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BIODEGRADATION OF SYNTHETIC BIPHASIC CALCIUM PHOSPHATE AND BIOLOGICAL CALCIFIED SUBSTRATUM BY CELLS OF HEMOPOIETIC ORIGIN

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Abstract

Different types of osteoclastic cells (authentic osteoclasts from human giant cell tumor and bone marrow of newborn rats; newly-formed osteoclasts from adult rat bone marrow), giant multinucleated cells and macrophages were studied for their effect on synthetic and natural mineralized substrata. Biphasic calcium phosphate ceramic consisted of hydroxyapatite and beta tricalcium phosphate was chosen for *in vitro* experiments, and dentine served as a positive control for cell-resorbing activity. Our results show the limited capacity of authentic and newly-formed osteoclasts to resorb synthetic ceramic as compared to that of natural substrata. *In vitro* cell-mediated biodegradation included also modifications of the synthetic substratum surface caused presumably by phagocytosis of the material.

Key Words: Biphasic calcium phosphate ceramic, cell culture methods, osteoclasts, multinucleated giant cells, macrophages, biodegradation, scanning electron microscopy, transmission electron microscopy.

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Introduction

Theoretical assumptions about the use of calcium phosphate ceramics in surgical practice are based on data made available during the last two decades. These ceramics are non-immunogenic, biodegradable, biocompatible and osteoconductive. After *in vivo* implantation, bioceramics are subjected to processes of partial dissolution and precipitation (Daculsi *et al.*, 1990a) as well as cell degradation of the material. The latter process could be mediated by two main cell mechanisms: phagocytosis by macrophage/fibroblast cells (Kwong *et al.*, 1989; Kawaguchi *et al.*, 1992) and resorption by osteoclast (osteoclast-like) cells (Jones *et al.*, 1984; Baslé *et al.*, 1993; de Bruijn *et al.*, 1993). However, both *in vivo* and *in vitro* data about the mode of action of different cells of hemopoietic origin (monocytes, macrophages, osteoclast-like cells) are sometimes controversial (for details, see **Discussion**). In the present study, we investigated the effect of different types of osteoclastic cells, giant multinucleated cells, and macrophages on synthetic calcium phosphate substratum and dentine (used as a positive control for cell-resorption activity).

Biphasic calcium phosphate (BCP) ceramic composed of hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) was chosen for these investigations. This material has been applied successfully in human surgery (Passuti *et al.*, 1989; Daculsi *et al.*, 1990b, 1992) and has certain advantages compared to monophasic ceramics (Nery *et al.*, 1992). The biphasic nature of the material is conducive to equilibration of ceramic dissolution after *in vivo* implantation (Daculsi *et al.*, 1989). To our knowledge, this material has not been used for *in vitro* co-culture studies with cells responsible for biodegradation.

Materials and Methods

Characterization of materials used as substrata for cells

The BCP used in this study (Triosite, Zimmer, Rungis, France) consisted of HA and β-TCP in 60:40 proportions as determined by X-ray diffraction. BCP

granules 40 to 80 μm in size were selected. Ten mm diameter and 1 mm thick disks were produced by compacting 200 mg of preselected granules at 130 MPa for 20 seconds (Specac, Kent, England). Disks were sterilized by heating at 180°C for 2 hours. All ceramic samples were preincubated in 24-well plates (Greiner OSI, Paris, France) for 18 hours in alpha minimum essentail medium (α-MEM) with antibiotics (37°C, 5% CO_2 in atmosphere) before cell seeding.

Transverse sperm whale dentine slices (about 100 μm thick) were cut with a water-cooled diamond saw (Isomet, Buehler, Lake Bluff, IL, USA), stored in a 70° ethanol/water solution and then washed three times in phosphate buffer solution and culture medium before culturing. Preliminary preincubation of dentine slices in α-MEM showed neither precipitation nor erosion; therefore, preincubation procedure was judged unnecessary for this experiment.

In some experiments, cells were seeded on Thermanox® coverslips (Nunc, Roskilde, Denmark).

All cultures were performed in 24-well plates containing the investigating substrata.

Animals

Wistar rats (8 to 10 weeks old or newborn animals 4 days old) were used for experiments after anesthetization and cervical dislocation.

Isolation and culturing of newborn rat's osteoclasts

The system used to study osteoclastic resorption *in vitro* is similar to that described by Chambers *et al.* (1984). Long bones were removed from 4-day-old rat pups. Bones were dissected free of adherent soft tissues and scrapped into HEPES-buffered (Flow ICN, Puteaux, France) MEM (Gibco BBL, Eragny, France) supplemented with an antibiotic mixture (penicillin-streptomycin solution, Gibco). The suspension was stirred with a pipette, and the larger fragments were allowed to settle for 1-2 minutes. Cells were aspirated through a 26-G needle, centrifuged and dissolved in a small volume of complete culture medium consisting of α-MEM (Gibco), 15% fetal calf serum (FCS, Dominique Dutscher SA, Brumath, France), 2 mM L-glutamine (Flow ICN) and 1% antibiotic mixture (subsequently designated as medium A). Cells in 20-μl aliquots were dropped onto the surface of substrata (1 x 10^6/well). After 10 minute incubation at room temperature, 1 ml of complete culture medium A was gently added to each well to avoid detachment of cells from the substrata, and plates were then incubated for an additional 30 minutes (37°C, 5% CO_2 in atmosphere). After this short-term incubation, all of the medium was replaced with fresh medium, and the cultures were incubated for an additional 3 days (37°C, 5% CO_2 in atmosphere, 90% relative humidity: standard incubation conditions), with replacement of medium on the second day of culturing.

Culturing of suspension fraction cells from rat Dexter-type long-term bone marrow cultures (LTBMC)

Cultures were performed as described previously (Gan *et al.*, 1994). Briefly, adult rat bone marrow cells were seeded according to the Dexter method (Dexter *et al.*, 1977) into 25 cm^2 culture flasks (Nunc) (15 x 10^6 cells/flask) in 10 ml of complete medium consisting of α-MEM, 1% antibiotic mixture, 2 mM L-glutamine, 10^{-6} M hydrocortisone sodium hemisuccinate (Sigma), 7% FCS (Gibco) and 13% horse serum (D.A.P., Brumath, France). Flasks were incubated at 33°C, with weekly replacement of half of the complete culture medium.

Suspension fraction cells from 2-week cultures were counted, centrifuged, examined for cell morphology and placed on ceramic substratum (10^5 cells/well) in culture medium consisting of α-MEM, L-glutamine, 10% FCS and antibiotic mixture for an additional 3-4 days in standard incubation conditions.

Culturing of adult rat bone marrow cells

Bone marrow cells were obtained and processed as described elsewhere (Ogura *et al.*, 1991; Gan *et al*, 1994). One million cells in 20-μl aliquots were deposited on the surface of each substratum for 10 minutes before addition of culture medium A.

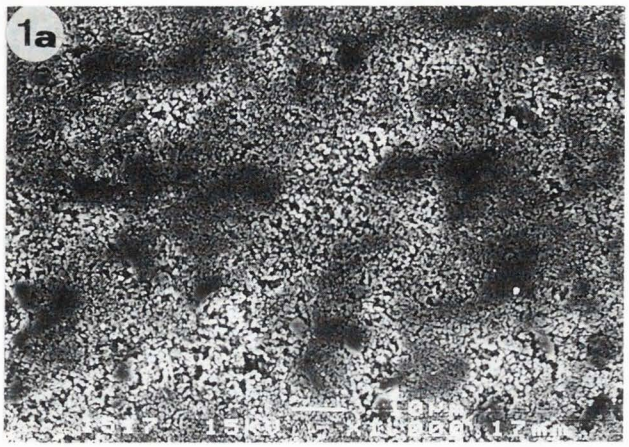
In some cultures 1,25-dihydroxyvitamin D3 (VD3) was added at a concentration of 10^{-7} M. Cultures were incubated 21 days in standard incubation conditions, and half of the medium was replaced every 3 days.

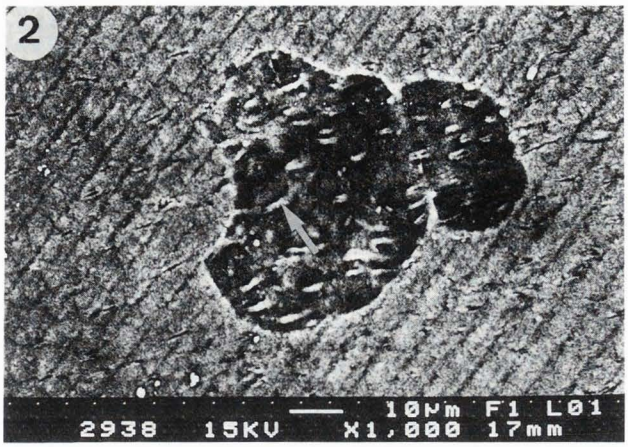
Cell culture of human giant cell tumor of bone

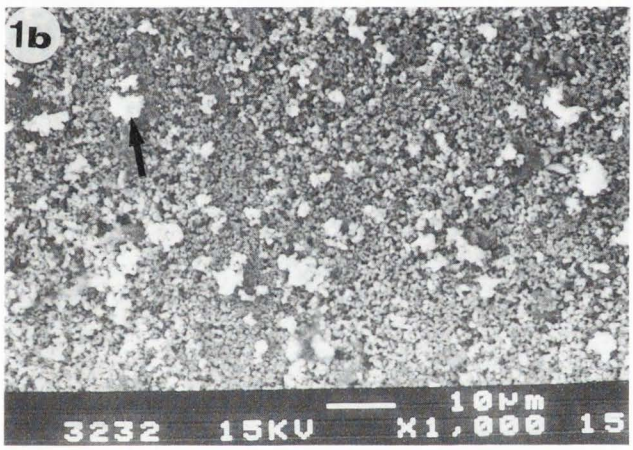
Tumor specimens were surgically obtained from a patient with a giant cell tumor in **ischium** and stored for 1 hour in sterile α-MEM supplemented with antibiotics and heparin (50 U/ml). Necrotic and hemorrhagic areas were removed before tissue dissection. Three subsequent trypsinization procedures were performed with a mixture of trypsin 0.05%, ethylenediaminetetraacetic acid (EDTA) 0.02% in special salt solution (Flow INC), and the resulting cell suspensions were then washed. Cells were processed, as described above for osteoclasts from newborn rats, and cultured in medium A in standard incubation conditions for 2 days.

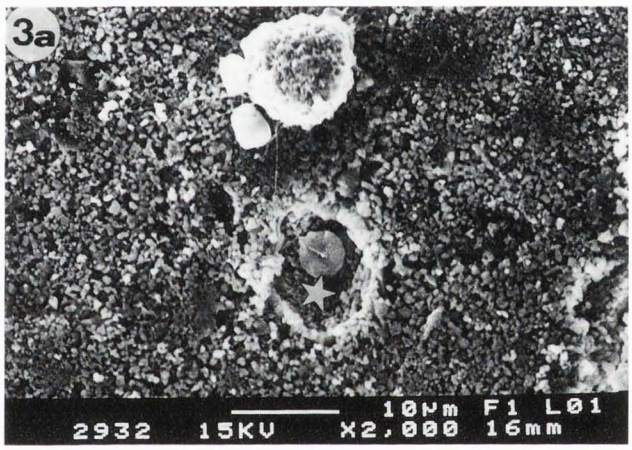
Treatment of substrata for morphological observations

Biphasic calcium phosphate (BCP) After culture termination, BCP disks were processed for scanning electron microscopy (SEM) cell morphology investigations. Briefly, BCP disks were placed in 4% glutaraldehyde in PBS (1 hour, 4°C) and postfixed with 1% osmium tetroxide (1 hour, room temperature). Samples were









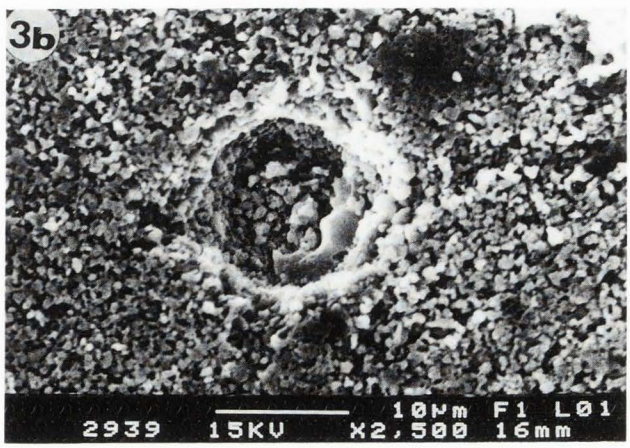


Figure 1. Scanning electron micrographs of BCP disk surface. (a) Disk before incubation; (b) Disk incubated for 3 days in culture medium without cells. Treatment with hydrazine. Note the presence of precipitated particles (arrow) and the absence of any erosions.

Figure 2. Scanning electron micrograph of a multilobular resorption lacuna on dentine slice co-cultured with bone marrow of newborn rats. Note the relative resistance of peritubular dentine (arrow) to resorption.

Figure 3. Scanning electron micrographs of a BCP disk co-cultured with bone marrow of newborn rats. (a) Critical point-dried preparation. Lacuna-like structure (asterisk) close to a small-sized cell. (b) Treatment with hydrazine. Small, bright-border erosion. Note the similar morphological features inside lacuna and on the original disk surface.

dehydrated through a graded ethanol series, critical point dried and sputter-coated with gold-palladium.

For investigation of the surface state of BCP disks, cells were eliminated from the surface. Disks were incubated for 15 minutes at 55°C with hydroxyl-hydrazine, followed by absolute ethanol treatment (1 hour,

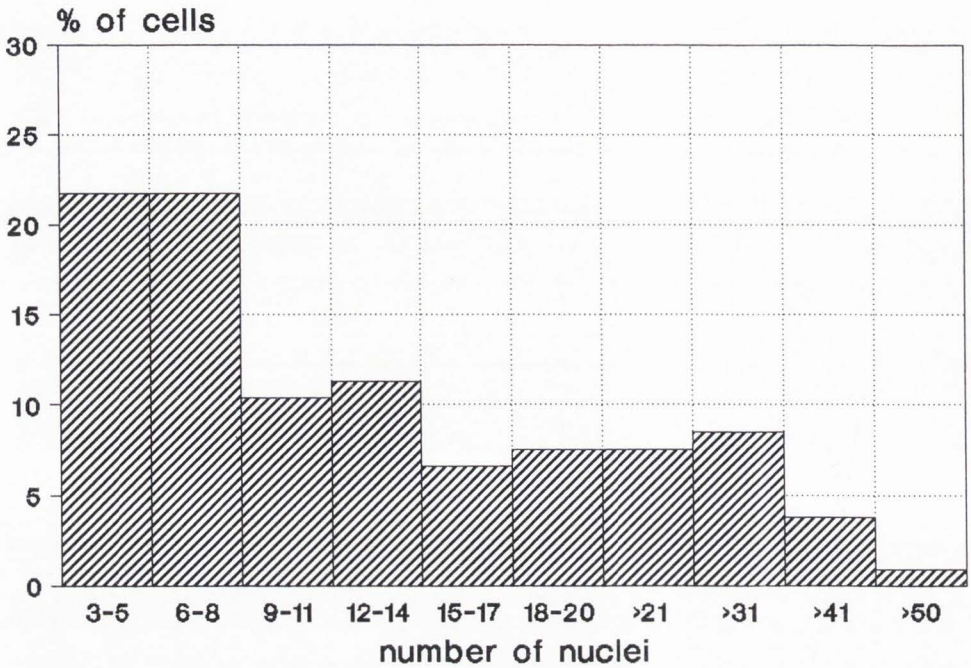


Figure 4. Distribution pattern of nuclei number per multinucleated cell. Human giant cell tumor cultured for 2 days on Thermanox coverslips.

room temperature), drying and preparation for SEM observations by gold-palladium coating. This treatment resulted in minimal damage to the surface morphology of BCP disks (see **Results**).

Dentine For SEM cell morphology investigations, dentine samples were processed similarly to BCP disks.

To examine resorption lacuna features, dentine slices were placed in distilled water, ultrasonicated for 1-2 minutes, dried and prepared for SEM by gold-palladium coating.

Thermanox coverslips Cell morphology was investigated on Thermanox coverslips by routine light-microscopy methods (May-Grünwald-Giemsa staining) and SEM (using a procedure similar to that for BCP disks and dentine slices).

Cells on coverslips were also studied for their tartrate-resistant acid phosphatase (TRAP) activity using the Sigma kit (Cat. No. 387-A, Sigma, St. Louis, MO, USA) for leukocyte acid phosphatase.

The cells containing three or more nuclei were counted as multinucleated cells.

SEM observations were performed by means of secondary and backscattered electrons (BSE) in a JEOL 6300 SEM operated at an accelerating voltage of 15 kV.

Results

Physicochemical features

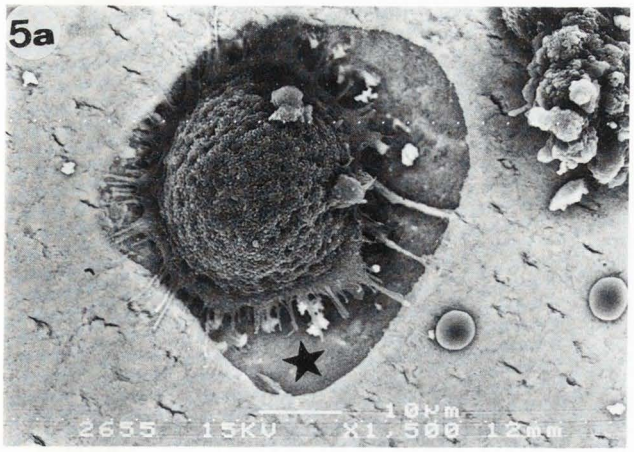
SEM observations of the BCP disk surface before incubation showed individual separate grains and micropores (Fig. 1a). After 18 hours of preincubation in culture medium and during the culturing, precipitation has occurred on the disk surface. The number of precipitated particles and their distribution pattern were irregular (random) (Fig. 1b). This process of precipitation occurred the first day of BCP disks incubation in medium without cells and did not change noticeably in course of following incubation. We have observed 3-weeks-incubated disks with similar pattern of precipitation as in 1- and 3-day-incubated disks (data not shown).

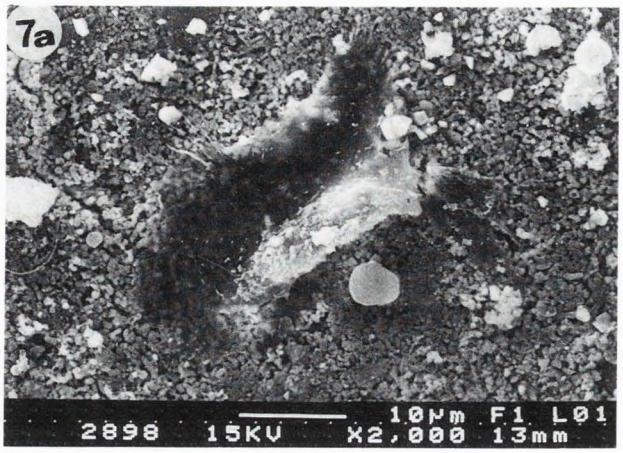
Dissolution/precipitation processes during preincubation of BCP disks increased medium pH after preincubation from 7.4 to 7.8. For all culture types, preincubated samples (with subsequent change of culture medium) were used to decrease medium alkalinity.

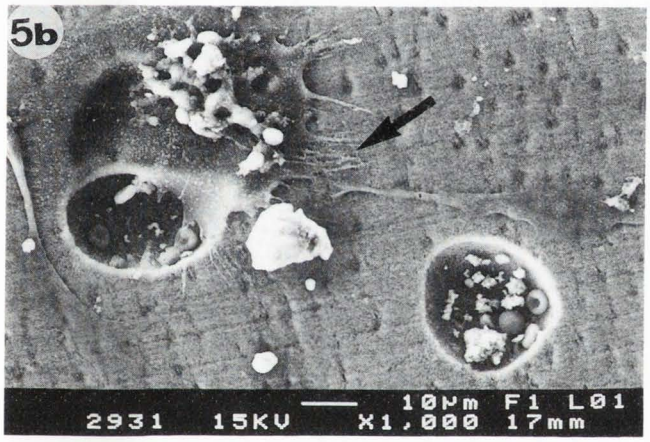
Co-cultures of BCP with osteoclastic cells

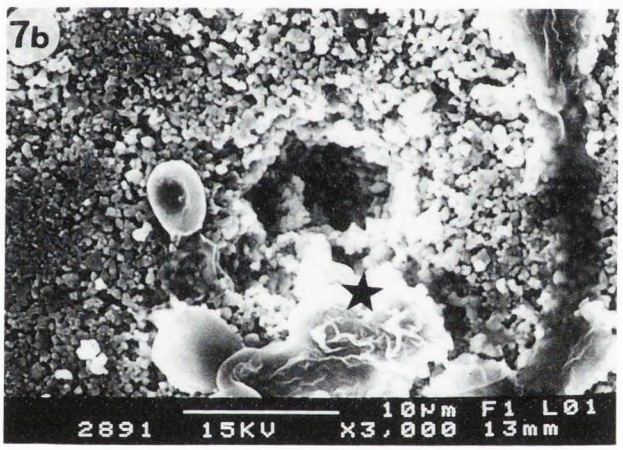
Osteoclasts isolated from long bones of newborn rats Bone marrow cells from 4-day-old rats were seeded in plates containing Thermanox coverslips, dentine slices and BCP disks since it is well-known that intensive bone remodeling occurs during this period of rat ontogeny. Two-day culturing of the cells on Thermanox coverslips followed by TRAP staining revealed the presence of some (mostly mononucleated) TRAP-positive cells. About 7% of all TRAP-positive cells were multinucleated containing less than 10 nuclei per cell.

The osteoclastic activity of the cells was verified by the appearance of resorption pits on dentine slices (Fig. 2). The size of resorption lacunae was usually about 10-30 μm. Most pits were round, but multilobular pits were occasionally observed. The relative resistance of highly mineralized peritubular dentine to resorption was noteworthy compared to less mineralized zones.









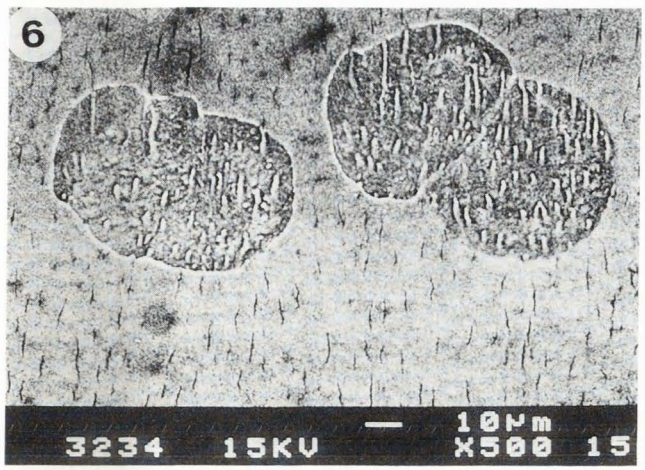
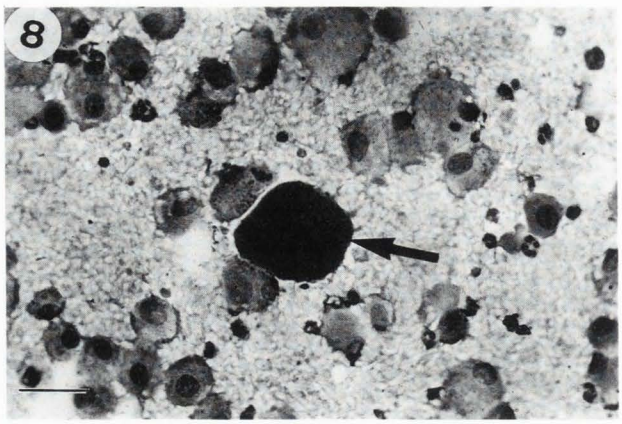


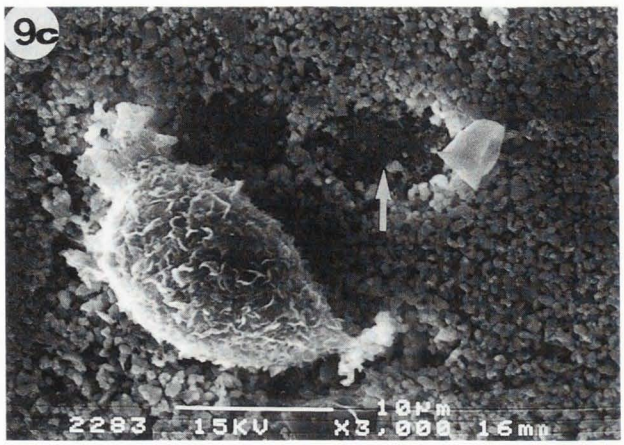
Figure 5. Backscattered scanning electron micrographs of a dentine slice co-cultured with human giant cell tumor cells. (**a**) "Dome"-shaped osteoclast inside resorption lacuna (asterisk). (**b**) Resorption lacunae and a flattened osteoclast with filopodia (arrow).

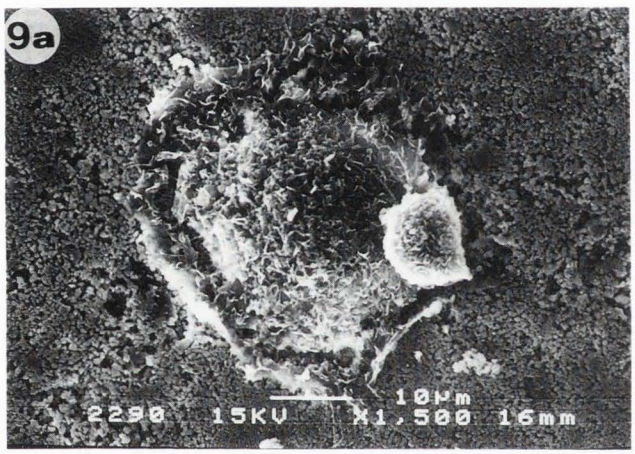
Figure 6. Scanning electron micrograph of a dentine slice co-cultured with human giant cell tumor cells. Treatment by ultrasonication. Simple and complex resorption lacunae.

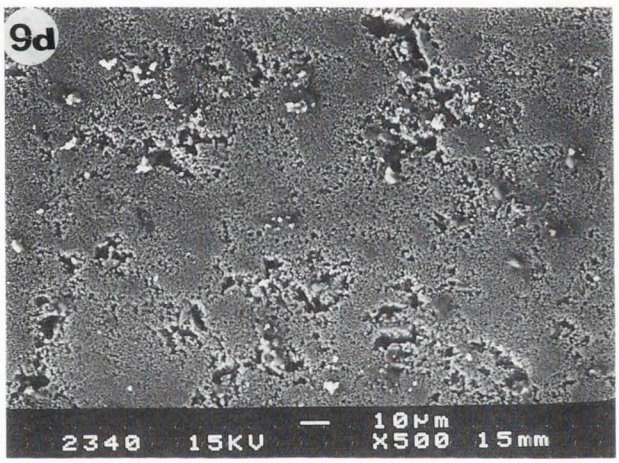
Figure 7. Scanning electron micrographs of a BCP disk co-cultured with human giant cell tumor cells. (**a**) Critical point dried preparation. Flattened osteoclast. (**b**) Treatment with hydrazine. Note the presence of a small lacuna-like structure close to the remnants of the cell (asterisk).

On the BCP surface, some small (about 10 μm), circular, well-delimited lacuna-like structures were observed close to small round cells (Fig. 3a). The inner surface of these pits displayed the same morphological features as the original substratum surface, except that









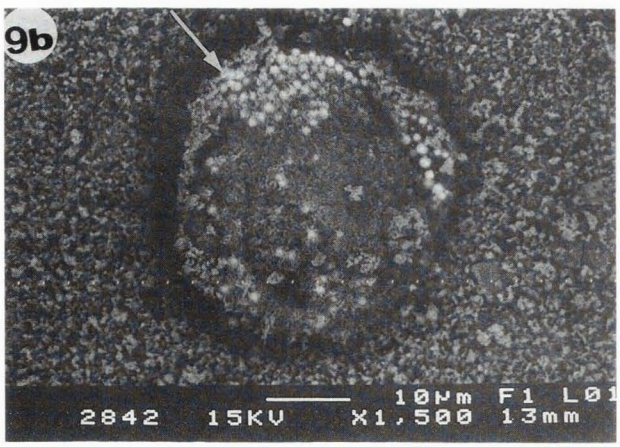


Figure 8. Light microscopy observation of a cytocentrifuge smear prepared from a suspension fraction of 2-week rat long-term bone marrow culture. TRAP staining. Note the presence of a TRAP-positive multinucleated cell (arrow) and TRAP-negative macrophages. Bar = 50 μm.

Figure 9. BCP disks co-cultured with cells from a suspension fraction of 2-week rat long-term bone marrow culture. (**a**) Scanning electron micrograph of a giant cell with numerous microvilli. (**b**) Backscattered electron micrograph of a giant cell with abundant osmiophilic inclusions (arrow). (**c**) Scanning electron micrograph of a macrophage close to two erosion areas (arrow) on the BCP surface. (**d**) Treatment with hydrazine. Scanning electron micrograph of irregular shallow erosions on the disk surface. (a), (b), and (c) are critical point-dried preparations.

the border was brighter (secondary electron observations). The number of lacuna-like structures observed on the BCP surface after cell elimination (Fig. 3b) was less than the number of the resorption pits on dentine (semi-quantitative analysis per field of observation).

Moreover, they were systematically smaller than the pits produced by osteoclastic cells on dentine. Control BCP samples incubated in culture medium and treated with hydrazine failed to reveal similar surface modifications (Fig. 1b).

Osteoclasts from human giant cell tumor (GCT) Cells from GCT were seeded for 2 days in cultures containing investigated substrata. This culture period was chosen since preformed osteoclasts are known to produce resorption lacunae *in vitro* within 24 hours (Piper *et al.*, 1992).

TRAP staining of Thermanox coverslips containing cultured cells revealed the presence of multinucleated TRAP-positive cells (mean: 112 per Thermanox coverslip) as well as numerous TRAP-positive mononucleated cells. Practically no TRAP-negative multinucleated cells were observed. The distribution pattern of nuclei number (determined on routine May-Grünwald-Giemsa preparations) showed that most multinucleated cells (about 65%) contained fewer than 15 nuclei (Fig. 4).

SEM observations of dentine slices showed that fairly small cells as well as giant cells were implicated in the dentine resorption process. Morphologically, two types of giant cells were observed: a first type of round "dome"-shaped cells with microvilli, usually aggregated near the "body" of a cell lacking lamellipodia (Fig. 5a); and a second type of flattened cells with lobulated and folded pseudopodes in some areas of the cell periphery. The cell margin of the second type showed fine filopodia which were irregular in length and diameter. Giant cell polarization was sometimes characterized by broad pseudopodia at one pole and filopodia at the other (Fig. 5b).

The resorption pits produced by GCT osteoclasts on dentine surface showed the relative resistance of peritubular dentine, the feature similar to those caused by osteoclasts from neonatal bone marrow. The shape and size of the resorption pits produced by GCT osteoclasts were very variable but of three main types: circular and relatively deep lacunae of small diameter; larger and shallower elongated excavations; and complex, generally shallow, pits with irregular contours (Fig. 6).

SEM observations of GCT cultures on BCP showed a giant cell morphology similar to that on dentine (Fig. 7a). The resorption activity of cells on BCP (after cell elimination) was less evident than on dentine. Well-delimited, circular small-sized (10-15 μm) lacuna-like areas were observed (Fig. 7b), but giant/elongated/complex lacunae were never present. Some areas of irregular shallower erosions (evidently due to cell activity) were also observed on BCP surface.

Co-cultures of BCP with giant multinucleated cells and macrophages, pre-formed in primary LTBMC

After two weeks of rat bone marrow culturing in conditions of "Dexter"-type long-term bone marrow culture, multinucleated cells appeared in suspension fraction of these cultures. There were 3-5% multinucleated cells, with similar proportions of TRAP-positive and TRAP-negative cells (Fig. 8). Other cells in suspension were macrophages.

These cells were seeded on BCP surface for four days to compare the material degradation pattern with that produced by preformed "authentic" osteoclastic cells.

SEM observations showed numerous cells of hemopoietic origin dispersed over the surface of BCP disks. Although medium-sized cells (15-20 μm) were predominant, some giant cells (more than 40 μm in diameter) were observed (Fig. 9a). These cells usually contained numerous osmiophilic inclusions preferentially distributed on the periphery of the cytoplasm (Fig. 9b). Our attempts to perform microanalysis to estimate the mineral content of inclusions in cells on BCP disks failed due to the strong signal from the surrounding Ca environment. Some erosions of BCP surface were observed in the proximity of hemopoietic cells (Fig. 9c). The shape of these areas was predominantly elliptical, and they were smaller than the cells involved.

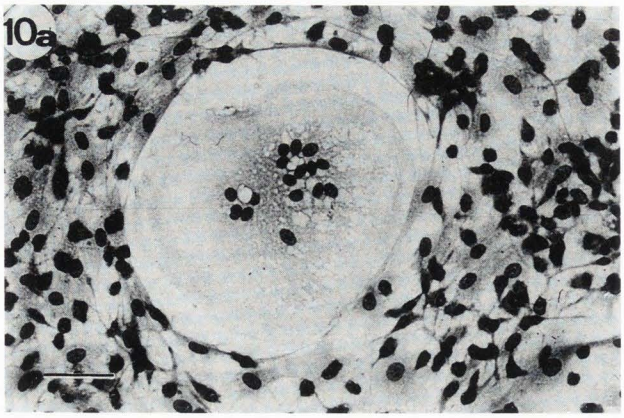
After elimination of cells from the BCP surface by hydrazine treatment, different areas showed a more or less clearly delimited eroded surface with various patterns and the extent of material degradation, similar to the features observed for GCT cultures. No lacuna-like structures were noted (Fig. 9d).

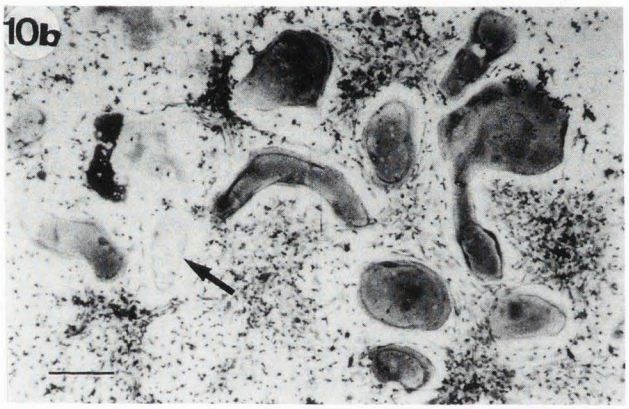
Long-term co-culturing of BCP with adult rat bone marrow cells

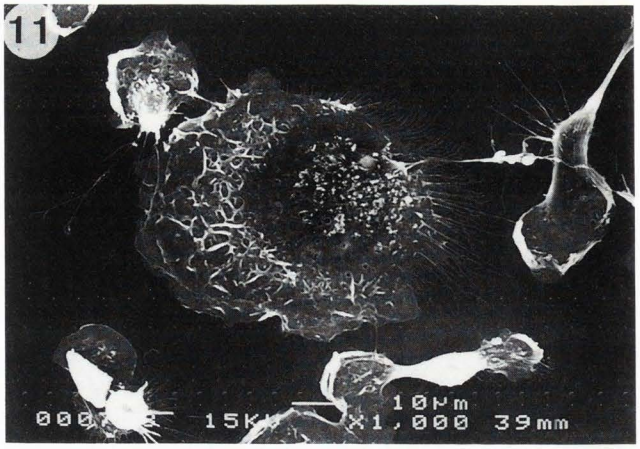
Long-term culturing of rat bone marrow cells was performed to determine the cell behaviour of residual osteoclasts, and mainly, of newly-forming cells with potential osteoclastic activity on different substrata.

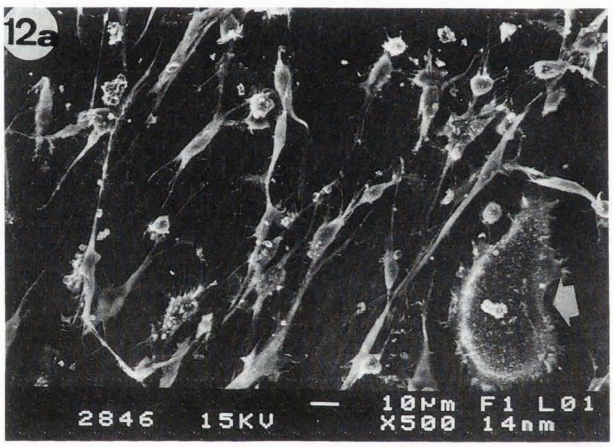
Culturing of rat bone marrow cells on Thermanox coverslips led to the appearance of a few multinucleated cells at day 7 of incubation and an increase in their number at day 12. These cells contained a variable number of nuclei (up to 92; mean = 15.5) distributed in the central or peripheral part of the cell (Fig. 10a). TRAP staining showed mononucleated and multinucleated positive cells (Fig. 10b).

The addition of vitamin D3 produced cultures with a constant and predictable amount of multinucleated cells. In cultures seeded with 1 x 10^6 cells/well their number increased about 30-fold (30-62 per well on day 12) compared to control (without VD3) cultures. Most giant cells were TRAP-negative. The percentage of multinucleated TRAP-positive cells ranged from 8 to 36%, and TRAP-positive mononucleated cells were also observed.









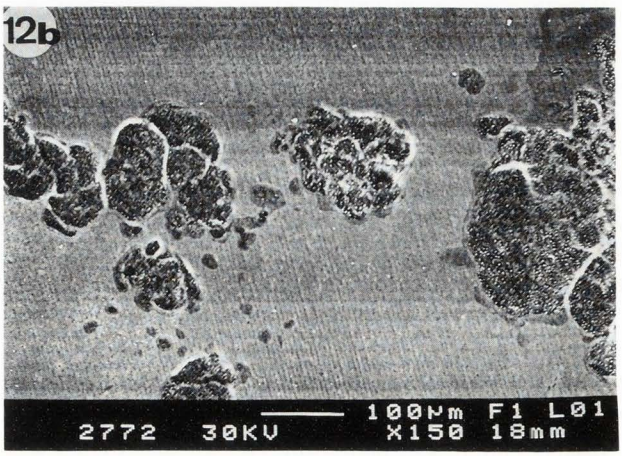
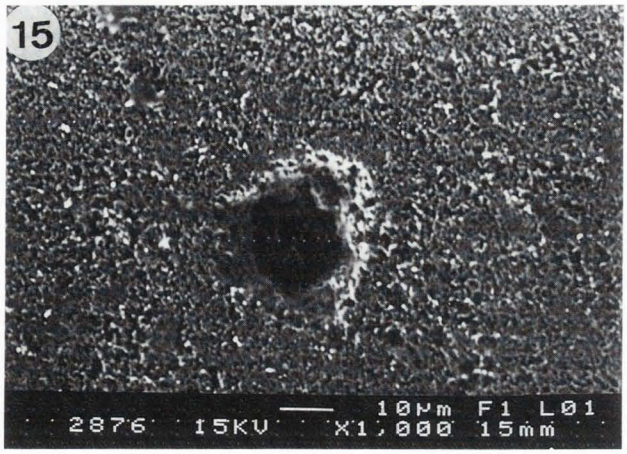


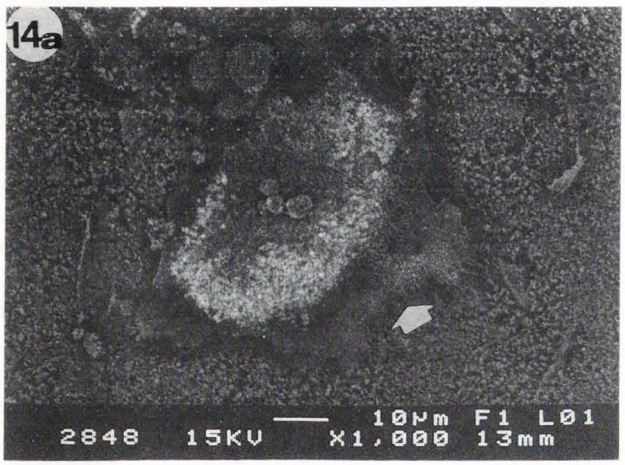
Figure 10. Light microscopy observation of a multinucleated giant cell in adult rat bone-marrow culture on a Thermanox coverslip. (**a**) May-Grünwald-Giemsa staining. Bar = 100 μm. (**b**) TRAP staining. Note the presence of TRAP-positive and TRAP-negative (arrow) cells. Bar = 50 μm.

Figure 11. Scanning electron micrograph of a giant cell. Adult rat bone marrow culture on a Thermanox coverslip. Note the presence of numerous microvilli on the cell surface and filopodia at one cell pole.

Figure 12. Scanning electron micrograph of a dentine slice co-cultured with adult rat bone-marrow cells. (**a**) Critical point-dried preparation. Giant cell (arrow), hemopoietic cells and orientated stromal fibroblast-like cells at day 12. (**b**) Day 21 of culturing in the presence of VD3. Treatment by ultrasonication. Complex and simple resorption lacunae.

SEM observations of Thermanox coverslips showed the presence of fibroblast-like cells as well as small and giant cells of hemopoietic origin. Some giant cells were polarized with filopodia at one pole and exhibited microvilli on the surface (Fig. 11).





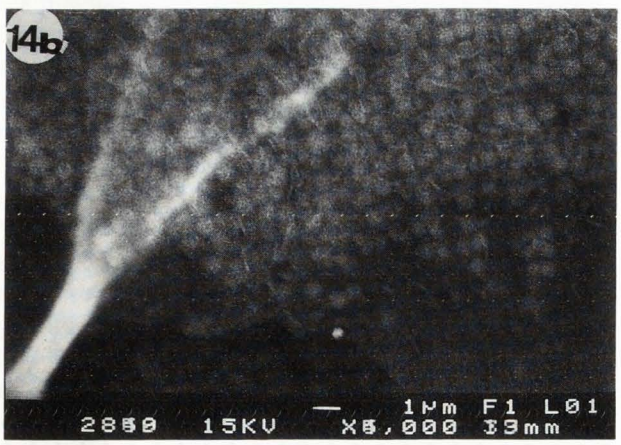


Figure 13. Scanning electron micrograph of a BCP disk co-cultured with adult rat bone marrow cells (day 2). A small, round, "dome"-shaped cell near a substratum excavation.

Figure 14. Backscattered electron micrograph of a BCP disk co-cultured with adult rat bone marrow cells for 12 days. (**a**) Giant cell with abundant osmiophilic inclusions and filopodia at one cell pole (arrow). (**b**) Part of a giant cell with osmiophilic inclusions on the periphery.

Figure 15. Scanning electron micrograph of a BCP disk co-cultured with adult rat bone marrow cells in the presence of VD3 for 12 days. Treatment with hydrazine. Lacuna-like erosion on the substratum surface.

SEM observations of dentine slices at various time-points during culture revealed different types of fibroblast-like and hemopoietic cells. At day 12, giant cells appeared. Their surface was "rough," whereas fibroblast-like cells were flattened with a smooth surface and were orientated preferentially (Fig. 12a). The osteoclastic nature of multinucleated cells grown in the presence of VD3 was proven by the following observations: multinuclearity, their TRAP-positivity, and numerous resorption lacunae observed on the surface of dentine on day 21. Resorption pits differed in size, appearance and depth (Fig. 12b), being similar to those produced by GCT cells at day 2 of culture. Some were much larger, and multilobular lacunae were also noted.

As on dentine slices, hemopoietic and fibroblast-like cell growth was observed in bone marrow cultures on BCP disks. No preferential orientation of fibroblastoid cells was seen. At day 2 of incubation, small, round "dome"-shaped cells with lamellipodia at one pole were observed near substratum excavations (Fig. 13). Most giant cells apparent at day 12 of culture contained a large number of cytoplasmic inclusions (1-2 μm in diameter) corresponding to osmiophilic material (Figs. 14a and 14b).

Elimination of cells from the surface of BCP disks at day 12 and 21 enabled us to observe some small lacuna-like erosions ($<$ 10 μm in diameter) (Fig. 15). Shallower extended and irregular alterations of the surface were much more typical.

Discussion

Calcium phosphate bioceramics are becoming more routine in surgical practice today. However, *in vivo* investigations to clarify the mechanisms of action and the origin of the cells participating in ceramic biodegradation/resorption processes have sometimes given contradictory results (Kawaguchi *et al.*, 1992; Baslé *et al.*, 1993; Dersot *et al.*, 1993). An understanding of the mechanisms of *in vivo* biodegradation could benefit from the use of an adequate *in vitro* model. In the last decade, several groups have introduced different types of culture systems to study events occurring during biodegradation of Ca-P synthetic materials (Jones *et al.*, 1984; Ogura *et al.*, 1991; Benahmed *et al.*, 1994). Although there is distinct *in vitro* evidence of osteoclast resorbing activity against dentine or bone (reviewed by Boyde and Jones, 1991), data about the ability of osteoclasts to resorb Ca-P ceramics are controversial (Jones *et al.*, 1984; Shimizu *et al.*, 1989; de Bruijn *et al.*, 1993; Gomi *et al.*, 1993a).

In our study, multinucleated cells from human giant cell tumor, osteoclasts from newborn bone marrow and newly-forming osteoclastic cells from adult bone marrow showed strong resorbing activity against dentine slices. Multinucleated cells from human giant cell tumor possess common features with authentic osteoclasts (Chambers *et al.*, 1985; Kanehisa *et al.*, 1991) and the resorbing activity is considered as the primary function of osteoclasts (Hattersly *et al.*, 1989). However, for more complete understanding of the resorption processes taking place in our culture systems, the enrichment of marrow cultures for osteoclast precursors by animals maintained on a calcium-deficient diet for 1 week (Prallet *et al.* 1992) would be helpful. Despite some variations in the size and shape of lacunae produced in these three types of cultures, a common feature was observed for all resorption pits: the relative resistance of peritubular dentine. Similar observations by Jones *et al.* (1984) showed that authentic mammalian and avian osteoclasts were unable to resorb highly mineralized areas of peritubular dentine.

The culturing of cells from GCT and newborn bone marrow on the surface of BCP disks allowed us to observe the osteoclastic cells with typical morphological features. Elimination of cells from disks revealed the presence of some small ($< 15\ \mu m$ in diameter) round lacuna-like structures, whereas multilobular pits were never observed. These data suggest the limited capacity of authentic osteoclastic cells to resorb biphasic calcium phosphate ceramic.

Why the osteoclasts, which are able to perform lacunae of resorption on natural substratum, do not absorb synthetic mineral material? Osteoclastic differentiation is very complex biological process depending on the presence of stromal (osteoblastic?) cells, cytokines, osteotropic hormones and substrata (bone matrix), it is possible that the synthetic substrata was unable to orientate the progenitors towards the osteoclastic phenotype. It is also conceivable that the absence of an organic component in cultures seeded with preformed osteoclasts reduces the resorbing capability of these cells. In this respect, the need for extracellular matrix components for osteoclast resorption activity has been demonstrated by Sasaki *et al.* (1989). Moreover, osteoclastic resorption of calcium phosphates has been observed in cultures with mineralized extracellular matrices previously produced by osteoblastic cells (de Bruijn *et al.*, 1993).

On thin layers of carbonated apatite precipitated on the surface of two different calcium phosphate substrata, the resorbing action of osteoclasts seemed absolutely identical to those on dentine (Yamada *et al.*, 1994). Erosions similar to lacunae were produced by osteoclast-like cells on submicron calcium-phosphate thin films (Davies *et al.*, 1993). It would appear that the thickness of resorbable synthetic substrata plays a role in the modulation of osteoclastic activity *in vitro*.

The physicochemical composition of synthetic substrata, i.e., the crystal structure, sintering time and temperature, and grain and inter-grain size governs osteoclastic resorption (de Bruijn *et al.*, 1993; Gomi *et al.*, 1993a; LeGeros, 1993). Another factor, which can modulate the pattern of osteoclastic action, is the concentration of calcium and phosphate ions: both systemic (in *vitro* conditions, in culture medium) and local. As a result of the dissolution of calcium-phosphate materials, the increasing concentrations of calcium and phosphate ions reduce osteoclast formation and decrease the activity of mature osteoclasts (Yates *et al.*, 1991). According to Zaidi *et al.* (1993), "elevated external concentration of Ca^{2+} in the mM range reduces bone resorption and results in motile changes in the cells." Due to the high content of calcium in BCP ceramics (and its dissolution, mainly at the expense of β-TCP; LeGeros, 1993), the action of osteoclasts could be altered soon after the beginning of the resorption process. A local change in the concentration of this key ion could cause partial detachment of osteoclastic cells from the substratum, with subsequent movement in thin Ca-P layer conditions or complete detachment (or arresting of the action) in thick BCP disk conditions.

The limited resorption of Ca-P synthetic materials can also be explained by an alteration in culture medium pH. Resorptive activity and the formation of an osteoclast clear zone have been stimulated in low pH conditions (Arnett and Dempster, 1986; Murrills *et al.*, 1993; Shibutani and Heersche, 1993). In the present study, due to the dissolution of the substratum, culture medium

pH tended to be slightly alkaline (at least at the beginning of culturing), and this alteration may have been responsible for limited osteoclast resorption.

We noted the limited efficiency of osteoclasts to resorb peritubular dentine because of its high mineral content. Not enough acidity of microenvironment produced by resorbing cells, their motility and resulting restricted action time could account for the failure of osteoclasts to dissolve highly-mineralized areas. The reduced number (and size) of lacuna-like structures produced by osteoclastic cells of different origin on BCP surface, as compared to those on dentine, could also be attributed to the high mineral content of synthetic material. The characteristics of the cells involved differed in the cultures used, varying in content of multinucleated cells, number of nuclei and TRAP activity. Despite the different nature of the osteoclastic cells (human GCT, newborn or adult bone marrow), lacuna-like erosions on BCP had common features. We cannot exclude the possibility that oligo-nuclear cells were mainly involved in the resorption of synthetic material and production of small-sized lacunae. The reasons for the restricted action of larger osteoclasts have been discussed above.

In vitro BCP degradation included not only the appearance of small lacuna-like structures but also shallower irregular surface modifications caused probably by phagocytosis of the material. Different cells were responsible for this effect, including macrophages and giant cells in which numerous osmiophilic inclusions (presumably phagolysosomes) were observed. The participation of TRAP-negative giant multinucleated cells in the phagocytosis of Ca-P material *in vivo* has been reported by Baslé *et al.* (1993).

The availability of culture techniques to elucidate the role of hemopoietic cells in biomaterial degradation would be useful for understanding *in vivo* events after ceramic implantation. The use of the "dentine" model in investigations of osteoclastic resorption is now fairly routine. However, additional experiments with secondary cultures of cells forming on BCP and re-seeded on dentine are necessary to prove osteoclastic activity of cells formed. Our future studies are being focussed on problems caused by the physicochemical "behaviour" of synthetic Ca-P materials in culture conditions. This require a greater consideration of such parameters as ion concentrations, pH variations and precipitations.

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Discussion with Reviewers

A. Nanci: Why were the dentin slices not preincubated for 18 hours, similarly to the ceramic specimens? Could this difference have an impact on the results?

Authors: As stated in the **Results** (under **Physicochemical features**): "Dissolution/precipitationprocesses during preincubation of BCP disks increased medium pH after pre-incubation from 7.4 to 7.8. For all culture types, preincubated samples (with subsequent change of culture medium) were used to decrease medium alkalinity." Dentine is not soluble (in culture medium) material and it has no effect on pH of the medium. Also, as mentioned in **Materials and Methods**, no precipitation (or erosions) have been noticed in preliminary experiments with dentine slices incubated for 24 hours. The preincubation procedure for dentine was not necessary and could not have an impact on the results.

A. Nanci: Why were the disks used in Figure 1 incubated for 3 days whereas for the various experiments the incubation time was only 18 hours?
Authors: We agree that for the presentation of our data, the placement of the photo of 3-day-incubated BCP disk (instead of 18-hour-incubated disk) is not correct. Our observations allowed us to see that precipitation occurred during the first day of BCP disks incubation in medium without cells did not change considerably in during the subsequent incubation. As stated in **Results**, we have observed 3-weeks-incubated disks with similar pattern of precipitation as in 1- and 3-day-incubated disks (data not shown).

A. Nanci: BCP should generate a fairly strong backscatter signal. Why is the cell signal stronger than the surrounding mineral phase?
Authors: We suppose that strong cellular, and especially, inclusions signal on BSE could be explained by high concentrations of cellular components possessing osmiophilia (membranes of cytoplasmic inclusions, presumably phagolysosomes). We cannot exclude also the additive effect of Os and Ca in inclusions containing (?) phagocyted Ca/P particles. Our attempts to perform microanalysis to estimate the mineral content of inclusions in cells on BCP disks failed due to the strong signal from the surrounding Ca environment.

J.L. Saffar: In **Results**, why were "preformed giant multinucleated cells and macrophages" not seeded on dentine surfaces for an accurate comparison with BCP as in the other experiments?
Authors: In the experiments, we used two different sources of genuine osteoclasts (GCT, rat newborn long bones) as well as adult bone marrow as a source of progenitors of newly-forming in cultures osteoclasts (in the presence of VD3). We investigated also the ability of giant multinucleated cells and macrophages produced in primary rat LTBMC to modify the surface of synthetic Ca/P substratum. We **agree** that for the better comparison with other culture systems, it would be helpful to use dentine model for the latter source of cells.

J.L. Saffer: Some groups assume that TRAP activity is not a reliable marker for osteoclast *in vitro* (Hattersley and Chambers, 1989; Modderman *et al.*, 1991). To identify the cells as mono- and multinucleated osteoclasts, several tests are required today. These include binding of ^{125}I-calcitonin to the cells or challenge with calcitonin, pits formation in calcified matrices, challenges with antibodies assumed to be specific of the osteoclast phenotype. The authors have to clarify whether their cells fitted in with these criteria.
Authors: The formation of osteoclasts (osteoclast-like cells) in bone marrow cultures supplemented with vitamin D3 is now well-known culture system applied for bone marrow cells of different mammalian species. In Hattersley and Chambers (1989), the authors state "... have found that multinucleate cells, including TRAP-positive multinucleate cells, develop in cultures without 1a, 25-$(OH)_2D_3$ in which there is no bone resorption" (page 1694). At the same time, they concluded that "The primary function of the osteoclast is the excavation of bone. We have found that this function is detectable in cultures of mouse bone marrow cells after 7 days in culture with 1a, 25-$(OH)_2D_3$"; ... and "the resorptive activity was due to the differentiation of resorptive cells from bone marrow precursors in culture" (page 1694). In Takahashi *et al.* (1988), the authors postulated that "...TRACP-positive multinucleated cells formed in response of osteotropic hormones (vitamin D3 or human PTH) in mouse marrow cultures satisfy most of the criteria of osteoclasts" (page 1373). For our experiments with rat bone marrow cultures supplemented with vitamin D3, we used following criteria to identify osteoclasts: multinuclearity, TRAP-positivity and ability to resorb dentine. As stated in the text "The osteoclastic nature of multinucleated cells grown in the presence of VD3 was proved by successive observations: numerous resorption lacunae were observed on the surface of dentine on day 21".

J.L. Saffer: If one accepts the cells differentiated on the dentine slices as osteoclasts (and I am prone to do so provided that more information is given) there is no evidence that the cells differentiated in contact with BCP are genuine osteoclasts. Indeed osteoclast differentiation on artificial material is a complex succession in events involving interactions of the precursor cells with both the osteoblasts and the bone matrix. It is very likely that the osteoclasts and the giant cells differentiated on artificial materials arise from common precursors, but there is no evidence today that the ceramic substrate can orientate these progenitors towards the osteoclastic phenotype. This point must be discussed.
Authors: Osteoclastic differentiation is a very complex biological process depending on the presence of stromal (osteoblastic?) cells, cytokines, osteotropic hormones and substrata. In our experiments, on dentine the osteoclastic nature of newly-formed cells was evident due to the production of the resorption lacuna, but on BCP we have observed giant cells able to modify substratum surface possibly due to phagocytic mechanism. We **agree** with Dr. Saffar that latter cells could not possess characteristics of similar cells formed in the presence of natural mineralized substratum (dentine). Additional experiments with secondary cultures of cells forming on BCP and re-seeded on dentine are necessary to prove osteo-

clastic activity of cells formed. We wish to emphasize, that neither genuine osteoclasts (from GCT or newborn long bones), nor newly-forming giant cells were able to produce lacuna similar to those on dentine.

A.M. Gatti: Is the superficial roughness equal or similar in the BCP and dentine samples?
Authors: Superficial roughness of dentine and BCP are evidently different and this physical characteristic has an important influence on the generation of cell and tissue responses to biomaterials. Gomi *et al.* (1993b, 1993c) have demonstrated this effect especially concerning the fusion of osteoclast mononuclear precursors and simulation of the differentiation to TRAP positive cells.

A.M. Gatti: In Figure 7b, there is a lacuna. The surface surrounding that is the original surface? It appears different from the surface of Figure 5b. Could this difference involve different behaviour in different types of cells?
Authors: We observed on dentine different sizes and shapes of lacunae (simple, multilobular, complex) with smooth and well delimited surrounding border. On the other hand, for the BCP we observed only small, simple and round lacuna-like structure with irregular surrounding border. Although cellular ability to resorb BCP is limited, we cannot exclude that irregular shape is due (in part) to the behaviour of compacted BCP which can be slightly disaggregated in medium; indeed, some small particles can be separated from the disk. We are currently working on sintered material to avoid that kind of inconvenience by increasing of physical cohesion of the disk.

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