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VARIATION IN THE SIZES OF RESORPTION LACUNAE MADE IN VITRO

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Abstract

The assessment of in vitro osteoclastic activity has, until recently, been dependent on the analysis of organ culture experiments. We have developed a single cell resorption assay so that the resorptive function of individual osteoclasts could be studied. This paper examines the biological variation in the sizes of resorption lacunae produced by bone cell cultures derived from neonate rats and rabbits, and prehatch or hatchling chicks. Cultures were run for 24h for all species; and in addition for 48h for rat, 9 or 12 hours for rabbit and 3-7 hours for chick. The numbers of the nuclei of osteoclasts seeded on to plastic were counted for all three species. SEM stereophotogrammetry was used to measure areas, volumes, and maximum and average depths of the lacunae using specially designed instruments and software. Rat osteoclasts were smallest, and more chick osteoclasts were very large. There was a species difference in the onset of resorption and the sizes of pits produced, the chick osteoclasts being more vigorous resorbers than the rabbit ones, and the rat least so. For a given plan area, chick lacunae were deeper. There was a high correlation between area and volume. The range of maximum depths for a given area was high, however. Thus the mean of a few measurements of depths should not be used to calculate volume from area. At 24 hours , 77% of the rat, 47% of the rabbit and 28% of the chick lacunae were less than 1,000 μm^3 in volume; and 11% of the rat, 17% of the rabbit and 22% of the chick lacunae were between 1,000 and 2,000μm^3 in volume. The mean values at 24 hours were 981, 2796, and 4582 μm^3 for rat, rabbit and chick lacunae respectively.

KEY WORDS: In vitro bone resorption; SEM; stereophotogrammetry; osteoclasts; species differences; rat; rabbit; chick; dentine; bone disease.

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Introduction

The sizes and shapes of resorption lacunae made in dental and skeletal tissues in vivo vary greatly, and the factors that contribute to the variation are poorly recognized or understood. They fall into two main categories: those inherent in the characteristics of the cell and its biochemical response to its local environment; and those concerned with the physicochemical nature of the tissue being resorbed.

Estimations of the sizes of resorption lacunae in mammalian calcified tissue made by stereophotogrammetry of scanning electron micrographs (Boyde, 1968, 1970, 1973; Houghton et al, 1971; Boyde and Ross, 1975; Howell et al, 1984; 1986; Boyde and Jones, 1979) have provided us with valuable insight into the volumes of tissues that can be removed by one cell, but these certainly underestimate the total resorptive capacity of an osteoclast (or odontoclast). It is rarely possible to compute the amount of tissue removed by an osteoclast in a given time in vivo since single pit resorption loci are rare and the onset and cessation of resorption are difficult to determine.

In vitro studies (Boyde et al, 1983, 1984, Chambers et al, 1984a) paved the way for a better understanding of the cellular function because the material to be resorbed could be standardized and the time of the osteoclasts on the substratum controlled. In the course of general studies on the function of osteoclasts in vitro (Jones et al, 1985) we noted that the source of the osteoclasts had a large influence on the onset and amount of resorption and that there was a significant variation in resorption lacunar size on any one specimen, the variation being extreme in longer term cultures (Jones et al, 1986). The aims of this work were to quantify the species variation and the single source variation; and to investigate the relationship between resorption lacunar size and the number of nuclei in the resorbing osteoclasts.

Materials and Methods

Cells

Osteoclasts were harvested from the long bones of neonate rats and rabbits and 19d prehatch or hatchling chicks. The bones were

dissected out of the limbs and cleaned of any adherent periosteum and epiphyseal cartilage. They were then finely chopped in phosphate buffered saline and the fragments sucked up and down in a wide-barrelled pipette to dislodge osteoclasts. The milky fluid with cells was added to wells containing slabs of sperm whale dentine (SWD) and the cells allowed to settle for 30 minutes. The SWD was then rinsed in medium to remove non-adherent cells, and placed in fresh wells with 2 ml of medium with 10% added fetal calf serum, and cultured at 37°C in 5% CO_2.

Some cells from each species were seeded on to plastic dishes and allowed to settle and spread. The number of nuclei in each multinucleate osteoclast was counted and recorded, either whilst the cells were still alive using phase contrast microscopy, or after fixation and staining with toluidine blue.

Substratum

The standard substrate used for all the tests was a 200µm thick slab of SWD cut from a long rod using a low-speed, water-cooled diamond wheel. This resulted in a continuous surface with shallow parallel grooves. Sperm whale dentine was selected because of its greater homogeneity compared with bone and its lack of lacunae and vascular channels.

Culture Times and Preparation for SEM

All osteoclasts were cultured for 24hrs. Additionally, rat osteoclasts were cultured for 48hrs, rabbit osteoclasts for 9 or 12 hrs and chick osteoclasts for 3, 4, 6, or 7 hrs. The individual times were selected empirically to take account of the species difference in the onset of resorption that we had observed previously. The specimens were fixed with 3% glutaraldehyde in 0.15M cacodylate buffer at 37 degrees C at the end of the culture period. Some specimens were then stained with toluidine blue so that the location of osteoclasts and numbers of nuclei could be recorded (Fig. 1). All specimens were finally made superficially anorganic with a 10 minute treatment with 5% sodium hypochlorite (half strength of concentrated reagent), washed in water and then air dried. They were coated with gold by sputtering in argon.

Measurement of Resorption Lacunae

The specimens were positioned in the scanning electron microscope (Cambridge Stereoscan S4-10) so that the grooves lay perpendicular to the axis of tilt, and stereopair micrographs taken using backscattered electron imaging with a tilt difference of 10 or 14.4 or 20 degrees about the horizontal plane. They were analysed using an SFS3 stereocomparator (Fig. 2: Ross Instruments Ltd.), working with pairs in a 35mm negative format, and with data output automatically fed to a Sirius microcomputer (Boyde, Howell and Frank 1986).

We decided where to measure in relation to the features presented by each specimen, profiling along straight lines from side to side of the resorption pits (fig. 3). The sequence was started at any margin of the resorption pit complex, taking care to 'start' and 'finish' very close to the edge of the area covered by the complex, and at the exact level of the surrounding, flat, unaffected surface. Points were measured along a series of straight lines drawn from one edge of the pit to the other, to reconstruct planes cutting the surface perpendicularly (figs. 4-9). Each profile contained at least three points in order to be able to calculate the profile area. The first and last profiles are used mainly in the calculation of area and sometimes only contained three, closely spaced points. The profiles could be drawn in any direction, but profiles in X or Y were easier using SFS3.

As each profile is followed, the areas of succeeding triangles are calculated and are added (or subtracted, according to the sign of the curvature of the surface) by the method of determinants. At the end of each profile, therefore, the area below a straight line connecting the first and last points in the profile is known. Each new profile is assumed to begin on the side where the previous one finished. Sufficient points were selected to describe the shape of the profile. The high resolution graphics of the microcomputer display monitor were used to provide continuous plan and profile section views of the surface to help in judging the necessary density of measurement locations (figs.7 and 9). At the end of the second profile, the area of the quadrilateral in the XY plane, between this line and the previous one, is calculated. The volume of that section is calculated as the product of the mean of the two profile-section depths and the intervening plan area. In similar fashion, the third profile starts further along the starting side of the complex. The cumulative plan area and volume are known at the end of the second and subsequent profile sections.

The final printout registered (i) the number of points at which each pit was sampled, (ii) the number of loculi within a resorption complex, indicating intermittent phases of activity of a resorptive cell, (iii) the radius of curvature, (iv) the width, (v) the mean depth of the complex (volume/plan area), (vi) the maximum depth, (vii) the ratio of half the width/depth, as an indication of flatness, (viii) the plan area, (ix) the volume, (x) the true surface area of the scoop. In addition the ratio of the pit surface area/plan area was calculated.

Single cell function vs. cell volume

We used the same basic photogrammetric procedures to determine the volumes of osteoclasts, after $C_2Cl_3F_3$ to carbon dioxide critical point drying. We next determined the volume of the resorption pit associated with the same osteoclasts after washing the cells away with a WaterPik™. Finally, we determined the volumes of the same lacunae after dissolving the demineralized collagen fringe using sodium hypochlorite (demineralisation is the first step in osteoclastic degradation of bone or dentine). In deriving the volume of the osteoclast, we added the volume above the plane of the substrate to the volume below that plane which would have lain in the resorption pit - derived from the second step measurement.

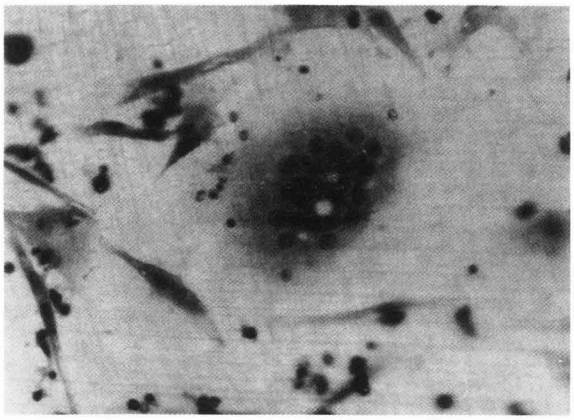


Figure 1. Light micrograph of toluidine blue stained sperm whale dentine slice with osteoclasts, showing method of viewing nuclei. Fieldwidth = 240 μm.

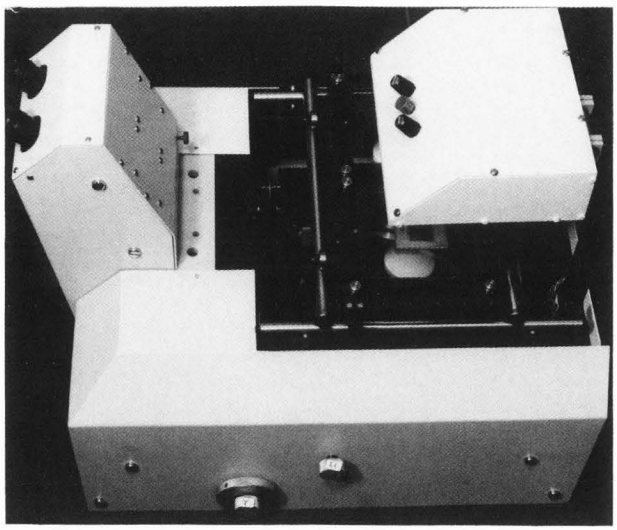


Figure 2. Photograph of right-hand-side of the Ross SFS3 3-axis recording stereo comparator. Two 5cm square format lantern slides containing pre-aligned stereo pair images are themselves aligned with the edge of a ruler crossing the photo carriages. The controls for movement in Y and parallax (Delta X) are seen on this side of the instrument.

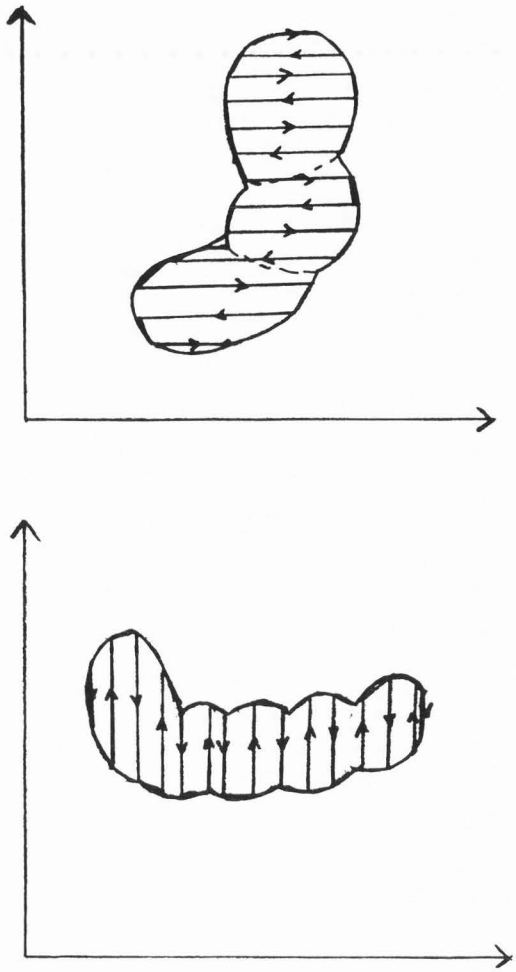


Figure 3. Diagrams of the plan view of a resorption lacuna complex to show the direction of progress in recording coordinates with the SFS3 system.

Results

Numbers of Nuclei

The numbers of nuclei in multinucleate osteoclasts harvested from the long bones of the neonate rats and rabbits and the prehatch chicks are shown in Figs.10-12. More than half the rat osteoclasts had 4 or fewer nuclei, the commonest nuclei number being 3 and the mean 4.84, whereas half the rabbit osteoclasts had from 4 to 8 nuclei, the commonest nuclear number being 6 and the mean 6.8. Chick osteoclasts generally had nuclei with a peak at 4 to 5. The mean for chick osteoclasts was 7.82, but when only cells with nuclei of 20 or less were considered this figure dropped to 6.69 (compared with 6.72 for rabbit and 4.84 for rat). Thus the rat osteoclasts were smaller, with fewer nuclei and a smaller range of nuclear numbers, those cells with more than 10 nuclei being rare. The main difference between the chick and rabbit osteoclasts was that there were more very large chick cells. Mononuclear osteoclasts, although some were undoubtedly present, were ignored since their identification was uncertain.

Settling time

We noted a difference in the adhesion and spreading times on plastic between cells from different species: the rat osteoclasts adhered and spread most quickly, and the chick ones least quickly.

Sizes of resorptive lacunae

The sizes of the resorption lacunae produced at the various times are given in Figs.13 to 18.

Volumes of resorption lacunae

At 24 hours, 77% of the rat, 47% of the rabbit and 28% of the chick lacunae were less than 1,000 μm^3 in volume; and 11% of the rat, 17% of the rabbit and 22% of the chick lacunae were between 1,000 and 2,000μm^3 in volume (Fig.14). The mean values at 24 hours were 981, 2796, and 4582 μm^3 for rat, rabbit and chick lacunae respectively. Thus by that time chick osteoclasts tend to remove a greater volume of dentine. This could be because more cells begin resorption early compared with rabbit or rat cells.

Areas of resorption lacunae

At 24 hours, 85% of the rat, 41% of the rabbit and 90% of the chick lacunae were less than 1,000um^2 in area (Fig.13). The mean values for the plan areas of the lacunae at 24 hr were 518, 1281 and 942μm^2 for the rat, rabbit and chick lacunae respectively. Thus chick lacunae at 24h tend to be deeper for a given plan area compared with rat and rabbit lacunae. When the true surface area of the lacunae was compared with its plan area, the same relationship was evident - the ratio was about 10% higher in chick than in rat and rabbit lacunae.

Correlation of Area and Volume (Figs. 15-18)

Within a given set (of species and time of culturing) the correlation coefficient was high - always greater than 0.9 (Fig. 15). The correlation of volume$^{0.33}$ and area$^{0.5}$ was also high (Fig.17). However, the range of maximum depths of the lacunae in any one set was large, so it would not be valid just to measure, say, 5 lacunar depths and use their mean to compute the volumes for a set of areas. The correlation of maximum depth with either area or volume is relatively poor (Fig. 16).

Variation

The variation was high for every set of species, time and measurement (Fig.18). It ranged between 70 and 190% and was, predictably, always greater for the volume than area.

Single cell function related to cell volume

The algorithm for measuring the pit volume can be used to measure the volume of the osteoclast. Since we also find that demineralisation of bone and dentine precedes matrix degradation and we can remove demineralized matrix after a first measurement of a resorption lacuna, and we have data on the shrinkage of cells in preparation for SEM: we have been able to determine that, to a first approximation, a 3pl rabbit osteoclast could "dissolve" 2pl of mineralized dentine and demineralize a further 1pl in 24h.

Discussion

The assessment of in vitro osteoclastic activity has, until recently, been dependent on the analysis of organ culture experiments, either by histological examination of the bone or by measuring the release of radiolabelled bone matrix constituents into the medium (Raisz and Niemann, 1969: Reynolds and Dingle, 1970). We have recently developed a single cell resorption assay so that the resorptive function of individual osteoclasts, isolated from bone and seeded onto slices of calcified tissue, could be studied. This paper examines the biological variation in the sizes of resorption lacunae produced by bone cell cultures derived from neonate rats and rabbits, and prehatch or hatchling chicks.

A purpose built stereocomparator, working with 10^0 to 20^0 tilt angle difference image pairs (figs. 5 and 10) in 35 mm negative format, and data output automatically to a microcomputer with special software, provided an efficient system for the measurement of the pits made by osteoclasts resorbing bone or dentine. We are now able to avoid shortcut methods in which assumptions about the specimen are made:- for example, that the curvatures of the surface are ellipsoidal (Boyde and Jones, 1979) or that the depth of one pit is an indication of the depth of another (Chambers et al. 1984b). Indeed, the extensive use of our system has demonstrated that such assumptions are fallacious.

There is obviously a species difference in the timing of the onset of resorption by osteoclasts separated mechanically from the bone and cultured on a new substratum. The volume of the calcified, collagenous tissues that they can destroy by 24h shows a progression of increasing osteoclastic resorptive vigour from rats to rabbits to chicks. The protocol for this experiment does not allow us to estimate whether, once all or most osteoclasts are resorbing well in culture, they would then all be resorbing at equal rates. This would be a difficult question to answer, because osteoclasts in vivo and in vitro change their size, and nuclear number. Fusion occurs between cells throughout the culture period, and we have also observed fission using video recording. These incidents would be influenced by the density of the bone (and marrow) cells on the substrate, and the relative density of osteoclasts and precursor osteoclasts compared with other cell types. Hence it would not be enough to take the increase in mean volume for lacunae at, say, 48h compared with 24h. The numbers of precursor cells from the mixed bone and marrow populations that would differentiate during the culture period may also vary between species. An extreme example of this can be seen in long term cultures, where the range of pit size is enormous (Fig. 19). Such cultures emphasize, too, the range of resorption pit depths for a given plan area.

The way in which one resorption pit grows may also differ from its neighbour. We have found from video cine recording osteoclasts whilst they

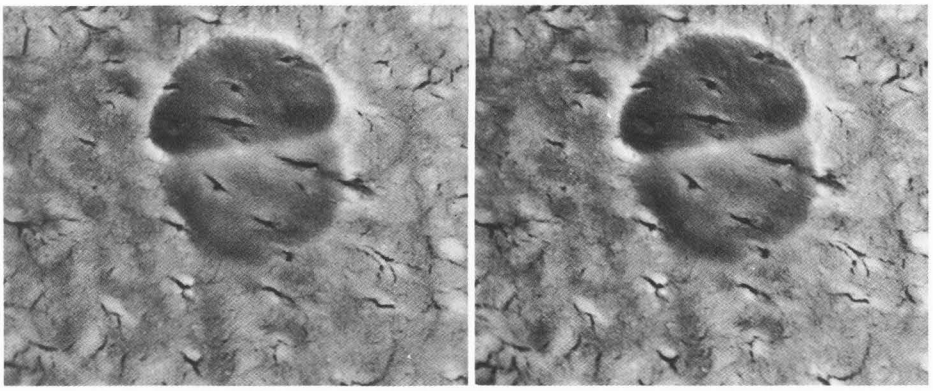


Figure 4. Stereo-pair SEM image of resorption lacuna complex made by chick osteoclast seeded for six hours on to the surface of a slice of sperm whale dentine (cut with a low speed diamond saw under water cooling). Tilt angle difference = 14.4 degrees. Scale identical with plan view in figure 5. Maximum horizontal width (W) of pit = 15μm: mean depth = 1.1μm: max depth = 1.6μm: coverage (C) = 364μm^2: surface area (S) = 381μm^2: volume (V) = 498μm^3.

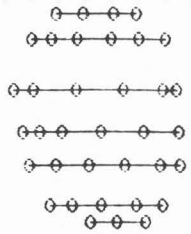


Figure 5. Printer output of the monitor display while measuring Fig.4.

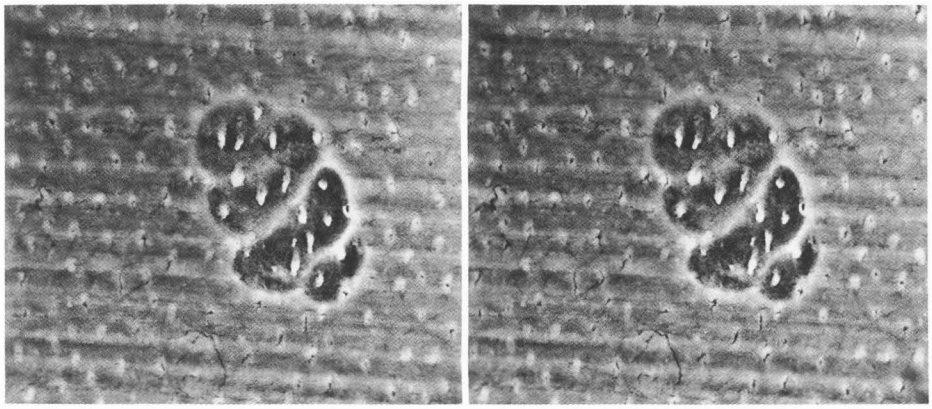


Figure 6. Resorption lacuna made by rabbit osteoclast in 24 hours on SWD. W = 23μm: mean depth = 1.0μm:max depth = 1.6μm: C = 1043μm^2: S = 1072μm^2: V = 1253μm^3.

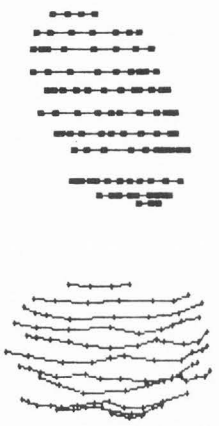


Figure 7. Printer output of monitor display relating to figure 6.

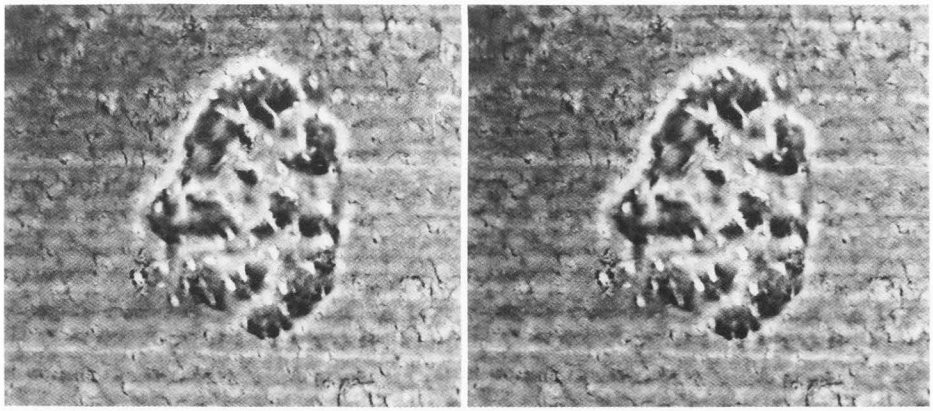
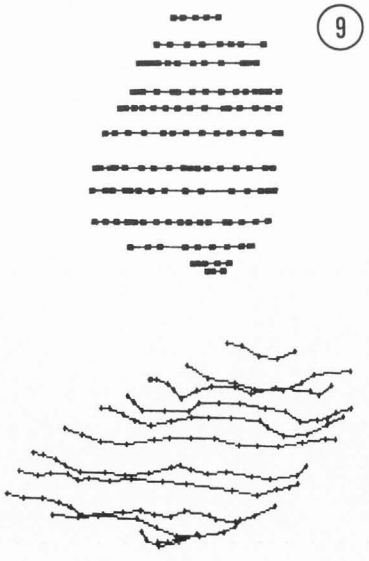
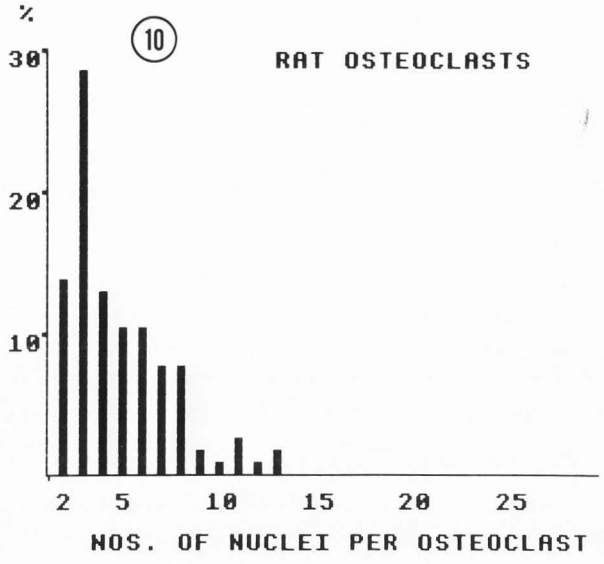
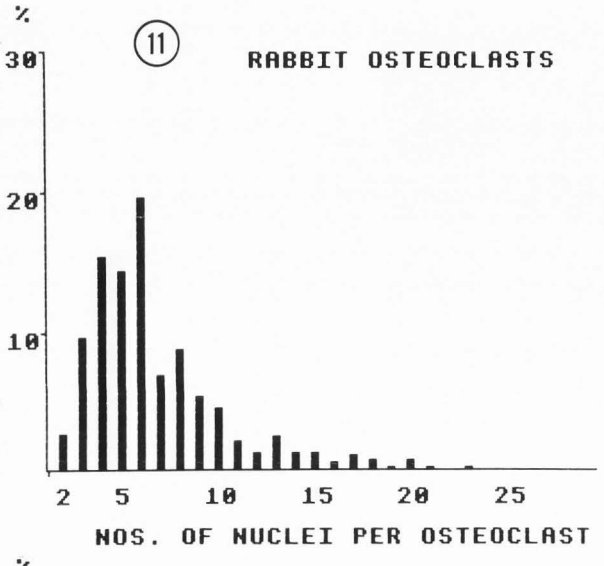
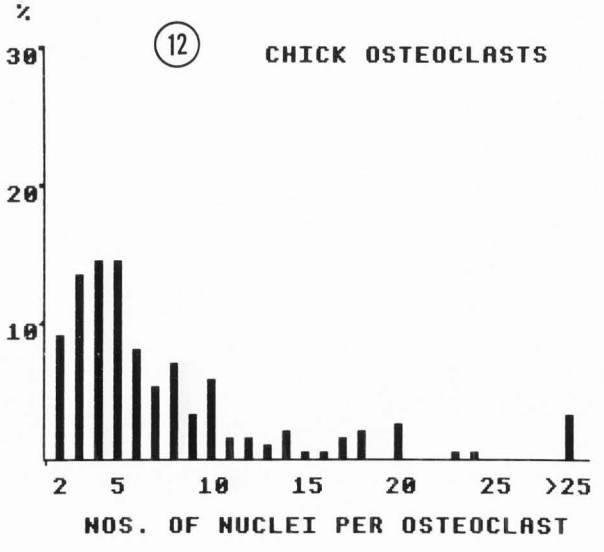


Figure 8. Resorption lacuna made by rat osteoclast in 48 hours on SWD. W = 20μm: mean depth = 0.9μm: max depth = 1.1μm: C =680μm^2: S = 690μm^2: V = 670μm^3. **Figure 9.**(right) Display relating to figure 8.









Figures 10, 11 and 12. (in the left column) Histograms showing the proportion of osteoclasts having particular numbers of nuclei, in rat, rabbit and chick respectively.

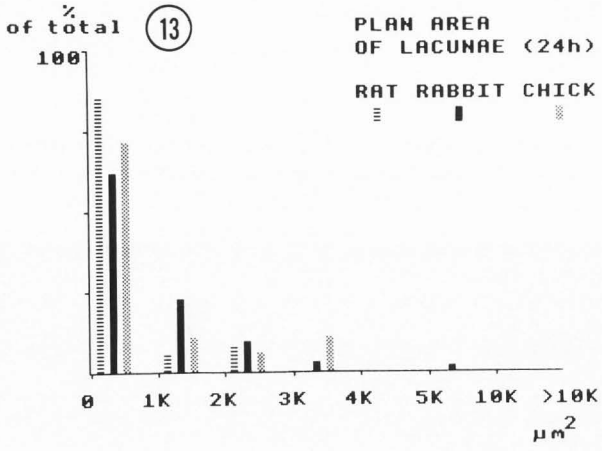


Figure 13. Histograms showing the areas of resorption lacunae at 24 hours for rat (cross-hatched), rabbit (solid bar) and chick (dotted bars) respectively. Areas are shown in thousands of square micrometres.

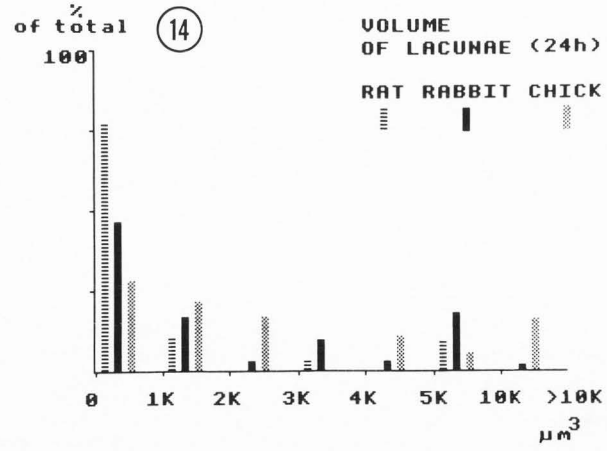


Figure 14. Histograms showing the volumes of resorption lacunae made by rat, rabbit and chick osteoclasts in SWD at 24 hours. Volumes are shown in thousands of cubic microns.

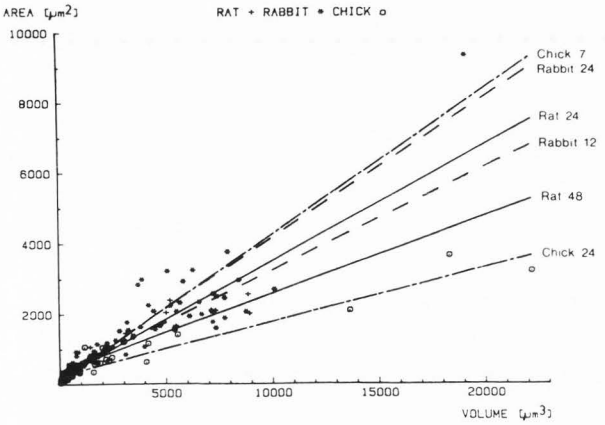


Figure 15. Regression lines of area of individual resorption lacunae against their volumes for chick 6 and 7 hours (labelled chick 7) and chick 24h, rabbit 12h and 24h and rat 24h and 48h groups. In the case of the chick and rat data, the slopes indicate that the pits are proportionately shallower at an earlier stage. This tendency appears to be reversed for the rabbit cells. Slopes and correlation coefficients for these six are:-

	Corr.Coeff.	Slope	Y Intercept
Chick 7	0.939	0.414	97
Chick 24	0.971	0.152	245
Rabbit 12	0.916	0.292	312
Rabbit 24	0.920	0.398	166
Rat 24	0.963	0.329	195
Rat 48	0.968	0.218	380

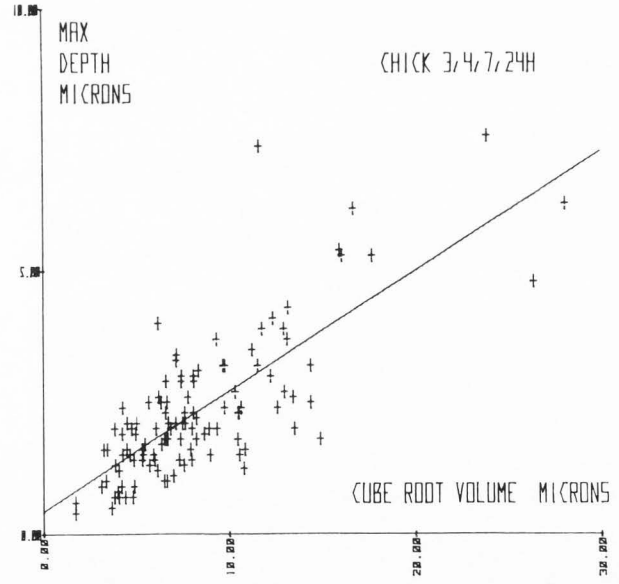


Figure 16. Correlation between the maximum depth of individual resorption pits made by chick osteoclasts (pooled data for 3, 4, 7,and 24 hours) and the cubic root of the volume: both axes labelled in microns. Correlation coefficient =0.586 . Slope =0.229.Intercept on y axis = 0.42.

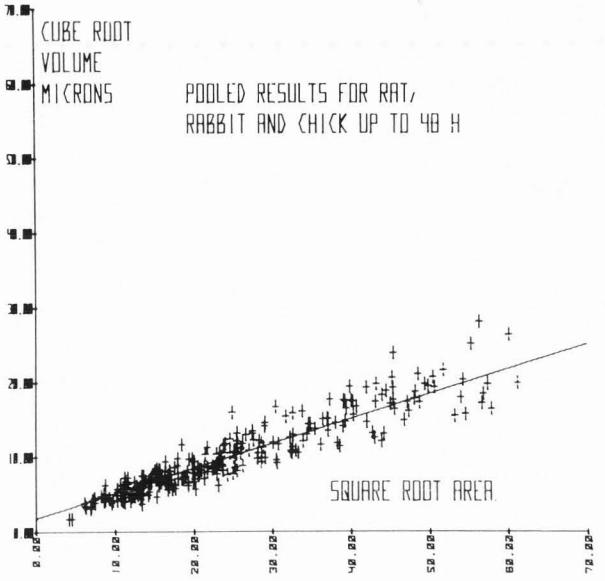


Figure 17. Correlation between the cubic root of the volume and the square root of the area of individual resorption pits made by rat, rabbit and chick osteoclasts cultured on SWD for up to 48 hours: both axes are labelled in microns. Correlation coefficient =0.862. Slope =0.333. Intercept on y axis = 1.77.

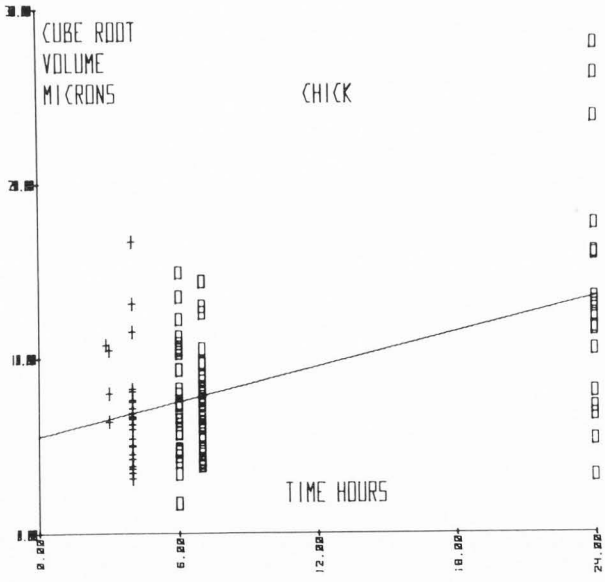


Figure 18. The distribution of resorption lacunar size (expressed as the cubic root of the volume in cubic microns - units are therefore microns) for chick osteoclasts at 3 , 4, 7 and 24 hours.

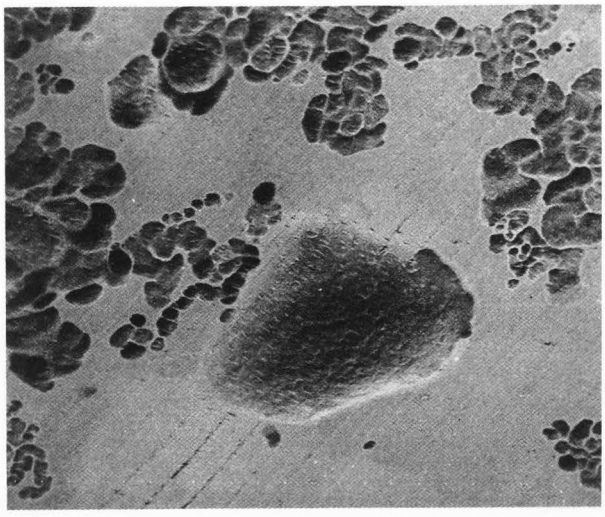


Figure 19.
Resorption lacunae made by chick osteoclasts cultured for 19 days on SWD. Note the very large range in areas, depths and volumes. The largest, central lacuna, measured as described in this text, has a volume of 369,760μm^3. Fieldwidth 415μm.

are resorbing SWD that some pits start with a small circular area that deepens and spreads centrifugally, whereas for others the plan extent of the ruffled border seems to be set early and the site is left before much depth of substrate has been excavated. More extensive, multilocular pits are almost infinitely varied in shape and size. The parameters that influence this variation are little understood. We have also found using video cine recording that osteoclasts in culture may appear as restlessly motile cells during resorptive episodes, or be relatively sedentary as they sink deeper into the substrate. Examples of both these patterns can be deduced from a study of surfaces resorbed in vivo.

There was a difference in the mean number of nuclei in osteoclasts isolated from the long bones. Rat cells were smaller and the range of numbers of nuclei was smaller. Our values for the rat osteoclasts are in general agreement with those reported by Ries and Gong (1982) and Hefley and Stern (1982), taking into account the different isolation procedures and that we disregarded mononuclear osteoclasts. However, although a small osteoclast cannot make a large, single pit, we have observed that very large osteoclasts may make enormous or very tiny single pits, or a series of tiny pits. We do not yet know whether it is the largest cells that begin to resorb earliest. But if that were true, then the greater number of chick osteoclasts with high nuclear counts and the quicker onset of resorption in the chick cultures would also be explained. Cell size is not only dependent on nuclear numbers but also on cytoplasmic volume and this would be increased in actively secreting cells. The rate of settling, adhesion and spreading would affect the time of onset of resorption though not the size of the lacunae subsequently made. The time the ruffled border has been active and the secretion rate of the individual cells will determine the volume of a particular tissue degraded in a culture period. It is possible, moreover, that the culture conditions that favour optimal osteoclasis in one species may vary from that for another, particularly when one compares mammalian with avian cells.

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Discussion with Reviewers

Reviewer I: One question which arises from the findings reported here is the relationship of sperm whale dentine as a substrate for osteoclastic bone resorption compared with bone. Sperm whale dentine has been used because there are less iregularities on the surface and it is technically easier to assess resorption. Do the authors know that the behaviour of osteoclasts on bone surfaces can be anticipated from their behaviour on sperm whale dentine? Is it possible that the difference in contour on the bone surface compared with the smooth surface presented to the cell by sperm whale dentine could influence osteoclastic activity?

Authors: Although we have not measured resorption lacunae made in bone and dentine in the very same culture, we have found that the behaviour and resorptive capacity cannot be distinguished visually when one compares, say, separate 24 hour cultures of chick osteoclasts on the two tissues. Equally, cells cultured on slabs of sperm whale tooth that have a region of cellular cementum (very similar to bone) as well as dentine make resorption lacunae of similar shapes and sizes in the two tissues. This is well shown where a resorption track or pit crosses the cement-dentine junction (see, for example, Fig 11, p. 248 in SJ Jones, A Boyde and NN Ali (1984) The resorption of biological and non-biological substrates by cultured avian and mammalian osteoclasts. Anat Embryol **170**, 247-256.) However, the rate of resorption in bone is influenced locally by the degree of mineralisation (SA Reid (1986) Effect of mineral content of human bone on in vitro resorption. Anat Embryol **174**, 225-234).

One cannot exclude the contour having some influence, because although osteoclasts appear to pay little attention to grooving, other cells, which may in turn influence the osteoclasts, are very responsive to this parameter.

Reviewer I: The authors have noted differences between the rat, chick and rabbit with respect to nuclear number and nature of the resorption pits. However, in the in vitro experiments reported here, the cells are not bathed in the normal hormonal environment to which they would be exposed in vivo. Some factors which influence osteoclast function presumably act as regulatory growth factors and some act as agents which stimulate differentiation and cause multinucleation. One example of the latter is 1.25 dihydroxyvitamin D. Do the authors have any information on whether regulation of nuclear number by osteotropic factors alters the pattern of resorbing activity?

Authors; We have not yet investigated how osteotropic factors alter the differentiation of osteoclasts or the pattern of resorbing activity in our system.

Reviewer I: MD Fallon et al (Lab Invest **49**, 159, 1983) have reported that multinucleation may, in itself, be a factor which enhances a cell's capacity to resorb bone. These experiments were performed with macrophage polykaryons using devitalized bone particles as a substrate. Using the more sophisticated techniques described here, do the authors consider that,for any individual cell, multinucleation is an important determinant in the capacity of the cell to resorb bone?

Authors: Our long term cultures (Jones, Ali and Boyde, 1986) suggest that the degree of multinucleation of osteoclasts certainly does change the volume of resorption pits and tracks produced, but not their shape. We do not know if it increases the rate (per nucleus) at which a cell can resorb, nor what the optimum size would be. As regards resorption of bone by macrophages, we do not believe that it has yet been demonstrated for particles larger than those which can be endocytosed. Neither macrophages nor monocyte polykaryons were able to make resorption pits in bone, dentine or cementum in our culture systems (IM Shapiro, SJ Jones, NM Hogg, M Slusarenko, A Boyde (1979) Use of SEM for the study of surface receptors of osteoclasts in situ. Scanning Electron Microsc. 1979;II:539-545.

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Reviewer II: The comparison of activity between species is quite interesting. There is some controversy on the role of calcitonin on inhibiting bone resorption, especially in chick. Have the authors carried out calcitonin dose response curves in the different species and looked for inhibition of resorption? Is there any indication that the chick osteoclast activity decreases with long term culture?

Authors: We have not constructed calcitonin dose response curves. As regards an effect on chick osteoclasts, TR Arnett, W Horbert and DW Dempster (Comparative effects of calcitonin on rat and chick osteoclasts in vitro. J Bone & Mineral Res **1 Suppl 1,** abst 7, 1986) working with a similar culture system - but using bovine cortical bone slices as a substrate and only counting the **number** of resorption pits - found chick osteoclasts were strikingly less sensitive to some (non-avian) calcitonins than rat osteoclasts.

With regard to osteoclastic activity in long term culture, our own experiments have not extended beyond 6 weeks, at which time active resorption was still continuing.

Reviewer III: The differences in species are of some interest, but in order to judge them we need additional information. These variations might be expected because of differences in viability.

Are the rat cells less active because only the small osteoclasts are recovered by this procedure and the large ones are left behind or die? The latter possibility is certainly consistent with the report from Hefley (Hefley and Stern, 1982).

Are the rat osteoclasts generally smaller than chick osteoclasts when they are examined in vivo?

What proportion of the cells from different species which adhere to dentine were osteoclasts?

Do the accompanying non-osteoclastic cells either increase or decrease resorptive activity?

Authors: We agree that species differences in viability may be an important factor even in short term cultures. In answer to your first two questions, we have not counted the nuclear numbers of osteoclasts before harvesting them from the bones. However, we would expect that large cells would be recovered at least as easily as small ones in our extraction procedure.

Our isolated "osteoclast" preparations are not "pure": there are many other cells present in cultures of all three species. We cannot now, retrospectively, go back to quantify the ratio of other cells to osteoclasts in the experiments reported here. This would have to be done with preparations seeded on to dentine and fixed at the beginning as well as at the end of the culture period. This is a very important and helpful suggestion for future studies: the other cells undoubtedly affect osteoclastic resorptive ability.