

# Osteoclast Formation from Mononuclear Phagocytes: Role of Bone-forming Cells

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**ABSTRACT** In a previous study, using co-cultures of embryonic bone rudiments stripped of periosteum, and mononuclear phagocytes of various sources, we found that multinucleated mineral-resorbing osteoclasts developed in vitro from radiosensitive mouse bone marrow mononuclear phagocytes (BMMP). (Burger, E. H., J. W. M. van der Meer, J. S. van de Gevel, C. W. Thesingh, and R. van Furth, 1982, *J. Exp. Med.* 156:1604–1614). In the present study, this co-culture technique was used to analyze the influence of bone-forming cells on osteoclast formation and bone resorption by BMMP or peritoneal exudate cells (PEC). BMMP or PEC were co-cultured with liver or dead bone, i.e., in the presence or absence of liver bone-forming cells. Mineral resorption and osteoclast formation were monitored via <sup>45</sup>Ca release from prelabeled live or dead bone followed by histology.

Osteoclasts developed from precultured BMMP as indicated by [<sup>3</sup>H]thymidine labeling, but only in live and not in dead bone. They formed readily from BMMP but only erratically, and after a longer culture period, from PEC. Macrophages from BMMP and PEC invaded live and dead bone rudiments but did not resorb the intact mineralized matrix. In contrast, ground bone powder was resorbed avidly by both cell populations, without formation of osteoclasts.

We conclude that live bone-forming cells are required for osteoclast formation from progenitors. Live bone is only resorbed by osteoclasts, and not by macrophages. Osteoclast progenitors are abundant in cultures of BMMP but scarce in PEC, which makes a direct descentance of osteoclasts from mature macrophages unlikely.

The nature of the mononuclear precursor cell of the osteoclast and the interactions between bone-forming and bone-resorbing cells are the subject of many recent studies (1, 2). In an earlier paper we presented evidence that multinucleated mineral resorbing osteoclasts will develop in vitro from cultures of proliferating bone marrow mononuclear phagocytes (BMMP)<sup>1</sup> (3). This was demonstrated by co-culturing adult mouse BMMP with noninvaded embryonic mouse long bones that had been stripped of adhering periosteum-perichondrium to remove the endogenous pool of osteoclast precursor cells (4). As the bones were dissected and stripped before cartilage invasion and osteoclast formation had occurred, the stripped bone rudiments essentially contained only chondrocytes and

some osteoblasts and osteocytes. By co-culturing such stripped bones with several populations of adult mouse cells, we demonstrated that proliferating cells of the mononuclear phagocyte series from bone marrow will form mineral-resorbing osteoclasts, but that nonproliferating bone marrow cultures, monocytes, or macrophages do not, at least not within 7 d of co-culture (3).

On the other hand, many in vivo studies in animals as well as in man have shown that mononuclear osteoclast precursors may be disseminated through the body via the blood vascular system (5–9). This has led several investigators to study the capacity of blood monocytes or tissue macrophages to resorb bone mineral by co-culturing them with devitalized bone tissue or particles of dried, ground bone (10–14). Although both cell types were able to resorb bone matrix, they did not form osteoclasts (10, 13, 14). However, in man chronic inflammatory lesions in or near bone tissue, as for instance in

<sup>1</sup> Abbreviations used in this paper: BMMP, bone marrow mononuclear phagocytes; EBSS, Earle's balanced salt solution; PEC, peritoneal exudate cells.

periodontal disease, may lead to localized bone loss (15). The capacity of monocytes and macrophages to resorb dead bone *in vitro* has opened up the possibility that they play a role in such pathological bone destruction (10).

In the present paper we studied the role of bone-forming cells in the process of osteoclast formation from phagocytic cells. For this purpose the osteoclast-forming capacity of proliferating BMMP and peritoneal exudate cells (PEC) was studied in long-term co-culture experiments with either viable intact bone rudiments, devitalized intact bone rudiments, or devitalized bone powder. The origin of *in vitro* formed osteoclasts was determined by [<sup>3</sup>H]thymidine labeling.

## MATERIALS AND METHODS

**Stripped Bone Rudiments:** Pregnant Swiss albino mice were subcutaneously injected with 30  $\mu$ Ci <sup>45</sup>Ca (sp act, 17.6 mCi/mg of Ca) on the 16th day of gestation. The next day the metatarsal rudiments of the 17-d-old fetuses were dissected and rigorously stripped of adhering perichondrium-periosteum by a combination of collagenase and mechanical treatment until all periosteal cells had been removed. As the 17-d-old metatarsal rudiments had not yet been invaded by blood vessels or any type of mononuclear or multinuclear cells, the stripped rudiments only consisted of a solid rod of cartilage (two noncalcified epiphyses and a diaphysis of noninvaded calcified cartilage without a primitive marrow cavity) surrounded by a thin layer of bone matrix with a few osteocytes (3, 4). Some of the stripped bones were killed by repeated freezing and thawing and subsequently severed in the center to expose the calcified cartilage as well as the calcified bone collar.

**Bone Powder:** Male albino rats weighing 100 g were injected subcutaneously on alternate days with 100  $\mu$ Ci <sup>45</sup>CaCl<sub>2</sub> for 14 d. At death, the long bones were removed, cleaned of adhering tissue, fragmented to expose bone marrow, and washed three times in sterile saline. After air-drying for 1 wk at 45°C, the fragments were ground to a coarse powder in a ball mill (Retch Inc., Federal Republic of Germany). Particles  $\leq$  20  $\mu$ m diam were collected by passing the powder through a sieve (Wilten, Etten Leur, The Netherlands). Before use the powder was sterilized by exposure to ultraviolet light, washed in 70% ethanol, and suspended in 10% rat serum in Earle's balanced salt solution (EBSS).

**BMMP:** Bone marrow cells from femora of 6-wk-old Swiss male mice were precultured for 7 or 8 d in Teflon culture bags (Teflon FEP film, 25- $\mu$ m gauge, Jansens M. and L., St. Niklaas, Belgium) in liquid culture medium consisting of Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) with 20% horse serum (Flow Laboratories, Inc., Irvine, Scotland) and 20% embryonic mouse fibroblast conditioned medium as the source of colony-stimulating factor (3, 16). Before co-culture with bone the cells were harvested, counted, and taken up in 10% rat serum in EBSS.

**[<sup>3</sup>H]Thymidine Labeling:** [<sup>3</sup>H]Thymidine (0.1  $\mu$ Ci/ml, 0.05 Ci/mmol), (Amersham International, Amersham, England) was added to BMMP cultures for the last 16 h of the 8-d preculture period. The cells were washed three times in Dulbecco's modified Eagle's medium with 10% fetal calf serum and co-cultured with nonlabeled stripped bone rudiments for 7 d as described below. The co-cultures were fixed for 3 h in 1.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer, decalcified in a saturated solution of EDTA in Tris buffer, pH 7, and postfixed for 1 h in 1% OsO<sub>4</sub> in phosphate buffer. After embedding in Epon, 1- $\mu$ m-thick sections were cut and exposed unstained for 10 wk to Ilford L4 dipping film emulsion (Ilford Ltd., Ilford, Essex, England). Autoradiographs were inspected with phase-contrast microscopy.

**PEC:** Peritoneal cells were harvested from 6-wk-old Swiss male mice, 4 d after intraperitoneal injection of 1 ml of newborn calf serum. After decapitation and desanguination of the animals, peritoneal cells were isolated by intraperitoneal injection of 2 ml EBSS which, after gentle kneading of the abdomen, was withdrawn with a pasteur pipette. The cells were centrifuged and suspended in 10% rat serum in EBSS.

**Co-culture Conditions:** Bone rudiments or bone powder were cultured with or without cells on top of Millipore filters (0.45- $\mu$ m pore size, Millipore Corp., Bedford, MA) in 24-well tissue culture plates (Flow Laboratories) containing 500  $\mu$ l of semisolid culture medium per well. The culture medium consisted of 10% rat serum, 20% chicken plasma, and 10% chicken embryo extract in EBSS. For co-culture experiments 20  $\mu$ l of suspension containing  $8 \times 10^5$  cells, was added to one stripped bone or to 0.1 mg of bone powder. The cultures were incubated in a humidified 5% CO<sub>2</sub> atmosphere at 37°C.

**Assay of Mineral Resorption:** Medium was changed after 1, 4, and 8 d of co-culture. After a total period of 11 d *in vitro* the co-cultures were fixed for 24 h in Lillie's acetic alcohol formalin solution which consisted of a 5:85:10 mixture of glacial acetic acid, 100% ethanol, and 40% formaldehyde (17). The fixed cultures were decalcified in 5% formic acid and processed for histology (see below). The semisolid culture media were digested overnight with 0.1% trypsin. The <sup>45</sup>Ca content of the 5% formic acid decalcification fluid and the trypsinized culture media was determined by liquid scintillation counting. The medium of the first co-culture day, containing only <sup>45</sup>Ca released by physico-chemical exchange, was excluded.

**Histology:** After fixation and decalcification (see above), the cultures were embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin or with Masson's stain for light microscopic examination.

## RESULTS

### Bone Rudiments with BMMP

Co-culture of live stripped bone rudiments with BMMP enhanced the release of <sup>45</sup>Ca from the bone tissue, starting after 4 d of co-culture and steadily increasing from then on (Fig. 1a). Killing the bone tissue by freezing and thawing completely inhibited this process (Fig. 1a).

Histology of the live stripped bones co-cultured with BMMP showed many multinucleated osteoclasts in the eroded center of the rudiment, but not in the stripped control bones that had been cultured without BMMP (Fig. 2, a-c). The osteoclasts of the 11-d BMMP co-cultures were usually quite large and seemed to contain more nuclei than in 7-d co-cultures (3), but no attempt was made to assess this quantitatively. In many cases the calcified bone collar around the center of the rudiment had also been eroded (Fig. 2, b and c). There was a strong positive rank correlation ( $r = 0.8378$ ) between the number of osteoclasts in 11-d co-cultures and the amount of <sup>45</sup>Ca released during the culture period (Fig. 3).

Autoradiography of co-cultures of [<sup>3</sup>H]thymidine-labeled BMMP and nonlabeled stripped bones showed that most nuclei of the *in vitro* formed osteoclasts were labeled. Table I summarizes the results. Cartilage nuclei usually contained zero or one grain per nuclear profile, which corresponded to background labeling. 50% of the BMMP-derived mononuclear cells and 89% of the osteoclast nuclei were labeled.

In the killed bone rudiments, co-culture with (live) BMMP did not cause formation of osteoclasts or release of <sup>45</sup>Ca. Mononuclear phagocytes did adhere to the dead bone surface

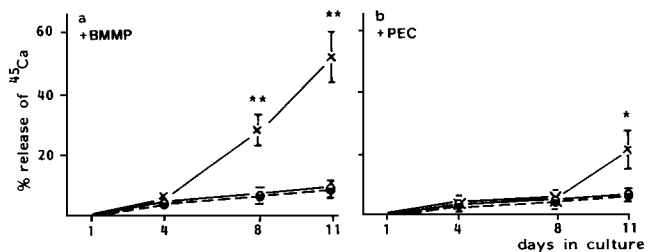


FIGURE 1 Cumulative release of <sup>45</sup>Ca from prelabeled, periostless bone rudiments by BMMP (a) or PEC (b). <sup>45</sup>Ca-labeled periostless bone rudiments, liver or killed by freezing/thawing, were cultured with or without BMMP (a) or PEC (b). <sup>45</sup>Ca was determined in culture media and in cultured bones. The medium of the first day, containing only <sup>45</sup>Ca released by exchange, was not considered. (a) ×, Live bones + BMMP; ○, killed bones + BMMP; ●, live bones without added cells. (b) ×, Live bones + PEC; ○, killed bones + PEC; ●, live bones without added cells. Values are means  $\pm$  SEM of 16 or more determinations. \*  $P < 0.05$ ; \*\*  $P < 0.001$  (Student's *t* test; group × compared with group ○ or ●).

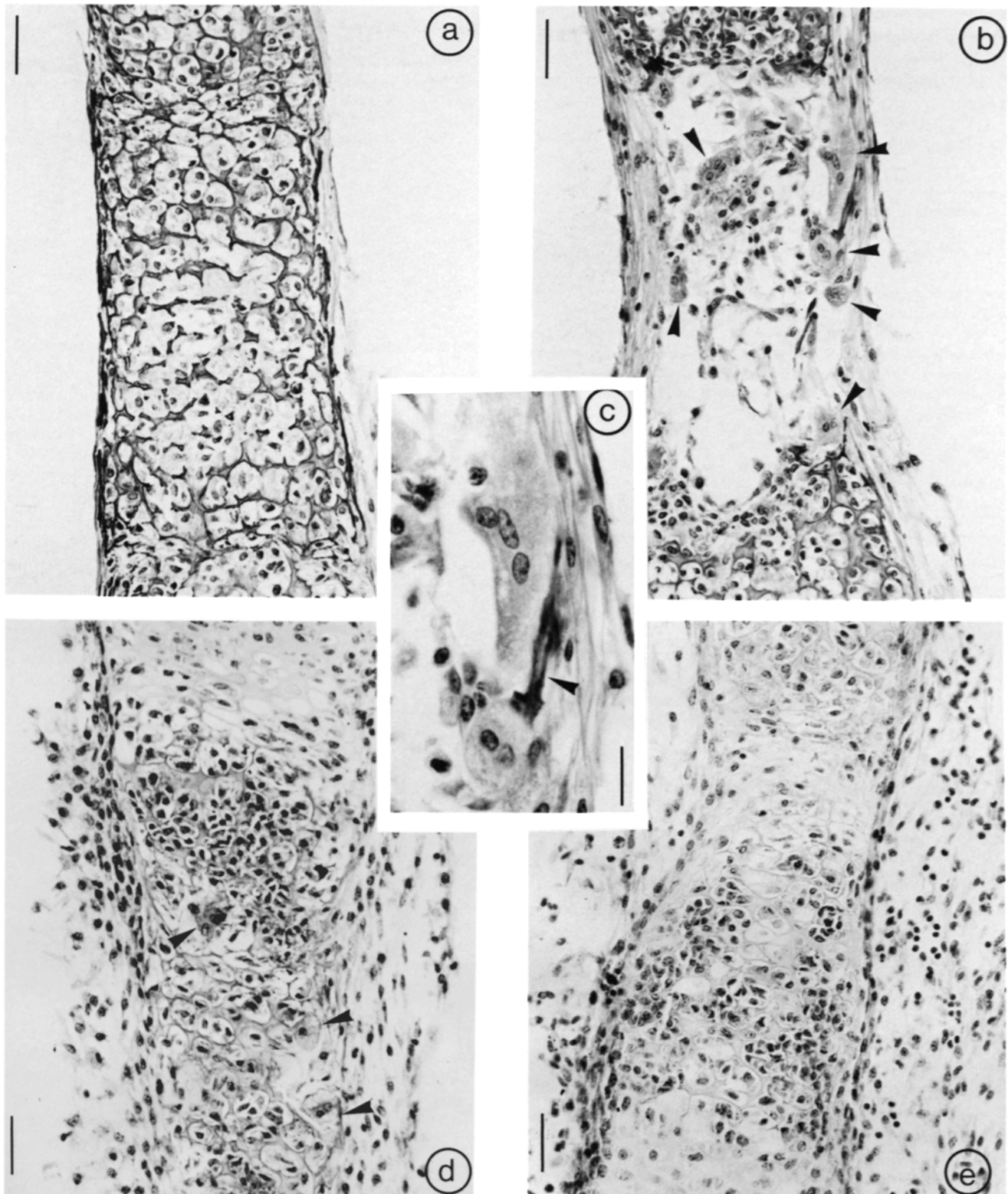


FIGURE 2 Central parts of live, stripped metatarsal bone rudiments cultured for 11 d in the absence (a) or presence (b and c) of BMMP phagocytes or PEC (d and e). (a) In the absence of extra cells, no osteoclasts develop in stripped bone rudiments. (b) In the presence of BMMP many osteoclasts (arrowheads) develop which erode the calcified center of the bone. (c) Detail of osteoclasts from the upper right part of b; two osteoclasts are in the process of resorbing remnants of the bone collar (arrowhead). (d) Three osteoclasts (arrowheads) have developed from PEC and have started to resorb the mineralized bone center. (e) In this case no osteoclasts have developed from peritoneal exudate cells. (a, b, d, e) Bar, 50  $\mu\text{m}$ ;  $\times 210$ . (c) Bar, 20  $\mu\text{m}$ .  $\times 520$ .

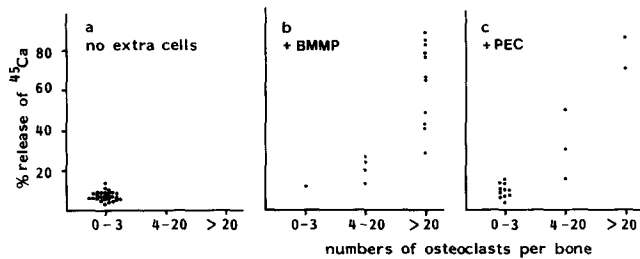


FIGURE 3 Correlation between release of  $^{45}\text{Ca}$  and development of osteoclasts in culture. Viable,  $^{45}\text{Ca}$ -labeled periosteal bone rudiments were cultured for 11 d without other cells (a), with BMMP (b), or with PEC (c). We determined the  $^{45}\text{Ca}$  released during culture and processed the bones for histology. The total number of osteoclasts per bone was counted in serial sections. Number of determinations: (Group a) 26; (group b) 17; (group c) 16. The coefficient of rank correlation of all determinations (number of osteoclasts versus  $^{45}\text{Ca}$  release) was 0.8378 ( $n = 59$ ) according to Spearman's rank correlation test, corrected for ties. Enhanced release of  $^{45}\text{Ca}$  in co-cultures with BMMP or PEC always coincided with formation of osteoclasts above background values.

TABLE I

Labeling Indices of Nuclei in Co-cultures of [ $^3\text{H}$ ]Thymidine-labeled BMMP and Nonlabeled Stripped Bone Rudiments

Cell type	Labeling index*	n*
	%	
Osteoclasts	88.6	141
Chondrocytes	0.0	200
Mononuclear phagocytes	50.2	200

\* A minimum of six grains per nuclear profile was taken as a criterion for "labeling" compared with backgrounds of zero or one grain per nucleus.

\* All 141 osteoclast nuclei and 200 chondrocyte and mononuclear phagocyte nuclei of four co-cultures were evaluated. Labeled and nonlabeled nuclei could be found within the same osteoclast.

and invaded the severed calcified stump, but osteoclasts were not formed.

### Bone Rudiments with PEC

Co-culture of live, stripped bone rudiments with PEC enhanced the release of  $^{45}\text{Ca}$  by the end of the co-culture period (Fig. 1b). This enhancement occurred in only 5 of the 16 cultures (Fig. 1b gives mean values of all 16 determinations), and started after a lag time of 8 d. In the histological sections osteoclasts were only present in those bones that also showed enhanced release of  $^{45}\text{Ca}$  (Figs. 2, d and e and 3). The osteoclasts were similar in appearance to those developed from BMMP in that they contained several nuclei and many vacuoles and were found closely apposed to mineralized matrix, usually in an eroded space. In bones that had not shown enhanced release of  $^{45}\text{Ca}$  during co-culture, no osteoclasts were found (Fig. 3).

As with BMMP, killing of the bone tissue inhibited osteoclast formation and mineral resorption (Fig. 1b). Here too, mononuclear phagocytes invaded the severed zone of calcified cartilage but this did not result in stimulated release of  $^{45}\text{Ca}$  or formation of osteoclasts (Fig. 4).

### Bone Powder with BMMP or PEC

BMMP and PEC avidly resorbed particles of devitalized powdered bone (Fig. 5). The resorption was similar for the two populations both in magnitude and time course. Contrary

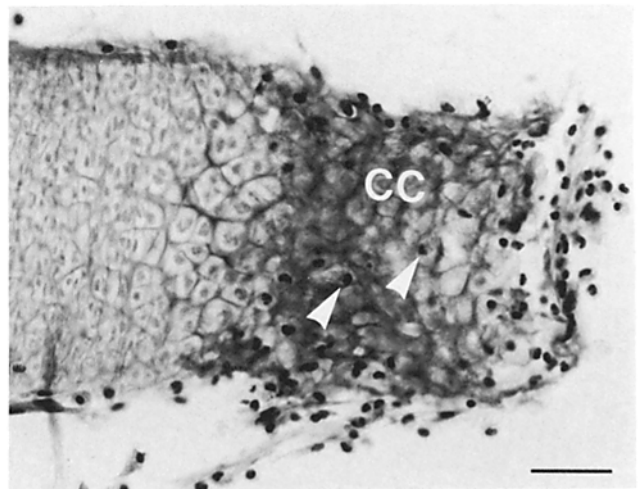


FIGURE 4 Stripped metatarsal rudiment killed by freezing and thawing and subsequently co-cultured with PEC. Live peritoneal cells (dark nuclei) have invaded the severed stump of calcified cartilage (arrowheads) without formation of osteoclasts or release of  $^{45}\text{Ca}$  (cf. Fig. 1b). CC, calcified cartilage. Bar, 50  $\mu\text{m}$ ;  $\times 210$ .

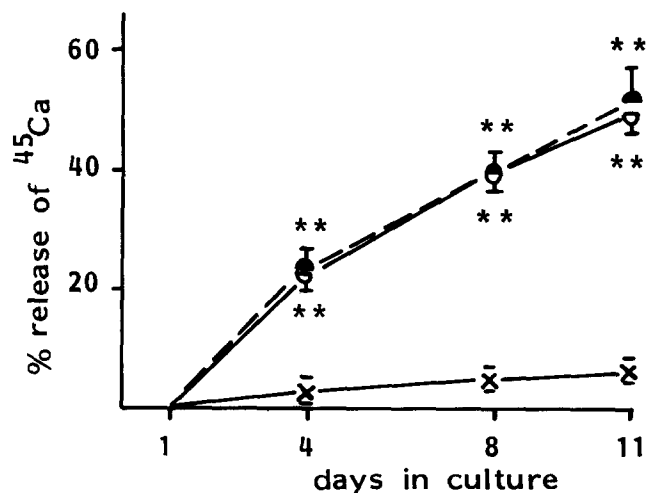


FIGURE 5 Release of  $^{45}\text{Ca}$  from devitalized bone particles by BMMP or PEC.  $^{45}\text{Ca}$ -labeled bone powder was cultured with or without BMMP or PEC,  $8 \times 10^5$  cells per 0.1 mg of bone powder.  $^{45}\text{Ca}$  was determined as in Fig. 1.  $\times$ , Bone powder without cells; O, plus BMMP;  $\bullet$ , plus PEC. Values are means  $\pm$  SEM of 12 determinations. \*\*  $P < 0.001$  (Student's  $t$  test; compared with group without cells).

to resorption of live rudiments it started immediately without a lag phase, and was very substantial after 4 d in vitro (500% increase over controls).

However, histology of the 11-d co-cultures revealed no evidence of osteoclast formation from either BMMP or peritoneal cells. In both cases the bone particles became surrounded by cells that were much smaller than osteoclasts and contained only one or occasionally two nuclei. (Fig. 6). Cells comparable to osteoclasts in size and number of nuclei were not encountered.

## DISCUSSION

### Origin of In Vitro-formed Osteoclasts

The high labeling index of the osteoclast nuclei in the autoradiographic experiments, when [ $^3\text{H}$ ]thymidine was used

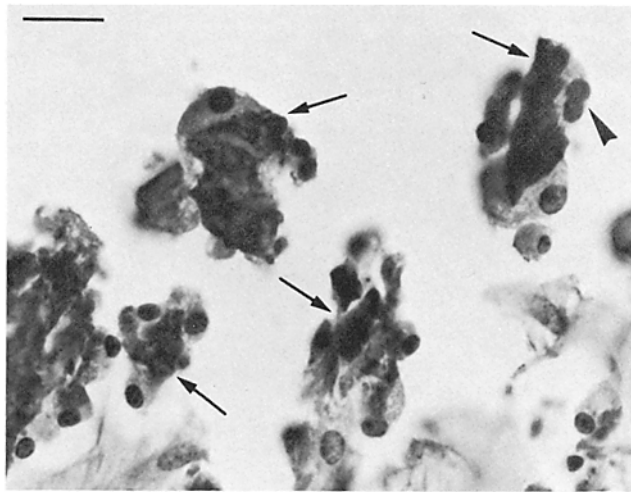


FIGURE 6 Powdered bone mineral cultured for 11 d with BMMP. Mononuclear and occasionally binuclear (arrowhead) cells adhere to the bone particles (arrows). Bar, 20  $\mu$ m;  $\times$  520.

as a nuclear marker of BMMP, indicates that the *in vitro* formed osteoclasts developed from co-cultured cells rather than from endogenous cells of the stripped bone. A descentance from the stripped rudiment itself is highly unlikely, in view of the absence of cartilage labeling in the autoradiographs and the absence of osteoclast formation in live, stripped bones cultured without added cells. Quail mouse chimera studies, which made use of the specific nuclear morphology of quail cells as marker, have led to a similar conclusion (4). The finding of a higher labeling index of osteoclast nuclei than of co-cultured mononuclear phagocytes is remarkable and may indicate that osteoclasts are selectively derived from the proliferating fraction of the BMMP population. This conclusion is also supported by our earlier finding (3) that osteoclasts develop from the radiosensitive, weakly adherent fraction of BMMP cells. Alternatively, the data may reflect an expansion of the osteoclast precursor pool during the co-culture time.

#### *Development of Osteoclasts from PEC*

The few cases where osteoclasts developed from PEC indicate that osteoclast precursors are potentially present among the cells that assemble in the peritoneal cavity as a result of inflammation. However, osteoclast formation needed a longer lag time and occurred erratically in comparison with BMMP. This probably means that osteoclast precursor cells are present, but at a considerably lower concentration among PEC than among BMMP. The longer lag time is similarly explained: in previous studies (3, 18) we showed by radiation experiments and [ $^3$ H]thymidine autoradiography that proliferation of osteoclast progenitors does occur at the surface of bone. A scarcity of precursor cells in peritoneal exudates is compatible with a longer period of cell proliferation to obtain the minimal density of precursor cells required for fusion and osteoclast formation. In precultured bone marrow the osteoclast-forming cells must be sought in the pool of proliferating macrophage precursors (3). Such cells are scarce in PEC populations (19–21) but abundant in 7-d-old bone marrow cultures (22), which is in line with the observed difference in osteoclast-forming capacity. Thus it seems reasonable to conclude that as in BMMP, the osteoclast-forming cells of PEC are not mature macrophages, but immature macrophage pro-

genitors. This view, when applied to the *in vivo* situation, would indicate that neither mature tissue macrophages nor their direct precursors in the blood, the monocytes, will form osteoclasts. Interestingly, in a recent *in vivo* study (23) no correlation was found between the kinetics of [ $^3$ H]thymidine labeling of monocytes and of osteoclast nuclei, arguing against a direct development of osteoclasts from blood monocytes.

#### *Role of Bone-forming Cells during Osteoclast Formation*

The strong positive correlation between resorption of live bone rudiments and formation of osteoclasts indicates that in live tissue, mineral resorption and osteoclast formation go hand in hand, and argues strongly for the osteoclast as the only cell type capable of resorbing mineral in live bone.

Osteoclast formation was inhibited by killing the bone tissue, i.e., the cartilage cells and few osteoblasts-osteocytes that the stripped rudiment contained. Killing the bone did not inhibit PEC or BMMP from making contact with the mineralized surface, and macrophages were even found in the lacunae of the chondrocytes of the calcified zone. Still, osteoclasts were not formed. Thus it seems that the presence of mineralized matrix is in itself insufficient for osteoclast formation and that viable mineral-forming cells are required as well, probably as regulators of osteoclast differentiation. Interactions between bone-forming and bone-resorbing cells have been postulated to play a role in both normal bone (re-)modeling and in parathyroid hormone-stimulated bone resorption (2, 24, 25). A regulatory role for mineral-forming cells during the formation of multinucleated osteoclasts from mononucleated precursors is also in keeping with the highly ordered sequence of formative and resorptive processes during normal bone development and in normal and experimental bone remodeling (26, 27).

#### *Resorption of Dead Bone*

Devitalized bone was only resorbed when the bone mineral was ground to a powder, and not when the killed tissue was left intact. However, the absence of a time lag, the similarity of the response of BMMP and PEC, and the absence of large, truly multinucleated cells even after 11 d of co-culture with BMMP indicate that this type of resorption is different from osteoclast-mediated resorption. Others (12, 13) have shown that it is effected by mature macrophages, which compose some 50% of both PEC and BMMP, as determined in cyto-centrifuge preparations stained either with Giemsa or for nonspecific esterase (data not shown).

Since the ground bone particles were approximately the same size as the spicules of mineralized matrix in the intact bone rudiments, the large difference in resorption of the two types of dead bone cannot be explained by differences in size. Rather, the intactness of the mineralized matrix seems to be important. In the intact, killed rudiment the mineral phase was still protected against digestion by macrophages, probably by an organic component of the matrix. In this respect the lamina limitans as described by Scherft (28) may be important. This organic electron-dense layer is found around areas of calcified cartilage and bone matrix (28, 29) and is the first structure to disappear in osteoclast-mediated resorption (28). If its intact presence protects bone mineral from digestion by macrophages, then the grinding of bone to produce bone

powder will, by rupturing this barrier, expose the mineral to macrophage recognition and digestion.

### Localized Bone Loss and Inflammation

In the previously mentioned studies on devitalized bone, some authors (11, 12) have interpreted the contact-mediated resorption of bone powder by macrophages as indicating that macrophages might be capable of transforming into osteoclasts. However, others (10, 13, 14) have stressed the differences between macrophage-mediated and osteoclastic bone resorption, and have linked macrophage-mediated resorption with localized bone loss that occurs in areas of chronic inflammation, on the argument that such lesions always contain an accumulation of mononuclear phagocytes. We have found no evidence for a direct transformation of macrophages into osteoclasts. Our experiments suggest yet another explanation for increased bone loss at inflammatory sites: in such lesions the population of blood-derived inflammatory cells contains an increased number of osteoclast progenitor cells which, when in contact with bone mineral and bone-forming cells, transform into osteoclasts. The demonstration of such cells in actual sites of inflammation must, however, await further investigation.

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