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IN VITRO FORMATION OF OSTEOCLASTS
FROM LONG-TERM CULTURES OF
BONE MARROW MONONUCLEAR PHAGOCYTES

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During the last two decades, ample evidence has been obtained that osteoclasts, the multinucleated calcified-matrix resorbing giant cells of bone, which form by fusion of mononuclear precursor cells, are of hematogenous origin. The evidence stems from experiments done in parabionts of labeled animals (1), studies on osteopetrotic animals and humans (2-4), and quail-chick and mouse-quail transplantation experiments (5-7). Essentially, these *in vivo* studies have shown that osteoclasts derive from bone marrow or other hematopoietic tissues and have indicated mononuclear phagocytes as the most likely candidates for the precursor cells which fuse to form an osteoclast. However, *in vitro* evidence for the direct transformation of monocytes and/or tissue macrophages into bone-resorbing osteoclasts is still lacking, although it has long been known that cultured macrophages can form foreign-body giant cells *in vitro* by fusion (8-10).

Several *in vitro* studies have dealt with the destruction of calcified bone matrix by mononuclear phagocytes; for this work, use was made of human peripheral blood monocytes (11, 12) or rodent macrophages (13) in combination with devitalized bone particles. Monocytes and macrophages were able to resorb mineral in a contact-mediated fashion, but did not form cells with the morphological characteristics of osteoclasts (13). Recent investigations, however, point to the importance of interactions between bone-forming and -resorbing cells during osteoclastic bone resorption (14). This means that studies done on devitalized bone without viable bone-forming cells might be of limited value with respect to the formation of osteoclasts and osteoclast-mediated bone resorption.

We recently found (7) that early removal of the perichondrium-periosteum from embryonic mouse long-bone primordia prevents the formation of osteoclasts during organ culture of such bones. In mouse-quail transplantation studies, such stripped bone rudiments are invaded by quail osteoclasts, but mouse osteoclasts are not formed because the stripping procedure has removed the osteoclast precursor cells.

In the present study, stripped live bone rudiments were used to assess the capacity of various populations of mononuclear phagocytes to form osteoclasts *in vitro*. Stripped bone rudiments were co-cultured with embryonic liver as well as with

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proliferating and nonproliferating populations of mononuclear phagocytes from the bone marrow, peripheral blood, or peritoneal cavity.

Materials and Methods

Animals. Swiss albino mice were used, i.e., 17-d-old embryos and 6-wk-old males.

Organ Culture of Embryonic Bones. The middle three metatarsal bone rudiments from 17-d-old embryos (day of vaginal plug discovery equals day 0 of gestation) were excised, and their adhering perichondrium-periosteum was removed by rolling the bone over a glass surface after a 10-min treatment with 1% collagenase (Sigma Chemical Co., St. Louis, MO) and 1% bovine serum albumin (fraction V; Sigma Chemical Co.) in Hanks' balanced salt solution (HBSS)¹ at room temperature. Enzyme activity was stopped in 10% rat serum in HBSS. Care was taken to standardize the age of the rudiments by selecting litters in which the first phalanges of the feet had not yet started to calcify. The stripped rudiments were cultured for 7 d on a piece of perforated cellophane (Serva, Heidelberg, Federal Republic Germany) placed on a semisolid medium (SSM) containing 20% cock plasma, 10% chick embryo extract (EE), and 10% rat serum in Earle's balanced salt solution (EBSS) (15), in a humidified 5% CO₂ atmosphere at 37°C.

Experimental Set-Up. Control cultures consisted of stripped bone rudiments cultured alone, and experimental cultures comprised stripped bone rudiments co-cultured with other cell populations. For studies with embryonic liver, whole pieces of tissue were co-cultured. In all other cases, suspensions of cells were centrifuged and resuspended in 10% rat serum in EBSS. EE and cock plasma were added to make a weak coagulum composed of 10% EE and 30% plasma containing $1-2 \times 10^7$ cells/ml. Pieces of coagulum containing some 5×10^5 cells were wrapped around stripped rudiments and co-cultured for 7 d.

Cell Populations Used

EMBRYONIC LIVER. 1-mm³ pieces of liver from 17-d-old embryos were cultured in contact with the bone rudiments (three pieces of liver for each bone rudiment).

CULTURES OF BONE MARROW MONONUCLEAR PHAGOCYTES. Bone marrow cells from femora of 6-wk-old male mice were precultured for 8 or 14 d in Teflon culture bags in liquid culture medium (LCM) containing embryonic mouse fibroblast-conditioned medium as the source of colony-stimulating factor (CSF) (16, 17). The medium consisted of Dulbecco's modified Eagle's medium with 20% horse serum and 20% conditioned medium (CM), as described elsewhere (17, 18). For 14-d-old cultures, fresh CM was added after 7 d. Culture in Teflon bags avoided adherence of mononuclear phagocytes, thus facilitating their recovery (16).

IRRADIATED 14-D-OLD BONE MARROW CULTURES. 11-d-old bone marrow cultures in Teflon culture bags were irradiated with 1,000 rad and cultured for another 3 d. After 14 d of preculture, irradiated and nonirradiated cultures were harvested and cocultured with stripped bone rudiments. Samples of 14-d-old irradiated and nonirradiated bone marrow cultures were incubated with 1 μ Ci [³H]thymidine (TdR)/ml (sp. act. 2 Ci/mmol) for 6 h, after which the incorporation of ³H into the trichloroacetic acid-insoluble cell fraction was measured.

WEAKLY/STRONGLY ADHERENT CELLS IN BONE MARROW CULTURES. Bone marrow cells were precultured for 8 d in LCM in plastic petri dishes (35 mm, Falcon Labware, Oxnard, CA) to allow adherence of mature mononuclear phagocytes. Cells that did not adhere strongly to the petri dish were removed by vigorous shaking and co-cultured with stripped bone rudiments. After vigorous washing with warm HBSS, the adherent cells were removed by incubating them overnight in a thin film of SSM (0.5 ml/35-mm dish) which, after the addition of HBSS, became detached together with the cells from the bottom of the dish, as could be readily observed in dishes stained with Giemsa before or after removal of the SSM film. The film bearing the cells was wrapped around a stripped bone rudiment and co-cultured. The concentration of cells in the film, as estimated in histological sections, amounted to $1-10 \times 10^6/$

¹ *Abbreviations used in this paper:* CM, conditioned medium; CSF, colony-stimulating factor; EE, chick embryo extract; EBSS, Earle's balanced salt solution; HBSS, Hanks' balanced salt solution; LCM, liquid culture medium; NBCS, newborn calf serum; PBS, phosphate-buffered saline; SSM, semisolid medium; TdR, thymidine.

ml.

BLOOD MONOCYTES. Peripheral blood from 6-wk-old mice was obtained by heart puncture, using heparinized syringes (20 U/ml blood). After dilution with 4 vol of phosphate-buffered saline (PBS), the erythrocytes were removed in a gradient of Ficoll-Hypaque (19). The cells were washed twice in PBS and once in LCM to remove the Ficoll-Hypaque, precultured for 1 d in LCM in Teflon bags, and co-cultured with stripped bone rudiments.

RESIDENT MACROPHAGES. 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.

EXUDATE MACROPHAGES. Cells were harvested as described for resident macrophages, 1.5 or 4 d after intraperitoneal injection of 2 ml newborn calf serum (NBCS) or 4 d after intraperitoneal injection of 1.5 ml 4% brewers thioglycollate broth (Difco Laboratories, Detroit, MI). Resident and elicited macrophages were co-cultured with stripped bones without preculture.

Histology. In most cases, cultured, co-cultured, and noncultured bone rudiments, pieces of embryonic liver, and cell-containing coagula were fixed and decalcified in Bouin's fluid for light-microscopical examination of serial paraffin sections which had been stained with hematoxylin and eosin. In the other cases rudiments were fixed with glutaraldehyde and osmium, decalcified with 2.5% EDTA, and embedded in Epon 812 for ultrastructural observations made in ultrathin sections stained with uranyl and lead (20).

⁴⁵Ca Release. To study the resorption of calcified matrix in vitro, embryonic bone rudiments were labeled with ⁴⁵Ca by injecting pregnant mice subcutaneously with 30 μ Ci ⁴⁵Ca (sp. act. 17.6 mCi/mg Ca), 1 d before the embryos were taken out, i.e., at 16 d of pregnancy (21). The stripped bone rudiments were cultured with or without other cells in 24-well tissue culture plates (Falcon Labware). For the experiments, 8×10^5 cells from bone marrow cultures or from peritoneal exudates in 40 μ l of semisolid medium were added per bone rudiment. The amount of ⁴⁵Ca released in the culture medium was measured after 1, 4, and 7 d of culture. The (semisolid) medium was digested with 1% trypsin in HBSS overnight at 37°C, and the ⁴⁵Ca content of the solution was determined in a liquid scintillation counter. ⁴⁵Ca remaining in the bone tissue was determined in a 5% trichloroacetic acid extract of the cultured rudiment.

Results

Morphology of Noncultured and Cultured Stripped Bones. Before culture, the bones consisted of a center of calcified cartilage surrounded by a thin shaft of calcified bone with hyaline cartilage at both ends. Invasion by a periosteal bud and formation of osteoclasts had not yet occurred. The dissected bones were treated with collagenase until all of the periosteum-perichondrium was removed, including many of the osteoblasts on the outer surface of the bone collar (Fig. 1). As a result, the width of the bone shaft did not increase much during the subsequent culture period, although the length of the core of calcified cartilage increased some 100–200%.

In control cultures, the hypertrophic chondrocytes remained healthy and were still surrounded by a network of calcified matrix whose spiculae had become considerably wider during culture (Fig. 2). The bone shaft contained some osteocytes but few osteoblasts. Osteoclasts were absent or very scarce in control cultures: of a total of 45 control cultures, 5 contained one to three osteoclasts, the others none (Tables I and II). The osteoclasts that did develop may have penetrated the bone collar before the collagenase treatment and so escaped stripping. Absence of osteoclasts was always accompanied by absence of mineralized matrix resorption.

Co-Culture with Embryonic Liver. In bones co-cultured with pieces of embryonic liver—this organ being the main site of hemopoiesis in the embryo (22)—many osteoclasts usually developed within the zone of calcified cartilage (Table I). The calcified cartilage matrix, and, to a lesser extent, the bone shaft were eroded, and a primitive stroma with many erythrocytes in sinusoid-like channels had formed in

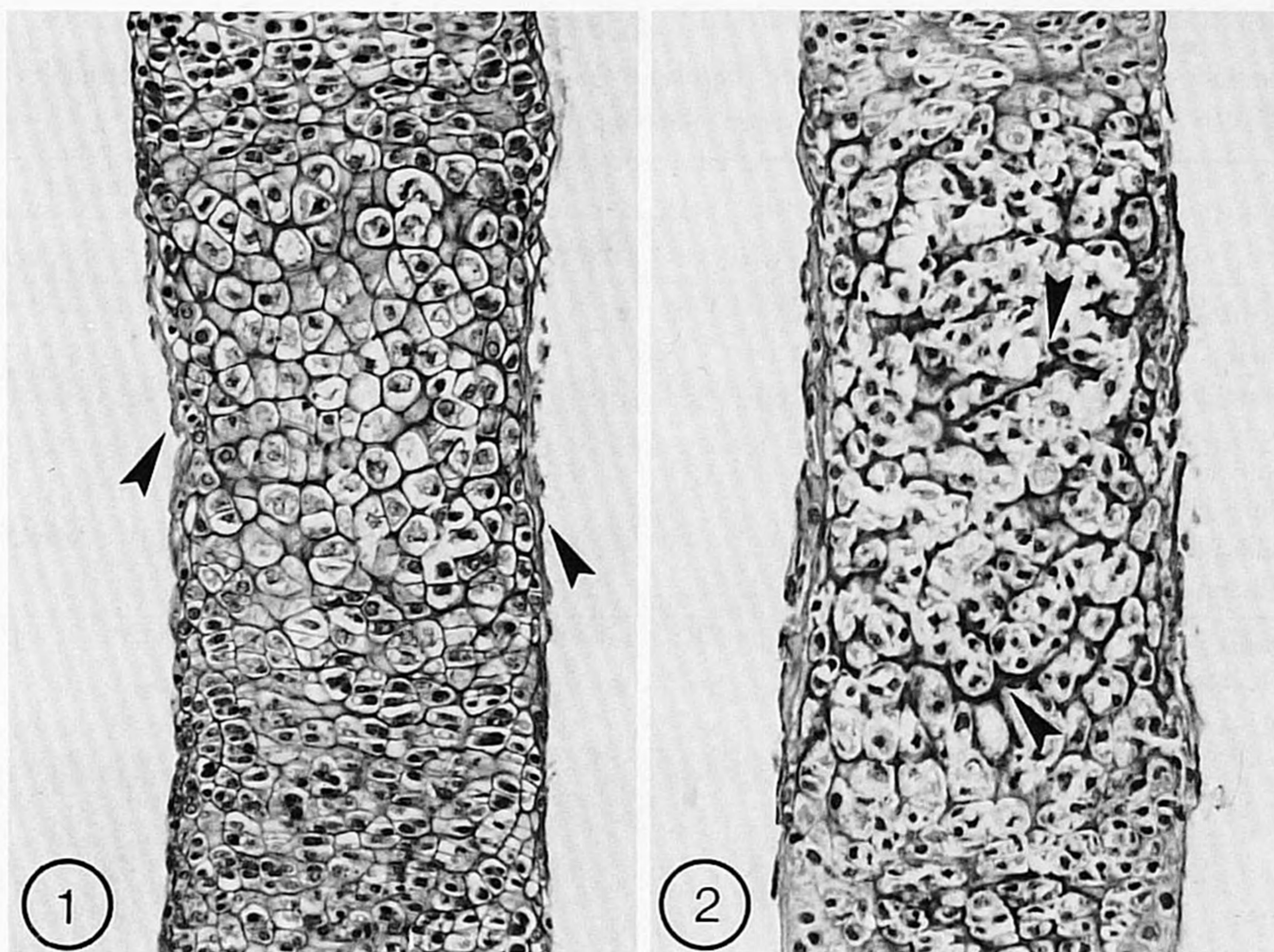


FIG. 1. Central part of a stripped metatarsal bone rudiment taken from a 17-d-old mouse embryo. The bone is still a solid rod of cartilage, surrounded by a thin bone collar (arrows). The stripping procedure has removed the periosteum-perichondrium. $\times 200$.

FIG. 2. Control bone, cultured for 7 d after stripping. The spiculae of the calcified cartilage matrix have increased in width (arrows) and have not been resorbed. Osteoclasts are not present. $\times 200$.

TABLE I
Occurrence of Osteoclasts in Stripped Bone Rudiments Co-Cultured with Embryonic or Adult Hemopoietic Cell Populations

| | Numbers of osteoclasts per bone rudiment* | | | | | (n) | P |
|----------------------------------|---|-----|------|-------|-----|------|---------|
| | 0 | 1-3 | 4-10 | 11-20 | >20 | | |
| Control cultures | 23 | 2 | 0 | 0 | 0 | (25) | |
| Co-cultured plus embryonic liver | 0 | 1 | 1 | 4 | 12 | (18) | <0.001‡ |
| Plus 8 d bone marrow cultures | 3 | 3 | 6 | 2 | 2 | (16) | <0.001‡ |
| Plus 14 d bone marrow cultures | 0 | 0 | 0 | 5 | 8 | (13) | <0.001‡ |

* Counts made in serial histological sections.

‡ Compared with control cultures; Wilcoxon's rank sum test.

vitro. In the liver tissue outside the bone shaft, erythropoiesis continued throughout the culture period.

Co-Culture with Cultured Bone Marrow Mononuclear Phagocytes. In Giemsa-stained cytocentrifuge preparations of the cells from 8-day-old bone marrow cultures, 60-80% of the cells were identified as immature and mature mononuclear phagocytes; the remainder were granulocytes. The granulocytes disappeared during prolonged culture (18), and the 14-d-old bone marrow cultures contained only immature and mature mononuclear phagocytes. Multinucleated giant cells were not encountered in these cultures. Co-cultivation of stripped bones with adult bone marrow precultured in the presence of CSF induced osteoclast formation: in 10 out of 16 rudiments co-cultured

TABLE II

Occurrence of Osteoclasts in Stripped Bone Rudiments Co-Cultured with Nonirradiated, Irradiated, Weakly Adherent, and Strongly Adherent Bone Marrow Culture Cells

| | Numbers of osteoclasts per bone rudiment* | | | | | (n) | P |
|---|---|-----|------|-------|-----|------|----------|
| | 0 | 1-3 | 4-10 | 11-20 | >20 | | |
| 14-d-old bone marrows cultures, nonirradiated | 0 | 0 | 0 | 5 | 8 | (13) | |
| 14-d-old bone marrows cultures, irradiated | 11 | 3 | 1 | 0 | 0 | (15) | <0.0001‡ |
| 8-d old bone marrow cultures, weakly adherent cells | 1 | 0 | 2 | 0 | 2 | (5) | |
| 8-d-old bone marrows culture, strongly adherent cells | 11 | 0 | 0 | 0 | 0 | (11) | <0.02‡ |

* Counts made in serial histological sections.

‡ Wilcoxon's rank sum test.

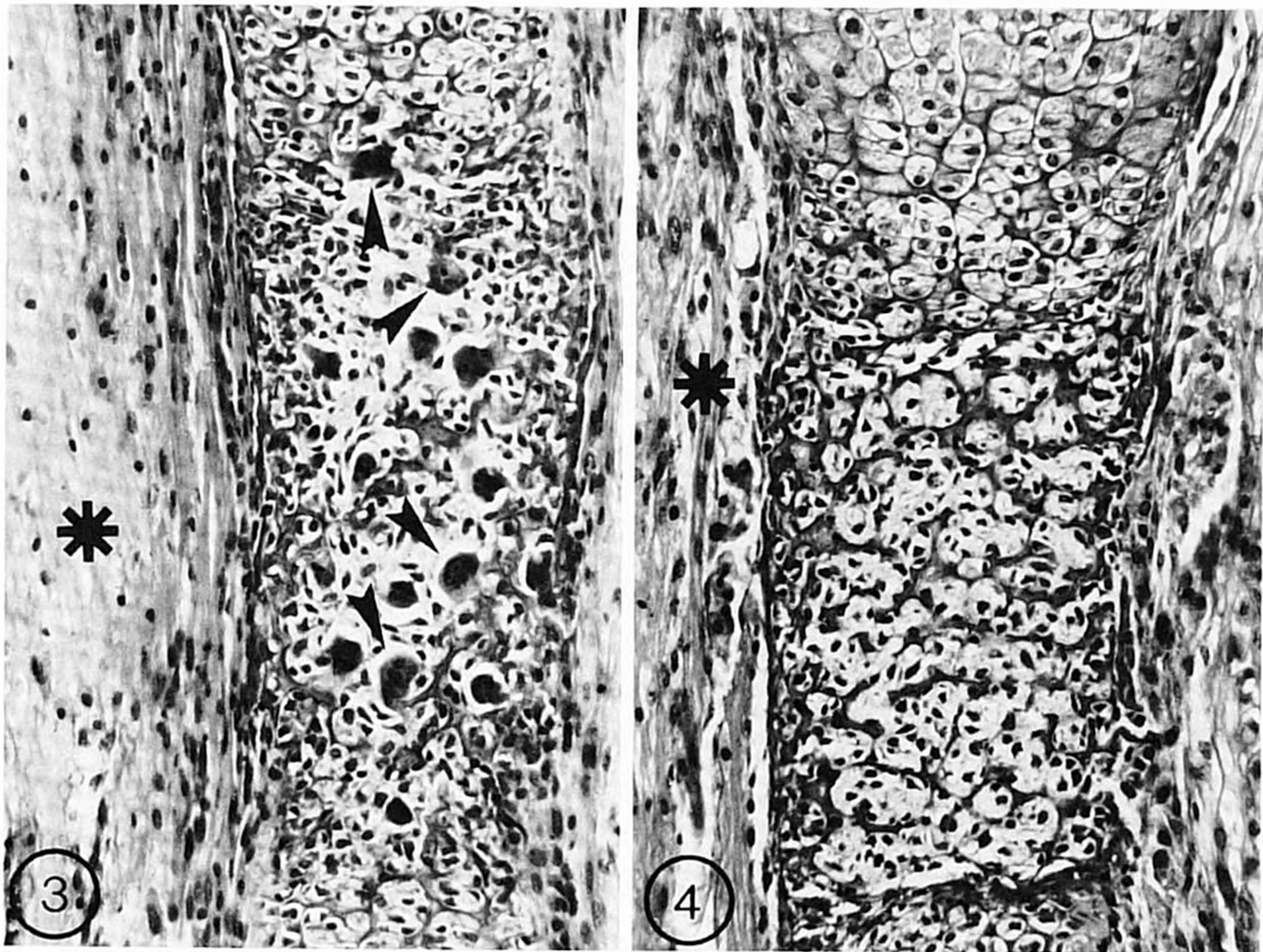


FIG. 3. Stripped bone co-cultured with bone marrow cells, precultured for 14 d. Many multinucleated osteoclasts (arrows) have eroded the calcified matrix. *, SSM with bone marrow cells. $\times 200$.

FIG. 4. Stripped bone co-cultured with precultured and irradiated bone marrow cells. No osteoclasts have been formed. *, SSM with bone marrow cells. $\times 200$.

with 8-d-old bone marrow, and in all of the 13 rudiments co-cultured with 14-d-old bone marrow, osteoclasts were formed in numbers higher than the control values (Table I). Osteoclasts were only observed within the eroded bone center or along the bone shaft, usually in close contact with calcified matrix (Figs. 3 and 4). Electron-microscopic observations confirmed their typical osteoclast-like morphology: giant multinucleated cells with dark-staining cytoplasm due to the presence of many free ribosomes and many small mitochondria. They adhered to the calcified matrix along

a clear zone and formed ruffled membranes with many invaginations and abundant small and large vacuoles at sites of matrix resorption. Matrix destruction was characterized by loss of stainability and disorganization of structural elements (Fig. 5 a, b, c). The eroded bone center also contained many macrophages.

Irradiation of Cultured Bone Marrow Cells. Irradiation of 11-d-old bone marrow cultures with 1,000 rad led to a severe reduction of [^3H]TdR incorporation 3 d later: 197 ± 4 cpm/ 2.10^5 irradiated cells ($n = 3$) compared with $5,764 \pm 770$ cpm/ 2.10^5 nonirradiated cells ($n = 3$). The total cell density did not differ between nonirradiated and irradiated cultures. The loss of proliferating cells from the irradiated cultures was accompanied by a dramatic reduction of osteoclast formation to almost background values (Table II, Fig. 4).

Co-Culture with Strongly and Weakly Adherent Bone Marrow Mononuclear Phagocytes. When 8-d-old bone marrow cultures were separated into two populations, one of strongly adherent and the other of weakly adherent cells, only co-culture with the latter population resulted in osteoclast formation (Table II).

Co-culture with Monocytes or Macrophages. After Ficoll-Hypaque separation, the leucocyte suspension contained 34% monocytes (range, 28–40%), 51% lymphocytes, and 15% granulocytes. The composition of the cell suspension from the unstimulated peritoneal cavity was 75–80% macrophages, 12–19% lymphocytes, and 1–4% granulocytes and mast cells. 1.5 d after an intraperitoneal injection of NBCS, the exudate contained 63% macrophages, 10% lymphocytes, 11% polymorphs, 15% eosinophils, and 1% mast cells; 4 d after NBCS i.p., the exudate showed 50% macrophages, 18% lymphocytes, 4% polymorphs, 24% eosinophils, and 2% mast cells. Thioglycollate-induced exudates comprised 74% macrophages, 18% lymphocytes, 1% polymorphs, and 6% eosinophils.

Co-culture of stripped rudiments with peripheral blood leukocytes, resident macrophages, or exudate macrophages did not lead to the formation of osteoclasts (Table III). However, macrophages were often found inside the bone shaft, among the chondrocytes in what remained of the lacunae. Only in 1 out of 16 bones co-cultured with blood leucocytes was the number of osteoclasts higher than the background (Table III). This rudiment contained five osteoclasts, but was also surrounded by an unusually large amount of periosteum, which suggests that it was not sufficiently stripped before co-culture.

Of the various cell types present in the cell populations, only the mononuclear phagocytes always remained healthy during co-culture. Lymphocytes always died, and granulocytes survived to a varying degree.

Release of ^{45}Ca . The results of the studies with ^{45}Ca -labeled bone rudiments are summarized in Table IV. Only co-culture with precultured bone marrow cells resulted in a significant increase in the amount of ^{45}Ca released from the bone rudiments during the second half of the culture period.

Discussion

The giant bone-resorbing cells that developed in co-cultures of stripped bone rudiments and embryonic or adult hematopoietic tissue showed all the characteristics of osteoclasts, at both the light and electron microscopic levels: dark-staining cells with many nuclei, always present in close relationship with mineralized tissue and resorbing the mineralized matrix in a manner characteristic of osteoclasts, i.e., by

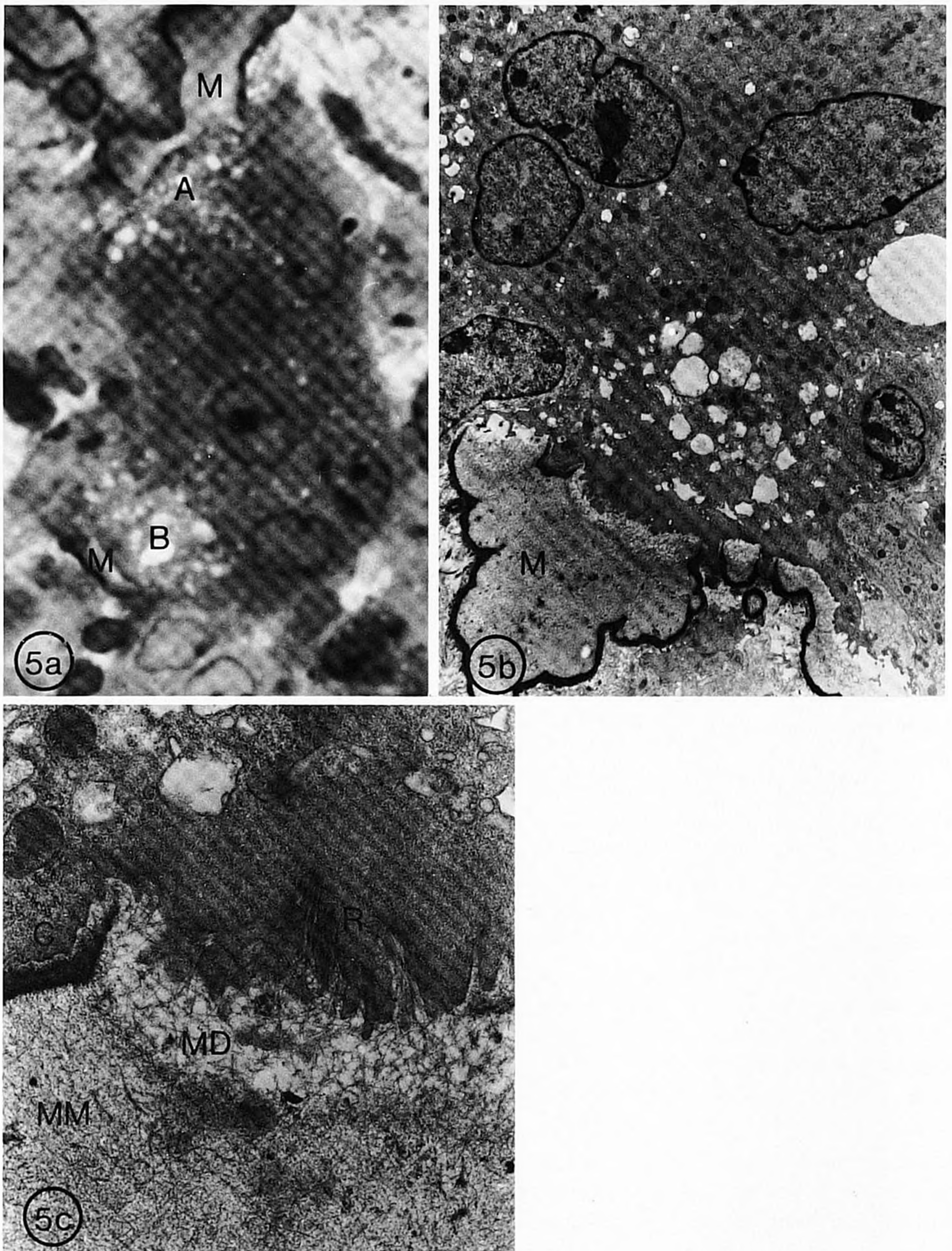


FIG. 5. Light micrograph (a) and low power (b) and high power (c) electron micrographs of the same osteoclast in a stripped bone rudiment co-cultured with precultured bone marrow cells. (a) Matrix resorption can be observed at two separate sites, A and B (semithin section; $\times 1,500$). M, mineralized matrix. (b) Detail of site B (ultrathin section; $\times 2,700$). The dark-staining cytoplasm contains many small mitochondria and many vacuoles. M, mineralized matrix. (c) Detail of the site of matrix resorption (ultrathin section; $\times 16,000$). Ruffling membranes (R) are present at the site of mineral degradation. The cell is attached to the mineral along a clear zone (c). MM, mineralized matrix; MD, matrix in the process of degradation.

TABLE III

Occurrence of Osteoclasts in Stripped Bone Rudiments Co-Cultured with Blood Leukocytes or Macrophages

| | Numbers of osteoclasts per bone rudiments* | | | | | (n) | P |
|---|--|-----|------|-------|-----|------|-----|
| | 0 | 1-3 | 4-10 | 11-20 | >20 | | |
| Control cultures | 17 | 3 | 0 | 0 | 0 | (20) | |
| Plus blood leukocytes | 15 | 0 | 1 | 0 | 0 | (16) | NS‡ |
| Plus resident macrophages | 18 | 3 | 0 | 0 | 0 | (21) | NS‡ |
| Plus exudate macrophages 1.5 d NBCS | 10 | 0 | 0 | 0 | 0 | (10) | NS† |
| Plus exudate macrophages 4 d NBCS | 9 | 1 | 0 | 0 | 0 | (10) | NS‡ |
| Plus exudate macrophages 4 d thioglycollate | 9 | 0 | 0 | 0 | 0 | (9) | NS‡ |

* Counts made in serial histological sections.

‡ Compared with control cultures, Wilcoxon's rank sum tests. NS, not significant.

TABLE IV

*Release of ⁴⁵Ca from Prelabeled Stripped Bone Rudiments in Culture**

| | Percent ⁴⁵ Ca released per rudiment | | | P |
|---|--|-------------|------|---------|
| | Day 2-4‡ | Day 5-7 | (n) | |
| Control cultures | 2.5 ± 0.8 | 1.5 ± 1.0 | (12) | |
| Co-cultured plus 8 d bone marrow cultures | 2.9 ± 0.6 | 18.5 ± 15.1 | (11) | <0.001§ |
| Plus exudate macrophages 4 d NBCS | 3.6 ± 1.3 | 2.4 ± 1.8 | (12) | NS§ |

* Values are means ± SD.

‡ Medium of day 1 was not considered, as it contained mainly ⁴⁵Ca released by physicochemical exchange.

§ Compared with control cultures; Wilcoxon's rank sum test. NS, not significant.

means of a ruffled border and vacuolar complex. Furthermore, the results of the studies on the release of ⁴⁵Ca show that these cells have bone-resorbing activity. Together, these findings support the conclusion that the multinucleated giant cells formed in vitro are identical to naturally occurring osteoclasts.

In vivo studies on the formation of osteoclasts from bone marrow and spleen cells (2-4) have proven that osteoclasts derive from migrating cells that invade the bone tissue and form osteoclasts on the surface of the mineralized matrix. Our chimera studies with mouse embryonic bone rudiments and quail embryos (7, 23) have shown that both in vivo, after transplantation on quail chorioallantoic membrane, and in vitro, after co-culture with quail spleen tissue, stripped bone rudiments are only invaded by osteoclasts of quail origin, that is, from an extraneous source. In the present experiments osteoclasts developed from cells of co-cultured embryonic liver tissue or bone marrow cells which invaded the stripped bone tissue. Moreover, in preliminary experiments, 8-d-precultured bone marrow cells were labeled with [³H]-TdR (0.1 μCi/mmol) for 24 h and after a thorough washing procedure co-cultured with stripped bone rudiments. Autoradiography revealed that ~50% of the precultured bone marrow mononuclear phagocytes used for the co-culture experiments and 75% of the osteoclast nuclei in the co-cultured bones were labeled.

With cytochemical and functional markers, it has been demonstrated that liquid cultures from murine bone marrow, cultured in the presence of embryonic mouse fibroblast CM, contain only immature and mature mononuclear phagocytes and granulocytic cells after 8 d of culture (18); in cultures incubated for 14 d only

mononuclear phagocytes are present (18). Multipotential stem cells are absent from bone marrow cultures older than 7 d, as shown by a spleen-colony assay (J. W. M. van der Meer, J. te Velde, J. S. van de Gevel, and R. van Furth, unpublished observations). Taken together, these findings indicate that the osteoclasts were formed by fusion of mononuclear phagocytes. However, no osteoclasts developed from peripheral blood monocytes and resident or exudate macrophages, which represent more mature, nondividing end-stages of the mononuclear phagocyte cell line. Moreover, the capacity to form osteoclasts was absent in the strongly adherent, mature cell population of cultured bone marrow, and was lost from bone marrow cultures, which had been irradiated to destroy the proliferating immature cells. These results point strongly to the proliferating, immature mononuclear phagocytes (monoblasts or promonocytes) as the cells that can be induced to form osteoclasts.

Whether the osteoclast precursor cells represent a subpopulation within the mononuclear phagocyte series remains to be established. It is also still possible that osteoclasts are derived from a separate subpopulation of bone marrow cells not belonging to the mononuclear phagocyte series. Although this possibility cannot be completely ruled out, we think it is unlikely in view of the characterization studies performed in these bone marrow cultures (18).

Failure of monocytes and macrophages to form osteoclasts *in vitro* has also been reported by others (11–13), but in those studies the cells were co-cultured with devitalized bone particles, which means that local changes resulting from the devitalization of the bone tissue might have interfered with the formation of osteoclasts. In the present study this can be ruled out, because the bone tissue itself was vital throughout the culture period. The mechanism that triggers the formation of osteoclasts is not known, but it seems certain that calcified tissue plays an important role, because osteoclasts developed only in immediate contact with calcified bone tissue and were never found among the bone marrow cells outside the bone shaft.

Summary

The origin of osteoclasts was studied in an *in vitro* model using organ cultures of periosteum-free embryonic mouse long-bone primordia, which were co-cultured with various cell populations. The bone rudiments were freed of their periosteum-perichondrium by collagenase treatment in a stage before cartilage erosion and osteoclast formation, and co-cultured for 7 d with either embryonic liver or mononuclear phagocytes from various sources.

Light and electron microscopic examination of the cultures showed that mineralized matrix-resorbing osteoclasts developed only in bones co-cultured with embryonic liver or with cultured bone marrow mononuclear phagocytes but not when co-cultured with blood monocytes or resident or exudate peritoneal macrophages. Osteoclasts developed from the weakly adherent, but not from the strongly adherent cells of bone marrow cultures, whereas 1,000 rad irradiation destroyed the capacity of such cultures to form osteoclasts. In bone cultures to which no other cells were added, osteoclasts were virtually absent.

Bone-resorbing activity of *in vitro* formed osteoclasts was demonstrated by ^{45}Ca release studies. These studies demonstrate that osteoclasts develop from cells present in cultures of proliferating mononuclear phagocytes and that, at least in our system, monocytes and macrophages are unable to form osteoclasts. The most likely candidates

for osteoclast precursor cells seem to be monoblasts and promonocytes.

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