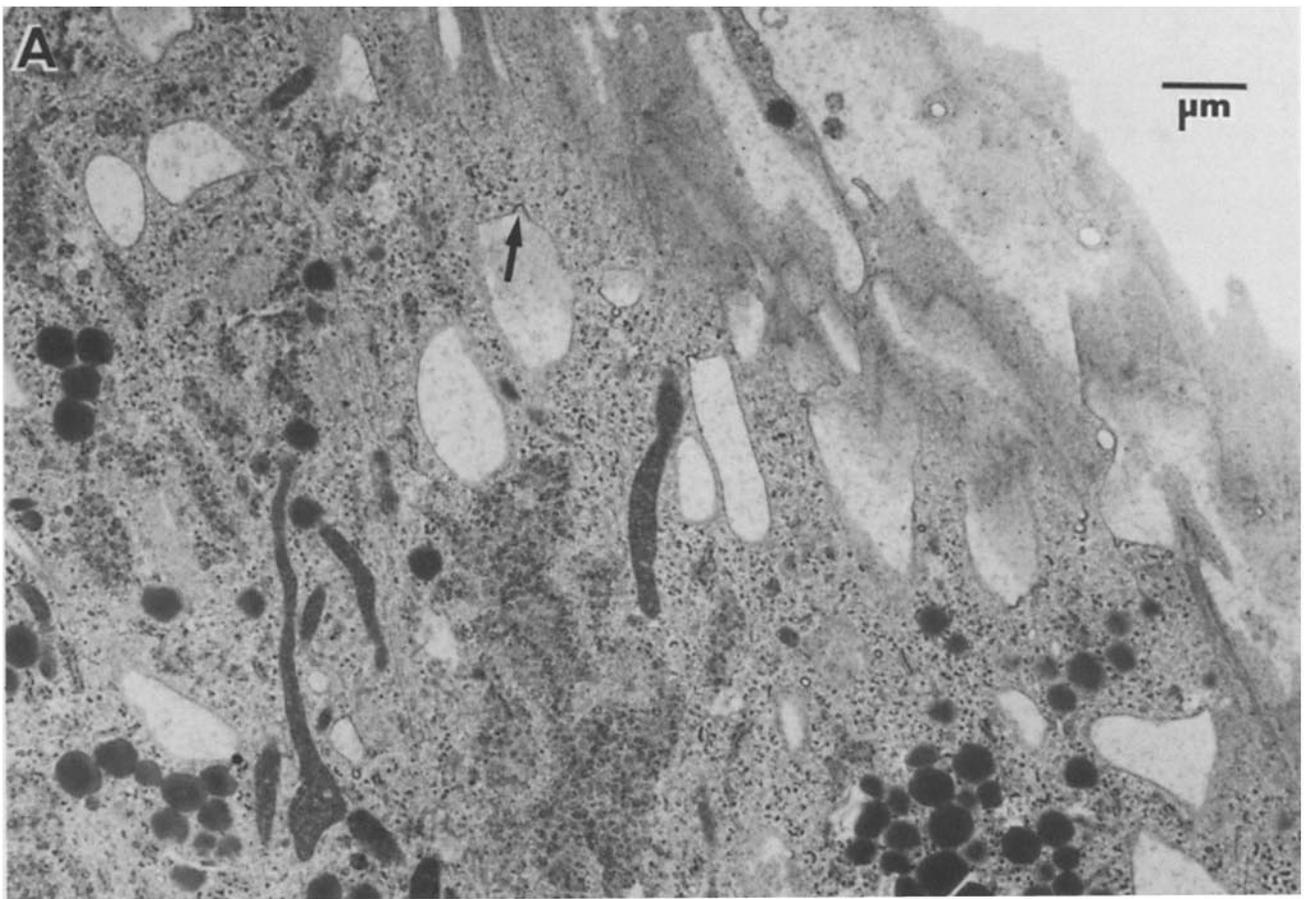


FIGURE 3 Cell surface specialization in cultured feline osteoclasts. (A) Extensive cytoplasmic involutions are present at the periphery of this multinucleated cell. Vacuoles present in this area contain granular material and have focally thickened areas of membrane that resembles coated pits (arrow). Bar, 1  $\mu\text{m}$ .  $\times 11,700$ . (B) Thin and delicate cytoplasmic extensions are present at the surface of this cell, which also contains numerous vacuoles. Bar, 1  $\mu\text{m}$ .  $\times 12,800$ . (C) In addition to surface folding, the plasmalemmal membrane of this multinucleated cell has focal, thickened areas resembling coated pits (arrowhead), several of which seem to be internalized (arrow). Bar, 1  $\mu\text{m}$ .  $\times 27,000$

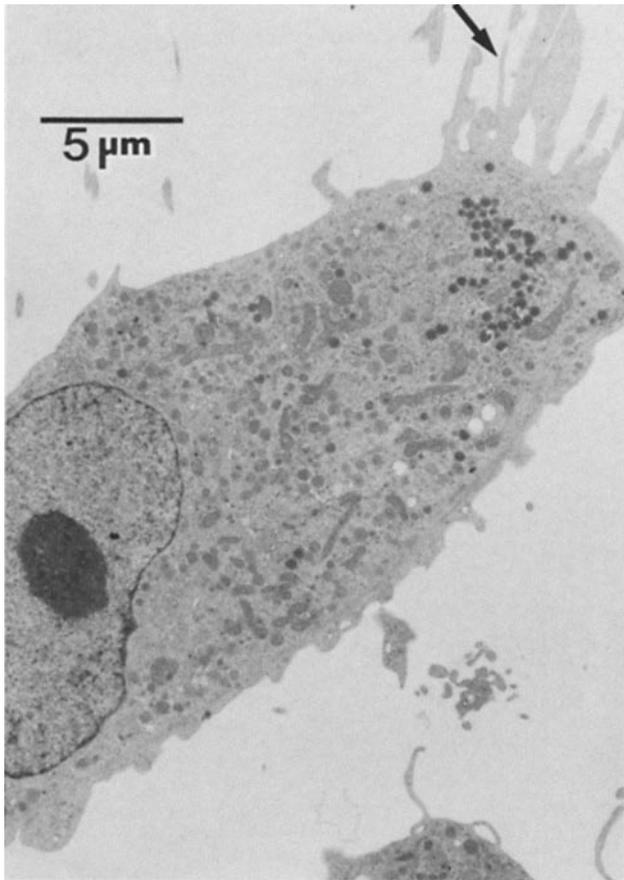


FIGURE 4 Ultrastructural features of one type of mononucleated cell present in unfractionated feline bone marrow-derived cultures. This population of cells is characterized by the presence of a single nucleus with peripherally displaced chromatin and a prominent nucleolus, pleomorphic and branched mitochondria, and a dense band of microfilaments adjacent to the cell surface. Extended cell processes (arrow) are relatively devoid of cytoplasmic organelles.  $\times 4,800$ .

mononuclear cells stained heavily with nonspecific esterase, a monocyte stain, but did not stain with granulocytic esterase, or periodic-acid Schiff stain, a lymphocyte stain. The progenitor cells were predominantly nonadherent to plastic. Cells initially adherent to plastic formed 26 multinucleated cells per 20 microscopic fields while nonadherent marrow cells formed 149 multinucleated cells per 20 microscopic fields. Removal of cells adherent to plastic from unfractionated marrow cells enriched progenitors threefold as compared to unfractionated marrow. The multinucleated cells that formed also were stained with nonspecific esterase but did not stain with periodic-acid Schiff stain or granulocytic esterase, a staining pattern seen in monocyte-macrophages. The nonspecific esterase staining was most pronounced in the smaller multinucleated cells containing three to six nuclei, and was decreased or absent in the larger more multinucleated cells. These multinucleated cells were phagocytic (Fig. 7, arrow), a characteristic of monocyte-macrophages.

The multinucleated cells resorbed bone *in vitro* as shown by the release of  $^{45}\text{Ca}$  from devitalized bone particles. Cultures enriched for multinucleated cells showed significant bone resorption.  $42 \pm 3\%$   $^{45}\text{Ca}$  was released by these cultures (total counts in bone particles added =  $1,290 \pm 77$  cpm) compared



FIGURE 5 Ultrastructural characteristics of monocyte-like cells present in unfractionated feline bone marrow derived osteoclast cultures. This type of cell contains a single lobulated nucleus and a variety of cytoplasmic granules. Bar,  $1 \mu\text{m}$ .  $\times 7,500$ .

TABLE II  
Effect of PTH and Calcitonin on the Formation of Multinucleated Cells in Unfractionated Feline Marrow Cultures

	Total cells scored	Multinucleated cells	Per-cent of cells scored	No. of nuclei per cell
Control	$1,541 \pm 181$	$9 \pm 1$	0.60	$4.43 \pm 0.35$
PTH	$1,509 \pm 181$	$19 \pm 3^*$	1.3	$4.55 \pm 0.56$
Calcitonin	$1,528 \pm 123$	$10 \pm 3$	0.65	$4.24 \pm 0.35$
Calcitonin and PTH	$1,596 \pm 183$	$7 \pm 2$	0.43	$4.67 \pm 0.49$

Unfractionated feline bone marrow cells were cultured in MEM containing 30% horse serum as described in Materials and Methods. After 2 wk of culture PTH (200 ng/ml), calcitonin (100 ng/ml), or PTH (200 ng/ml) and calcitonin (100 ng/ml) were added. The cultures were incubated an additional 24 h, and then fixed with glutaraldehyde and stained with Wright's stain. The number of cells containing 3 or more nuclei were scored in 20 microscopic fields ( $\times 400$ ). Results represent the mean  $\pm 1$  SEM of triplicate determinations from three separate experiments. Differences were determined by one way analyses of variance for repeated measures and a Newman-Keuls multiple comparison procedure.

\* Significantly different from control group at  $P < 0.01$ .

to  $20 \pm 3\%$  (total counts added =  $1,725 \pm 170$  cpm) for cultures not containing cells. Addition of PTH did not affect bone resorption and calcitonin inhibited resorption in two

TABLE III

Effect of Prostaglandin E<sub>2</sub> and 1,25(OH)<sub>2</sub> Vitamin D<sub>3</sub> on Formation of Multinucleated Cells from Unfractionated Feline Marrow Cultures

	Total no. of cells	Multinucleated cells	Percent of cells scored	No. of nuclei cell
Control	1,769 ± 168	13 ± 6	0.7	5.11 ± 0.57
Prostaglandin E <sub>2</sub>				
10 <sup>-5</sup> M	2,508 ± 633	160 ± 6*	6.4	4.1 ± 0.69
10 <sup>-6</sup> M	3,072 ± 249	120 ± 6*	3.9	5.04 ± 0.49
10 <sup>-7</sup> M	2,126 ± 204	100 ± 12*	4.9	5.37 ± 0.52
1,25(OH) <sub>2</sub> vitamin D <sub>3</sub>				
10 <sup>-6</sup> M	2,225 ± 245	280 ± 80*	12.6	5.09 ± 0.55
10 <sup>-7</sup> M	2,182 ± 356	240 ± 87*	11.0	4.26 ± 0.14
10 <sup>-8</sup> M	1,952 ± 149	100 ± 53*	5.1	5.36 ± 0.58

Unfractionated feline marrow cells were cultured for 2 wk and then prostaglandin E<sub>2</sub> or 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub> were added in various concentrations. The cells were incubated an additional 24 h and then were fixed and stained. The number of cells containing 3 or more nuclei were scored in 20 microscopic fields (× 250). Results are given as mean ± 1 SEM for triplicate determinations from three separate experiments.

\* Significantly different from control ( $P < 0.01$ ).

TABLE IV

Effect of PTH (200 ng/ml), Calcitonin (100 ng/ml), and Tartrate (27 mM) on Acid Phosphatase Activity of Multinucleated Cells from Feline Marrow Cultures

	Total acid phosphatase	Percent increase
Control	1.17 ± 0.04	—
PTH	2.13 ± 0.11	+82 ± 7*
Calcitonin	0.84 ± 0.23	-28 ± 16 <sup>†</sup>
Calcitonin and PTH	0.71 ± 0.06	-40 ± 5*
PTH + tartrate	2.41 ± 0.05	+105 ± 5

Acid phosphatase activity was detected histochemically and cells were scored by the intensity of granular staining. Cells were graded from 3 (intense granulation) to 0 (no discernible activity). All cells were scored at random without knowledge of the treatment group. Scores were totaled and divided by total number of multinucleated cells scored to give mean score per cell. Cells were counted in 20 random fields in each well (at least 60 cells per group) at × 1,000. Results are mean and ± 1 SEM of triplicate determination from three separate experiments. Differences were assessed by one way analysis of variance for repeated measurements and a Newman-Keuls multiple comparison procedure.

\*  $P < 0.01$ , compared to control group.

<sup>†</sup> Significantly different,  $P < 0.05$ .

‡  $P < 0.01$ , compared to PTH-treated group.

§ Significantly different,  $P < 0.05$ .

out of five experiments.

When examined by light microscopy, multinucleated cells appeared in close apposition to bone particles within 24 h after the addition of these particles to the cultures (Fig. 8). Ultrastructurally, multinucleated cells cultured together with <sup>45</sup>Ca-bone particles exhibited all of the characteristics of cells cultured in the absence of bone. However, of particular interest, the cells developed enhanced and much more extensive and elaborate cytoplasmic membrane folds or ruffled edges when bone was included in the culture media (Fig. 9).

## DISCUSSION

We have demonstrated that the multinucleated cells in long-term feline marrow cultures have many of the characteristics of osteoclasts. The cells in our cultures were large and multinucleated with ultrastructural features of osteoclasts, including a peripheral cytoplasmic clear zone devoid of subcellular organelles, and extensive cell surface folds and branched pleomorphic mitochondria. These particular ultrastructural features are found in freshly isolated osteoclasts and in osteoclasts not lying adjacent to an endosteal bone surface (7). Although numerous plasmalemmal folds and indentations

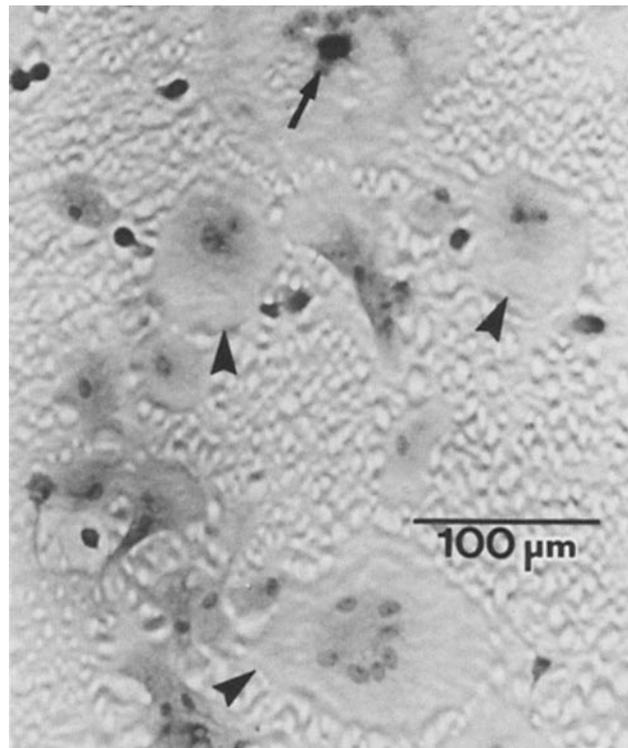


FIGURE 6 Representative pattern of [<sup>3</sup>H]thymidine nuclear labeling in cultured feline bone marrow-derived multinucleated cells. Bone marrow derived mononuclear cells enriched by Percoll density centrifugation and obtained from a density layer of 1.07 g/ml were labeled with [<sup>3</sup>H]thymidine for 24 h and cultured for an additional 18 h before processing for autoradiography and staining with hematoxylin. Although one multinucleated cell contains a labeled nucleus (arrow), most do not (arrowheads). The remaining nuclei of small cells are not labeled. They are intensely stained with the hematoxylin that was used to visualize the cells. × 250.

were also regularly observed in our cultured cells, we did not identify the classical ruffled borders as reported for osteoclasts found adjacent to bone in Howship's lacunae in vivo (17). The absence of a classical ruffled border in our cultured cells may be due to our inability to mimic the true endosteal bone surface in vitro. Nonetheless, the multinucleated cells did demonstrate a marked increase in cytoplasmic membrane folds in the presence of bone (Fig. 9).

The multinucleated cells in our cultures had other charac-

TABLE V  
Enrichment of Feline Marrow Multinucleated Cell Progenitors, by Percoll Density Gradient Centrifugation

Density (g/ml)	Cell per layer ( $\times 10^{-6}$ )	% Total	Initial predominant morphology	Multinucleated cells	Fractionated/ unfractionated
1.050	11.1	1.4	Myeloblasts	0	0
1.055	10.5	1.3	Myelocytes	0	0
1.060	15.4	2.1	Myelocytes	6	1
1.065	632.0	86.7	Metamyelocytes	86	2
1.070	36.4		Myeloid bands, monocytes	1,000	23
1.075	12.7	1.7	Myeloid bands, monocytes	1,400	32
1.080	8.0	1.0	Normoblasts	160	4
1.085	3.1	0.4	Normoblasts	NC	—
1.090	—	—	Erythrocytes	NC	—
Unfractionated	—	—	—	44	1

Feline marrow cells were layered over Percoll density gradients and the cells from each fraction cultured for 14 d as described in Materials and Methods. The marrow cultures from each layer were fixed, stained, and the number of cells containing three or more nuclei in 20 microscopic fields ( $\times 250$ ) were scored. Cytospin cytology was done on each layer prior to culture. The results are from one experiment. In four separate studies multinucleated cell progenitors were enriched 4- to 32-fold. Lymphocytes were seen in all layers of the gradient. NC, not cultured because of insufficient cell numbers.

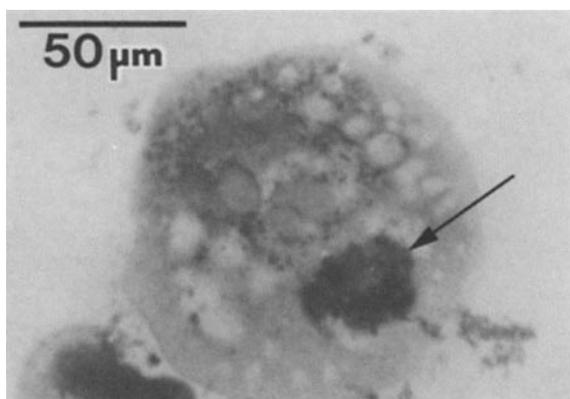


FIGURE 7 A multinucleated cell in feline marrow cultures stained for nonspecific esterase. Bone marrow cells were cultured for 14 d before processing for nonspecific esterase staining as described in Methods. Arrow denotes a phagocytosed cell. Bar, 50  $\mu$ m.  $\times 300$ .

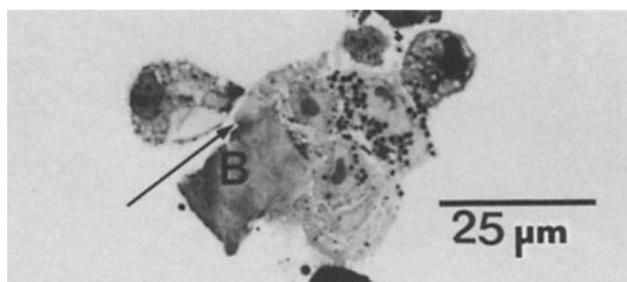


FIGURE 8 Light microscopic appearance of cat osteoclasts cocultured with divalitized bone particles. Bone marrow cells were cultured for 14 d; bone particles were then added and the cultures incubated for 48 h prior to fixation decalcification and processing for electron microscopy. Thick sections (1  $\mu$ m) were cut and stained with toluidine blue. A multinucleated cell is shown attached to a bone particle (B). Note the numerous lipid droplets (small, dark cytoplasmic granules) in these cells and their relative paucity in areas adjacent to the bone particle (arrow). Bar, 25  $\mu$ m.  $\times 800$ .

teristics of osteoclasts. They were responsive to treatment with hormones that influence osteoclastic activity. PTH increased the numbers of multinucleated cells in the cultures. This effect

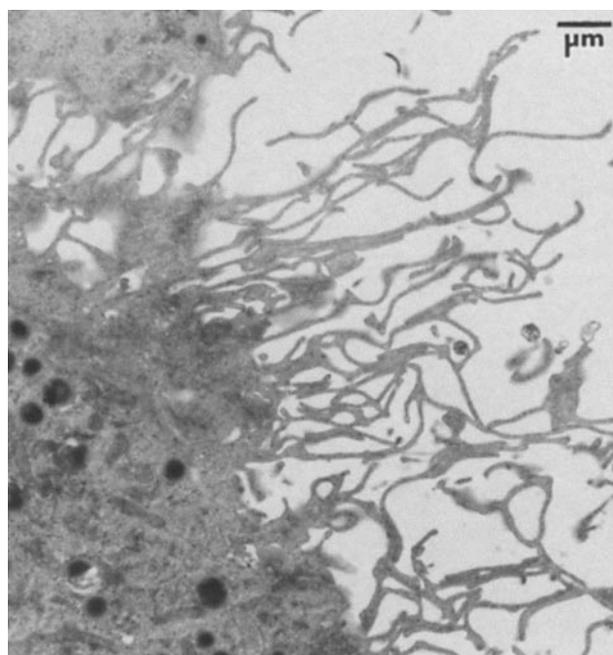


FIGURE 9 Extensive membrane ruffling of a multinucleated cell grown in the presence of bone particles for 72 h. Bar, 1  $\mu$ m.  $\times 6,800$ .

was inhibited by calcitonin. Osteoclasts have been shown before to form by fusion (18), and the results of the current investigation suggest that this process is probably stimulated by PTH and inhibited by calcitonin (Table II). The multinucleated cells in our cultures also contained an acid phosphatase which was tartrate-resistant and which was increased in the presence of PTH and was inhibited by calcitonin (Table IV). This property is also characteristic of the osteoclast (14, 19, 20).

Our data suggests that the mononuclear precursor cell of these multinucleated cells is immature marrow monocyte-macrophage. The progenitor cells stained heavily with nonspecific esterase, were phagocytic and appear similar to other monocytes-macrophages with Wright's-Giemsa staining. These data agree with studies of Burger et al. (21), who also

found that the precursor to their multinucleated cells were marrow monocytes that initially were not adherent to plastic. Similarly, *in vivo* studies of Gothlin and Ericsson (5) also suggested that marrow monocytes give rise to osteoclasts.

The progenitor of the osteoclast-like cell in our cultures appears responsive to osteotropic hormones since PTH, vitamin D, and prostaglandin E<sub>2</sub> increased multinucleated numbers while calcitonin blocked the effects of PTH (Tables II and III). Bingham et al. (22) and Tatevossian (23) have shown that administration of parathyroid extracts to rabbits or mice increases the number of osteoclasts *in vivo*. Similarly administration of vitamin D and prostaglandins increase osteoclast numbers *in vivo* (24). These data suggest that osteoclast progenitors are responsive to osteotropic hormones, and are consistent with our *in vitro* observations.

The multinucleated cells appear to form by fusion of mononuclear precursors based on time-lapse cinemicrography and autoradiographic studies. However, such studies cannot differentiate between phagocytosis of labeled mononuclear cells and fusion of mononuclear cells to form multinucleated cells. Fusion is the more likely mechanism for osteoclast formation, since the nuclei of the multinucleated cells are not pyknotic and appear normal ultrastructurally, whereas a phagocytosed cell should be degraded. Multinucleated cells form within 18 h after addition of PTH to the cultures, suggesting that it is unlikely that endomitosis is the mechanism responsible for the formation of the osteoclasts. If endomitosis is the mechanism of multinucleated cell formation, the cell cycle time of the progenitors must be extremely short. Further supporting that endomitosis is not responsible is the observation that the nuclei in the majority of the multinucleated cells were not labeled by [<sup>3</sup>H]thymidine, and therefore are not actively synthesizing DNA. The multinucleated cells which did contain labeled nuclei predominantly (>90%) had only one labeled nucleus with the other nuclei unlabeled. Previous *in vivo* as well as *in vitro* studies have suggested that osteoclasts arise from fusion of mononuclear precursors (1, 2, 18).

It appears that the progenitors of the multinucleated cells in our cultures behave differently from mature monocyte-macrophages. The progenitor cell is initially nonadherent to plastic and responds to osteotropic hormones. Mundy et al. (25) demonstrated that peripheral blood monocytes could resorb bone but were unresponsive to osteotropic hormones. Osdoby et al. (7) cultured chick peripheral blood monocytes and demonstrated that they gave rise to multinucleated cells that differed ultrastructurally from osteoclasts. Taken together, these data suggest that the osteoclast progenitor is not identical to monocytes found in peripheral blood, but may be a less differentiated marrow monocyte precursor that can give rise to osteoclasts in the appropriate microenvironment. The nonspecific esterase staining of the osteoclast-like cells and their phagocytic ability further supports their monocytic origin. Ultrastructurally, these progenitor cells may be the monocytoïd cells with branched mitochondria present in our cultures. Further studies will be required to confirm whether these cells are the progenitors of these osteoclast-like cells.

Enriched populations of cells released <sup>45</sup>Ca from devitalized bone particles, and the membranes of cells grown in the presence of bone particles showed extensive folding and ruffling. However, we could not consistently demonstrate hormonal control of <sup>45</sup>Ca release. This lack of hormonal responsiveness may be due to the use of devitalized bone particles in our studies. Devitalized bone may not have been suitable

for demonstrating such effects because of the absence of other bone cell types or changes in matrix structure in devitalized bone. The mechanism by which PTH stimulates bone resorption by osteoclasts is unknown. Possibly PTH acts on other cells in bone such as osteoblasts that in turn affect osteoclast function (26). The variable effect of calcitonin in inhibiting resorption may be due to loss of calcitonin sensitivity, by the osteoclast-like bone cells in our cultures. Wener et al. (27) have shown that bone cultures treated with calcitonin lose sensitivity to the hormone and resorb in the continued presence of calcitonin.

Microenvironmental factors appear important in osteoclast formation. The culture system we employed results in formation of multinucleated giant cells responsive to osteotropic hormones capable of bone resorption and possessing many of the physical and ultrastructural characteristics of osteoclasts. This culture system is dependent on the formation of a marrow adherent cell layer which mimics the marrow microenvironment (28–30). However, using a similar technique Dexter and co-workers (28–30) did not report the formation of multinucleated cells in their bone marrow cultures. Their marrow cultures routinely contained hydrocortisone, while ours did not. When hydrocortisone was added initially to our cultures, no multinucleated cells formed (data not shown). Therefore, hydrocortisone may inhibit osteoclast formation *in vitro*, and may explain why these authors have not reported multinucleated cells *in vitro*. Suda et al. (31) have also observed that hydrocortisone inhibits feline osteoclast formation *in vitro*.

There are other approaches being developed for studying osteoclasts *in vitro*. Pre-existing osteoclasts stripped from the endosteal surfaces of growing bones have been used by Zallone et al. (32) and Osdoby et al. (7). However, these cells do not allow studies of the differentiation of the mature cell from its precursors, nor do they permit the identification of precursors. Furthermore, the viability of the cell preparations is often low. Cells have also been obtained from rodent calvaria by enzyme digestion (33) or prior culture with PTH (34). However, these techniques will not provide an approach for studying osteoclasts derived from normal humans or patients with diseases associated with bone resorption as the technique described in this report does.

The ability to grow osteoclasts *in vitro* and enrich their progenitors will permit basic studies on bone cell biology. Such culture systems may help in elucidating other factors that may affect osteoclast formation and activity in normal and pathologic states.

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