Anat Embryol (1992) 186:291–299

Anatomy and Embryology



# The relationship between the number of nuclei of an osteoclast and its resorptive capability in vitro

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Accepted June 15, 1992

**Summary.** This study examined the relationship between the number of nuclei in an osteoclast and its resorptive efficiency, as demonstrated by the size of the pit it can make in a mineralized tissue in 24 h in vitro. Osteoclasts released mechanically from prehatch chick long bones were cultured on dentine slices or on plastic dishes for periods of 6 or 24 h. The frequency distribution of the multinucleate tartrate-resistant acid phosphatase (TRAP)-positive cells with different numbers of nuclei was determined: the mean number of nuclei per cell was 6.92, with a mode of 4. 47% had 5 or fewer nuclei and only 11% more than 10 nuclei. The pits associated with 292 osteoclasts with known numbers of nuclei were measured using a confocal laser light microscope (Lasertec) and dedicated image analysis system, and depths, plan areas and volumes determined. There was a positive correlation between the number of nuclei per osteoclast and the volume of the pit made, but a trend for the volume resorbed per nucleus to decrease with increase in the number of nuclei per osteoclast.

**Key words:** Osteoclast – Nuclei – Resorption – Pit size – In vitro

## Introduction

The volume of an osteoclast varies throughout its life history, and may both increase and decrease. There are no published data giving values for this crucial parameter although one would expect intuitively that there must be some functional advantage gained by the cell by virtue of added size. At the very least, cell size may give some indication of resorptive fervour at a particular site or time, or in response to local or humoral factors (Holtrop et al. 1979), including matrix components (Groessner-Schreiber et al. 1991). This is recognised by investigators' descriptive comments on the sizes of osteoclasts seen on the bone surface in various pathological (Kukita et al. 1990) or experimental conditions (Katoh et al. 1991), although a negative relationship may also be present (Shapiro et al. 1988).

Large cells are associated with large or small pits and small cells with small pits. Of course, large pits may develop from small ones by maintaining the same form but growing in proportion; or grow by changing form radically, beginning with an extensive plan area and deepening to a greater relative amount; or they may vacillate between different patterns of growth (Jones and Boyde 1988). As osteoclasts are motile cells, and can move whilst resorbing, the sculpting of the surface by an osteoclast is a resultant of its lateral translation across the mineralised tissue and its ability to secrete protons (Baron et al. 1985) and degradative enzymes into the ruffled border zone: movement during formation therefore changes the form of pits. Motile but non-resorptive phases may intervene; and when resorption starts, or restarts, round pits are initially favoured. The best geometric measure of the work done by an osteoclast must, therefore, be the volume of tissue it has destroyed (Boyde and Jones 1987, 1991, 1992).

An osteoclast is a cell that may be uninuclear but is most commonly recognised at the bone or tooth surface in a multinuclear state. Increase in the size of an osteoclast, beyond a certain but unknown amount, is a consequence of cell fusion, and there is a turnover in the nuclei (Kember 1960; Young 1962; Zambonin Zallone and Teti 1981; Ries et al. 1987). The number of nuclei that are present in an osteoclast is often taken as an indication of total cell size. However, it is not known whether multinuclearity or the act of cell fusion confers some special ability on the cell, such as is reported for multinucleated macrophages (Vignery et al. 1991), in addition to the effect of increase in size alone: nor, if this were so, whether the benefit accrues with each fusion or in relation to the numbers of nuclei present. An increase in nuclear number could also be detrimental to resorptive efficiency, perhaps beyond a critical cell size or youth of component nuclei, or as a result

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of a reduction in RNA synthesis by some nuclei (Zheng et al. 1991).

An indirect measure of resorptive ability might be the ratio of the total volume of the cytoplasm to that of the nuclei, or the relation between the enzyme content of the cell and the numbers of nuclei. These cell features are, however, promissory, and could be misleading (Umita et al. 1991): proof of the pudding is in the eating.

The purpose of this study was to measure the volume of the pits made by osteoclasts of known nuclear number under standardised conditions in vitro in order to discover whether cell fusion resulted in increased overall resorptive activity. For example, is an osteoclast with seven nuclei more, or less, efficient at the removal of a calcified tissue than two cells with a combined total of seven nuclei?

## Materials and methods

*Culture of chick osteoclasts.* Long bones were removed from 19-day prehatch chicks, the cartilaginous epiphyses cut off, and the bones chopped in phosphate-buffered saline (PBS) with added 10% fetal calf serum (FCS). The release of osteoclasts from the bones was aided by flushing the fragments up and down a plastic pipette. The milky cell suspension was then seeded on to rectangular slices of sperm whale dentine, which measured 5 mm × 5 mm, and had one clipped corner in order to make relocation of the pits easier. The cells were allowed to settle for 45 minutes at 37° C. Each chick provided four aliquots for seeding, and a total of 21 chicks were used in two experimental sets for measuring the resorption pits. The slabs of dentine were then washed gently in PBS to remove non-adherent cells, and the cells remaining on the dentine were cultured for 24 h in Eagle's Minimum Essential Medium (MEM) containing 10% added FCS, 2 mM L-glutamine, 0.25 pg/ml fungizone, 0.2 U/ml penicillin and 0.2 pg/ml streptomycin, in 5% $CO_2$ at 37° C.

In addition to the cell seedings on to dentine for pit measurement, some were made on to plastic culture dishes for 6 or 24 h, and on to other dentine slices for 24 h, and cultured in an identical way before fixation as below. Cultures were not terminated at 6 h on dentine because few pits would have been formed at that time, and many of those present would have been at an early stage of development.

*Counting the nuclei of osteoclasts, and identifying the cells with their pits.* At the end of the culture period, the dentine slabs were washed gently in PBS and the cells fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer at pH 7.4 for 5 min. The slabs of dentine were then washed in distilled water and air dried for 10 min. The cells were stained for the presence of tartrate-resistant acid phosphatase (TRAP: Sigma kit 386) and stored in 0.15 M cacodylate buffer or 5% sodium azide in PBS until recordings of the nuclei counts and pit positions were made. Cultures on plastic were similarly treated.

For location of the pits and recording the number of nuclei in the associated osteoclast, each of the numbered dentine slices was drawn using an Olympus IMT-2 microscope for observation and a graticule lined up to one marked corner of the slice. All multinucleate TRAP-positive cells with a pit that could be ascribed to them were included in the study. The plan shape of the osteoclast and its pit were drawn to assist in the identification of the pit after the cells had been removed. This set included 292 osteoclasts and pits on 75 dentine slices.

The nuclei of all the multinucleate cells that were TRAP-positive were counted in the 6- and 24-h cultures on plastic, and parallel 24-h cultures on dentine. Nuclear counts were made of 227 osteoclasts at 6 h (on plastic) and 211 cells at 24 h (on plastic or dentine) in this set. Including the 292 osteoclasts above, the nuclei of a total of 730 osteoclasts were counted in this study.

The cells were then removed from the dentine by washing with a mild detergent, 1% chlorhexidine gluconate, pH 7, followed by immersion for 30 min in 30% $H_2O_2$, and the 292 pits identified again using the graticule and chart.

*Measurement of the pits.* The method which we used (demonstrated at the Davos Bone and Cartilage Cells and Cytokines meeting, Jan. 1992) is based upon a video rate, line confocal scanning light microscope (CSLM) manufactured by Lasertec Corporation, Japan. The ILM11 instrument was developed for the semiconductor industry market. A He-Ne laser beam (lamda = 633 nm) is broadened to a line using a cylindrical lens, which is scanned down the specimen field at TV frame rate. The signal corresponding to each point in the line is directed to a single detection element in a linear diode array. Thus images are acquired at full standard TV rate. The microscope and its special Z-axis controller provide a hard-wired solution to the problem of acquiring the *max* and the *map* images during multiple plane acquisition. Images derived from 256 planes are acquired in approximately 10 secs: an improvement in time and acquisition that allows us to achieve a better confocal Z resolution – a practical match to the SEM photogrammetric method at ten times the speed.

Volume data from the *map* image was acquired by tracing (with a mouse and a screen cursor) a line (one pixel wide) around the pit to be measured. Software, written by SIS Münster/Werst, then found the volume enclosed within that area, deriving the height at which the surrounding, reference surface was found from the values under the trace binary. The area of the pit was derived from a second screen cursor trace placed close to the edge of the pit. The depth recorded was the maximum depth of the pit. The mean depth was determined from computing volume/area. Analysis of the data was performed using Minitab statistical software (Minitab, 1989).

## Results

### *The total osteoclast population*

The numbers of nuclei present in the osteoclasts harvested by the standard procedure from the chick long bones and seeded on to plastic culture dishes or on to dentine for periods of 6 and 24 h are shown in the histograms (Fig. 1). All the osteoclasts in these cultures were

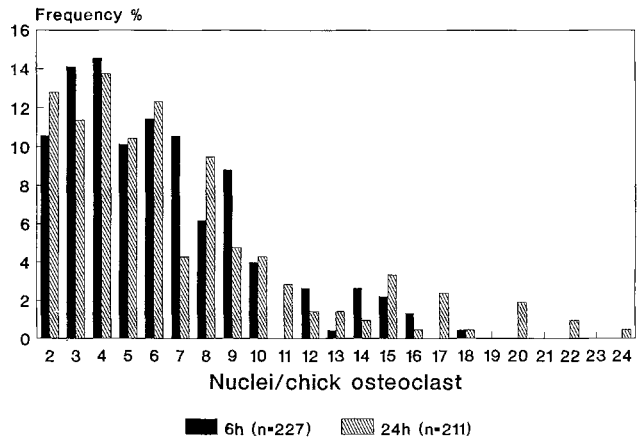


**Fig 1.** Histogram showing the relative frequencies of the chick osteoclasts with different numbers of nuclei, in 6 h (on plastic) and 24 h cultures (on dentine or plastic), excluding those dentine samples used for pit measurements

**Table 1.** Number of nuclei in chick osteoclasts

| *n* | Culture | Substratum | Mean | SD | Max. nuclei |
|---|---|---|---|---|---|
| 227 | 6 h | Plastic | 6.20 | 3.47 | 18 |
| 138 | 24 h | Plastic | 7.29 | 5.03 | 24 |
| 73 | 24 h | Dentine | 6.11 | 3.69 | 20 |
| 211 | 24 h | Plastic/ dentine | 6.88 | 4.64 | 24 |
| 292 With pits | 24 h | Dentine | 7.51 | 4.91 | 27 |
| 730 Total | 6 or 24 h | Plastic/ dentine | 6.92 | 4.54 | 27 |

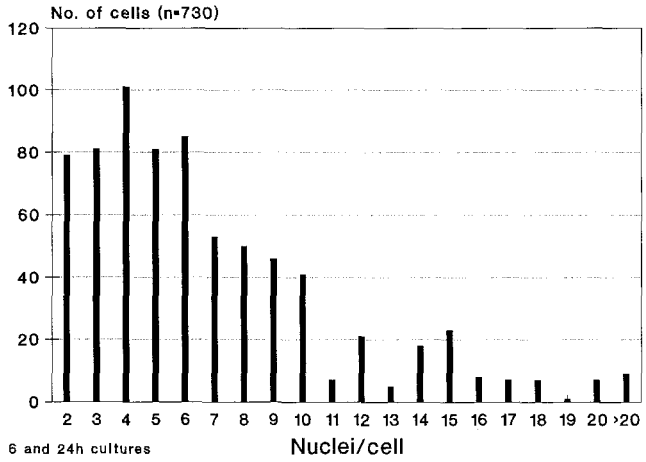


**Fig 2.** Histogram showing the number of nuclei/cell in all the osteoclasts counted, cultured for 6 or 24 h, including the 292 cells with associated measured pits

counted, a total of 227 cells at 6 h and 211 at 24 h. As there was no significant difference found between the proportion of the cells with different numbers of nuclei at 6 and 24 h, although there were more cells with high numbers of nuclei at 24 h (Table 1), the results were pooled. Figure 2 is a histogram of the distribution of nuclei for all the osteoclasts in the study. The frequency % and cumulative frequency histograms for the total number of osteoclasts (730) are shown in Fig. 3. Only 11% of the cells had 10 or more nuclei and 47% had five or fewer nuclei.

There was a significant difference ($P=0.0004$) between the numbers of nuclei/osteoclast in the 6-h cultures on plastic and the 292 cells associated with a pit at 24 h. This was because there were more very large cells in the second group, and a slightly lower frequency of cells with very low numbers of nuclei with a pit. However, the type of substratum did not appear to influence the frequency of attachment of cells of different nuclear number.

*The nuclei/cell in the population of the osteoclasts with measured pits*

Figure 4 shows the distribution of the cells according to the number of their nuclei for the 292 osteoclasts related to a measured pit. The number of nuclei per cell ranged from 2 to 27, with a mean of 7.514 and a median of 6. 44% of the multinucleate osteoclasts had 5 or fewer nuclei, and 81% had 10 or fewer nuclei. Only 11% had 15 or more nuclei.

*The sizes of the pits and the nuclei per cell*

The depths, areas and volumes of the pits measured are shown in the histograms of Fig. 4, and the means and medians of the maximum depths, mean depths, areas, and volumes in Table 2.

The correlation matrix for nuclei/cell, pit depths, areas and volumes is shown in Table 3. Whether considered as the total measured or in the smaller groups of the two individual experiments, the correlation between the number of nuclei/cell and the area of the matching pit was always greater than that between nuclei and volume, although both showed a positive correlation. The correlation between the number of nuclei per cell and

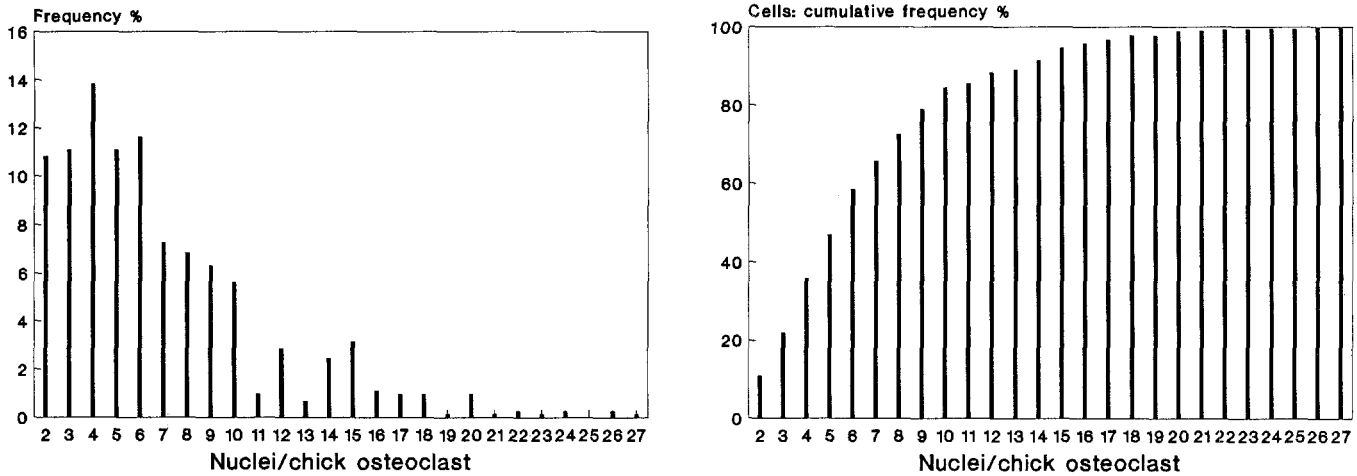


**Fig 3.** Relative frequencies and cumulative frequencies of the chick osteoclasts ($n=730$) with different numbers of nuclei

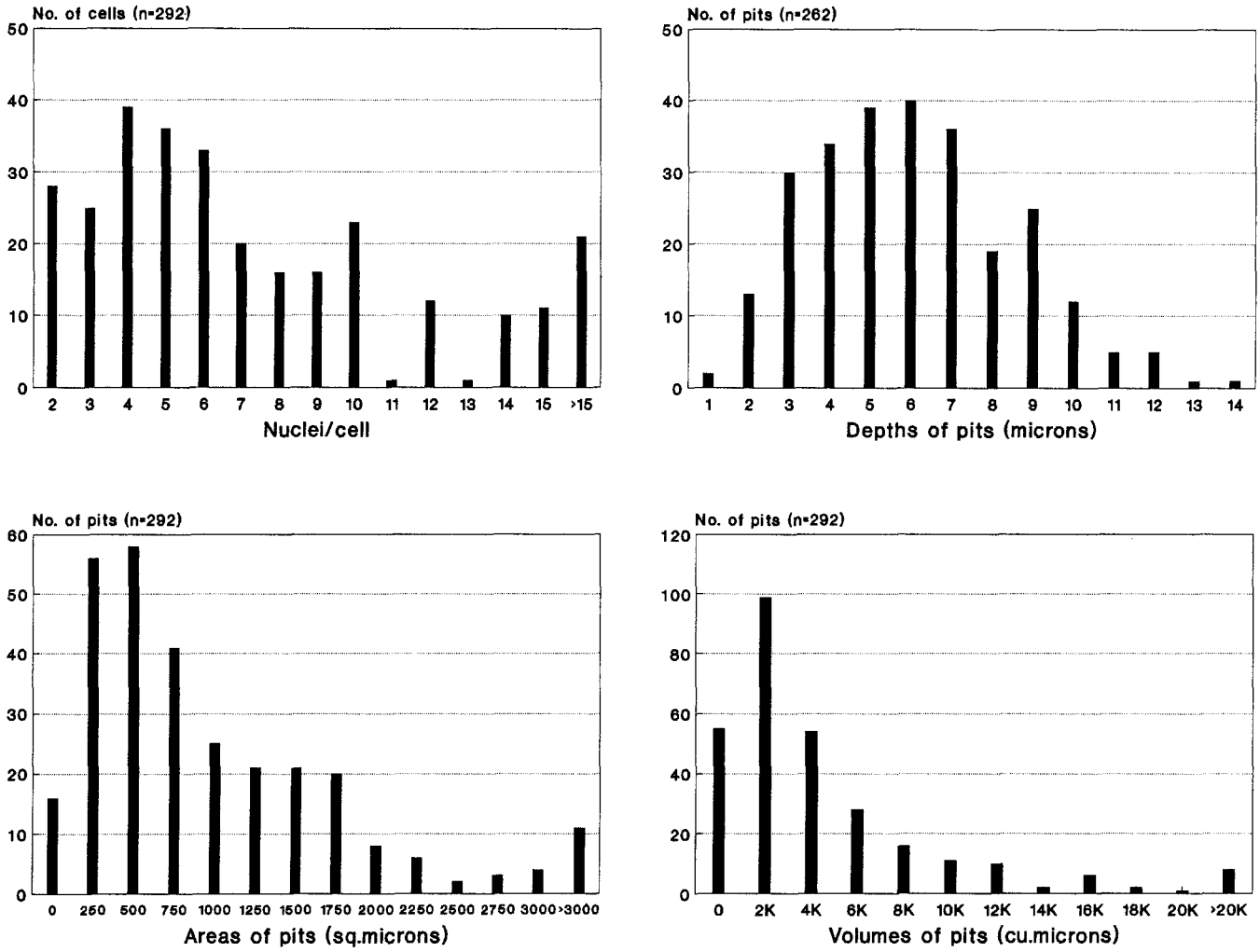


**Fig 4.** Histograms showing the distribution of the numbers of nuclei/cell of the 292 osteoclasts with associated measured pits, the maximum depths ($n=262$), the areas ($n=292$) and volumes ($n=292$) of the pits

the depth of its pit was always weakest, even when the 14 most dubious associations were excluded from the analysis (Table 3). As we have found previously, the correlation between the volume and the area of a pit formed under standard conditions and over short culture periods is high. As the size of the cells (as reckoned from their number of nuclei) increased, the correlation between size and volume within the group increased. For example, grouping the cells into three sets of those with 2–5 nuclei, 6–10 nuclei, or more than 10 nuclei, gave correlations of number of nuclei/cell with volume of associated pit, within the group, of 0.008, 0.049 and 0.347. This reflected the much greater diversity of size in the third group, comprising 19% of the total number.

Six random samples of 25 cell+pits from the total of 292 gave correlations between nuclei/cell and pit volume with a range of −0.108 to 0.751, and for nuclei/cell and pit area with a range of 0.060 to 0.847, showing the necessity for a large sample size. Only the correlations between pit area and volume were consistently high ($r=0.599$ to 0.916).

When the cells were grouped with regard for their size (number of nuclei) and the pits for their size (volume), the trend was obvious. Most small pits were made by small osteoclasts and larger osteoclasts were more frequently associated with larger pits (Fig. 5). The mean volume resorbed/nucleus/cell was 692 $\mu m^3$. There were weak negative correlations between the number of nuclei in a cell and the volume or plan area resorbed per nucleus, but a significant decrease in the depth of resorption per nucleus. Thus an increase in the number of nuclei did not appear to increase an osteoclast's resorptive activity per nucleus but to decrease it.

**Table 2.** The mean values of the sizes of 292 osteoclasts and related pits

| | Mean | Median | SEM |
|---|---|---|---|
| Nuclei/cell | 7.514 | 6 | 0.288 |
| Volume of pit | 4708 | 2819 | 363 |
| Area of pit | 1015 | 700 | 57.9 |
| Maximum depth of pit | 6.05 | 5.87 | 0.15 |
| Mean depth of pit | 4.26 | 3.88 | 0.16 |

Depths in μm; areas in $\mu m^2$; volumes in $\mu m^3$
SEM Standard error of the mean

**Table 3**

| | Nuclei/ cell | Volume | Area | Max. depth |
|---|---|---|---|---|
| *Correlation matrix for the 292 cells and pits* | | | | |
| Volume | 0.419 | | | |
| Area | 0.530 | 0.801 | | |
| Max. depth | 0.227 | 0.538 | 0.324 | |
| Mean depth | 0.058 | 0.613 | 0.138 | 0.577 |
| *Correlation matrix for the 175 cells and pits of Experiment 1* | | | | |
| Volume | 0.371 | | | |
| Area | 0.405 | 0.881 | | |
| Max. depth | 0.273 | 0.571 | 0.391 | |
| *Correlation matrix for the 117 cells and pits of Experiment 2* | | | | |
| Volume | 0.482 | | | |
| Area | 0.737 | 0.623 | | |
| Max. depth | 0.077 | 0.438 | 0.153 | |
| *Correlation matrix for 278[a] of the 292 cells and pits* | | | | |
| Volume | 0.517 | | | |
| Area | 0.566 | 0.865 | | |
| Max. depth | 0.264 | 0.502 | 0.269 | |
| Mean depth | 0.107 | 0.475 | 0.152 | 0.808 |

[a] 14 cells, with $<10$ nuclei but associated with pits of $>10000$ $\mu m^3$ omitted

Correlation coefficients for the 292 osteoclasts and pit size/nucleus

| | No. of nuclei/osteoclast |
|---|---|
| Volume/nucleus | −0.119 |
| Area/nucleus | −0.181 |
| Max. depth/nucleus | −0.589* |
| Mean depth/nucleus | −0.564* |

* $P<0.0001$

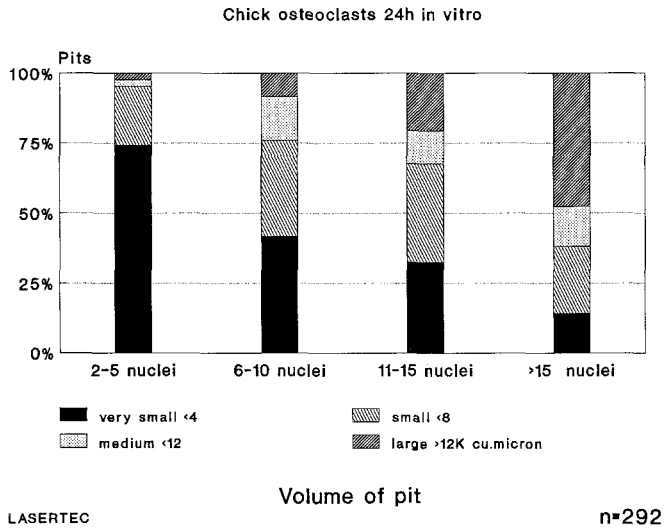


**Fig 5.** The volumes of pits made by osteoclasts of different sizes: small cells tended to make small pits and large cells large ones

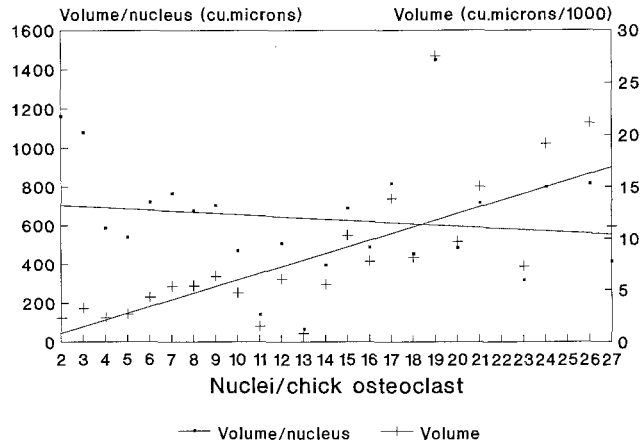


**Fig 6.** The mean pit volumes and the volume/nucleus for the cells grouped by the numbers of nuclei they contain. There is a trend for the pit volume to increase as the number of nuclei/osteoclast increases, but for the volume of tissue resorbed per nucleus to decrease

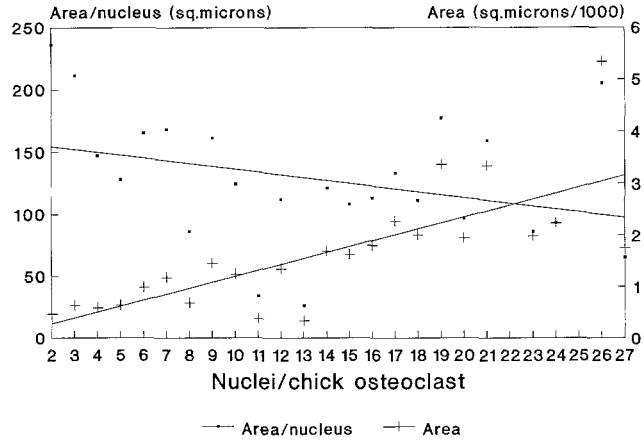


**Fig 7.** The mean pit areas and the area/nucleus for the cells grouped by the numbers of nuclei they contain. There is a trend for the plan area of the pit to increase as the number of nuclei/osteoclast increases, but for the plan area of tissue resorbed per nucleus to decrease

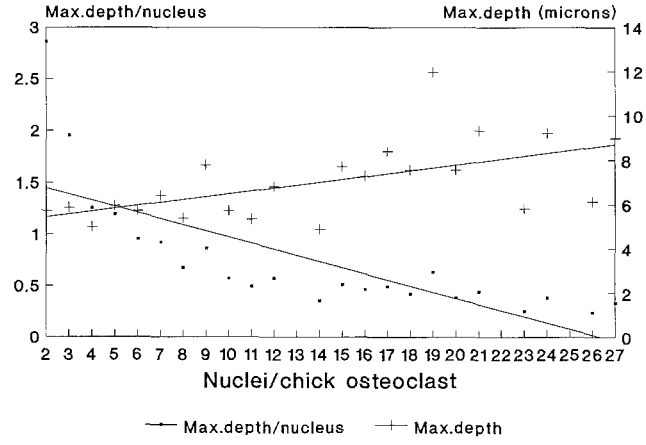


**Fig 8.** The mean pit maximum depth and the maximum depth/nucleus for the cells grouped by the numbers of nuclei they contain. There is a trend for the maximum depth to increase as the number of nuclei/osteoclast increases, but for the depth resorbed per nucleus to decrease

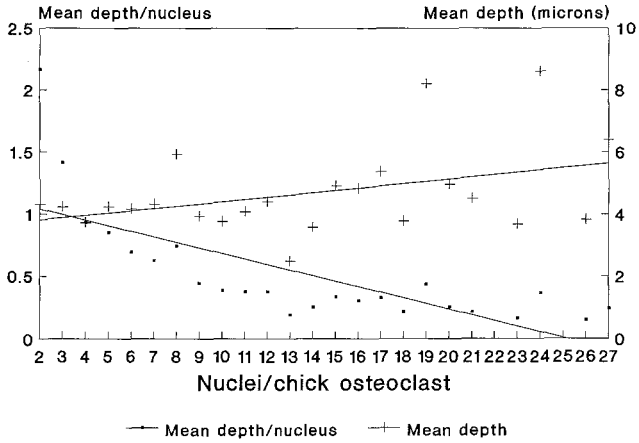


**Fig 9.** The means of the pit mean depths and the mean depth/nucleus for the cells grouped by the numbers of nuclei they contain. There is a trend for the mean depth of the pit to increase as the number of nuclei/osteoclast increases, but for the depth of tissue resorbed per nucleus to decrease

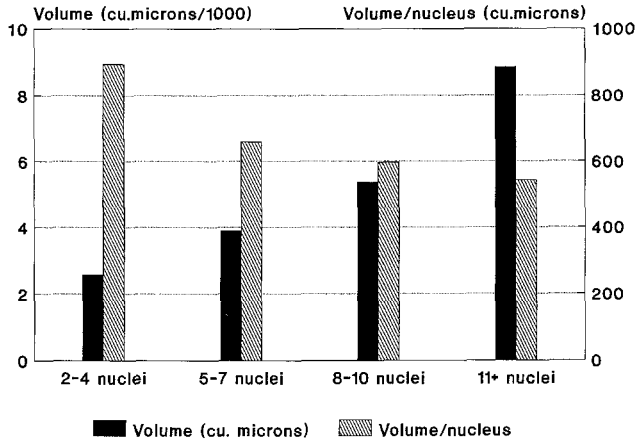


**Fig 10.** The relationship between the volume of the pit resorbed and the number of nuclei in the osteoclast, and the volume of tissue resorbed per nucleus and the number of nuclei per osteoclast. The numbers of cells in the four groups were: 2–4 nuclei $n=92$; 5–7 nuclei $n=89$; 8–10 nuclei $n=55$;11+ nuclei $n=56$. Student's $t$-test significance levels between steps were: for volumes $P=0.009$, $P=0.16$, $P=0.03$; for volumes/nucleus $P=0.12$, $P=0.64$, $P=0.70$ ($P=0.03$ between the first and last step)

## *Efficiency of resorption related to number of nuclei per osteoclast*

The depths, areas, and volumes resorbed per cell and per nucleus were assessed further by pooling groups of osteoclasts according to their number of nuclei, first in 27 sets (Figs. 6–9) and then in four larger sets (Figs. 10–13; Table 4). There was a clear trend for the depth, area, and volume of the pit to increase with an increase in the number of nuclei, but for the depth, area and volume resorbed per nucleus to decrease with increasing numbers of nuclei per cell.

When analysed in four sets so that there was an adequate number for statistical analysis in each group, the volume resorbed/nucleus and the area resorbed/nucleus were significantly less when the smallest (less than 5 nu-

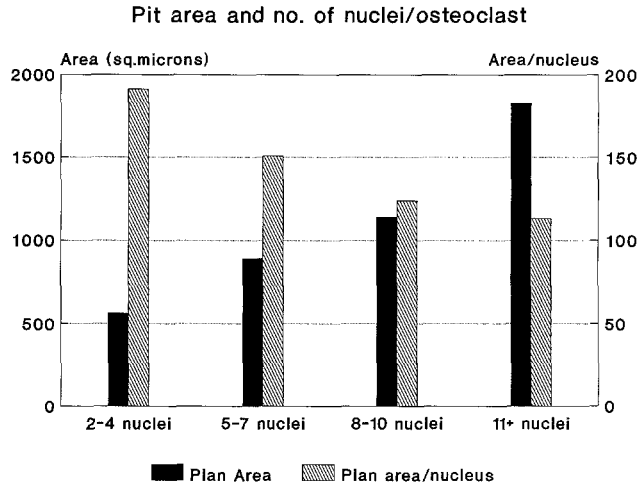


**Fig 11.** The relationship between the plan area of the pit and the number of nuclei in the osteoclast, and the plan area of tissue resorbed per nucleus and the number of nuclei per osteoclast. The numbers of cells in the four groups were: 2–4 nuclei $n=92$; 5–7 nuclei $n=89$; 8–10 nuclei $n=55$; 11+ nuclei $n=56$. Student's $t$-test significance levels between steps were: for plan areas $P=0.0001$, $P=0.09$; $P=0.005$; for plan area/nucleus $P=0.076$, $P=0.11$, $P=0.56$ ($P=0.002$ between the first and last step)

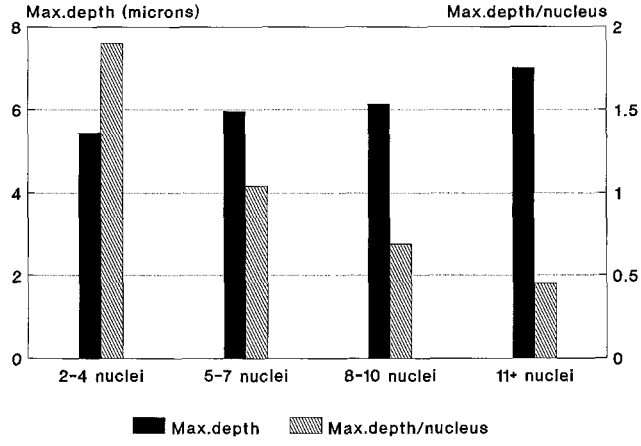


**Fig 12.** The relationship between the maximum depth of the pit and the number of nuclei in the osteoclast, and the maximum depth/nucleus and the number of nuclei per osteoclast. The numbers of cells in the four groups were: 2–4 nuclei $n=81$; 5–7 nuclei $n=81$; 8–10 nuclei $n=51$; 11+ nuclei $n=49$. Student's $t$-test significance levels between steps were: for maximum depths $P=0.14$, $P=0.58$, $P=0.15$ ($P=0.019$ between the first and last steps); for maximum depths/nucleus $P<0.0001$ between each step

**Table 4.** Work accomplished by the chick osteoclasts, grouped into four sets by their number of nuclei

| Nuclei $n$ | Cells $n$ | Mean vol. $\mu m^3$ | SEM | Mean vol./ nucleus | SEM |
|---|---|---|---|---|---|
| 2–4 | 92 | 2577 | 378 | 984 | 142 |
| 5–7 | 89 | 3895 | 329 | 657 | 51 |
| 8–10 | 55 | 5375 | 986 | 597 | 117 |
| 11+ | 56 | 8849 | 1250 | 543 | 75 |

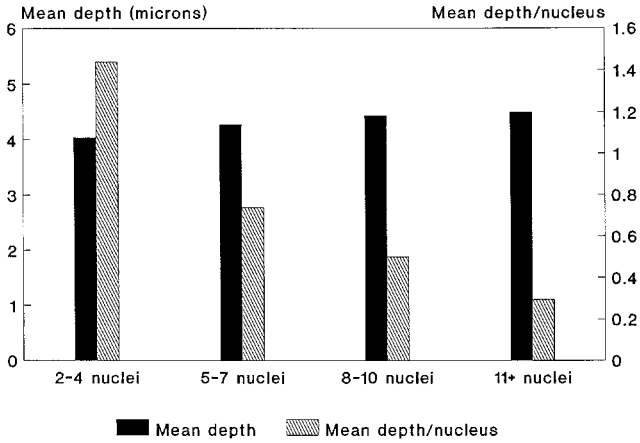


**Fig 13.** The relationship between the mean depth of the pit and the number of nuclei in the osteoclast, and the mean depth/nucleus and the number of nuclei per osteoclast. The numbers of cells in the four groups were: 2–4 nuclei $n=92$; 5–7 nuclei $n=89$; 8–10 nuclei $n=55$, 11+ nuclei $n=56$. Student's $t$-test significance levels between steps were: for mean depths $P=0.48$, $P=0.78$, $P=0.94$ ($P=0.17$ between the first and last steps); for mean depths/nucleus $P<0.0001$, $P=0.013$, $P=0.026$

clei) and largest (more than 10 nuclei) osteoclasts were compared (Figs. 10, 11). All sets were significantly different for the volumes and areas of the pits, except for the difference between the two middle groups. However each of these was significantly different from all other groups. There was no significant difference between the steps for depths (apart from the final group of maximum depths reaching significance), but each step was significantly different from the next as regards the depth/nucleus (Figs. 12, 13). The significance levels for each step are given in the captions to Figs. 10–13.

## Discussion

### *Sizes of osteoclasts*

The relative frequencies of osteoclasts with a given number of nuclei have been determined previously for a number of species. Finding the number of nuclei in such an irregular and often large cell efficiently requires it to be kept whole during the count rather than sectioned, especially as many cells need to be counted. Addison (1980), for example, used bone imprinting to obtain whole osteoclasts from kittens, and Jones et al. (1986) counted the nuclei in osteoclasts dispersed from chick, rat and rabbit bones by mechanically agitating bone fragments in fluid until the osteoclasts became dislodged, then allowing them to settle on to a dish or tissue slab. Ries and Gong (1982) studied rat osteoclasts obtained from periosteal smears, and Hefley and Stern (1982) isolated fetal rat osteoclasts by enzymatic digestion. All these methods rely on the released osteoclasts being representative of the whole population.

Remarkably, the relative frequencies of the numbers of nuclei in osteoclasts of different animals are quite similar, and not very different from counts of nuclei from human or kitten odontoclasts (Addison 1978). By counting very large numbers of cells, Addison (1980) was able to find a difference between the cumulative frequencies for nuclei counts in osteoclasts in control animals and those treated for 6 h with parathyroid extract, but the shift in the curve was small. This suggests that the number of nuclei in an osteoclast is normally controlled either by the life span of the constituent nuclei or of the cell for, although osteoclasts may reach a gigantic size, the great majority (81% in our present study; 80% of human odontoclasts in Addison's) have ten or fewer nuclei. The addition of extra nuclei does appear to parallel activation of the cells by, for example, PTH (Holtrop et al. 1979). This suggests that cell fusion may enhance resorptive activity, and not merely accompany it. The supply of preosteoclasts will affect the renewal rate of nuclei in osteoclasts, and the frequency distribution of the cells with different numbers of nuclei. It may also be that the addition of new nuclei acts as a survival factor, preventing the suicide of the cell (Raff 1992), but that there is a limit to the number of times this normally operates. Pagetic osteoclasts appear to disregard these restraints, whether produced in vivo (Basle et al. 1988) or formed in long-term cultures of Pagetic marrow (Kukita et al. 1990).

We deliberately excluded mononuclear osteoclasts or preosteoclasts (Athanasou et al. 1991) from our analysis of the distribution of nuclei in osteoclasts, although there is a growing body of support for these to be capable of resorption (Hattersley and Chambers 1989). This claim is sometimes based only on the small size of some resorption pits – a hazardous assumption given the tiny pits that may be made by any osteoclasts, and that are always made by binucleate cells at the initial stage of resorption. However some cultures with only mononuclear cells are reported to make pits, and we have found that many tiny pits as well as huge ones are produced in long term bone marrow cultures which are generating new osteoclasts (Jones et al. 1986). Amano and colleagues (1992) considered only two features as distinguishing mature osteoclasts: a ruffled border and a clear zone. We did not see pits below mononuclear cells in this study, but cannot claim to have especially sought them. Vignery and colleagues (1991) have suggested that macrophage multinucleation is accompanied by a change in gene expression and confers a specific function to macrophages. Whether this is true also for osteoclasts is not known.

It is not clear whether in vivo the addition of extra nuclei to a multinucleate cell is only by the fusion with it of mononuclear cells. If this is usually the case, the production of gigantic cells in pathological conditions might result from the unusual fusion of multinucleate cells, or from the rapid production of many new uninuclear osteoclasts and their addition to a multinucleate cell. Indeed, oversize osteoclasts generated in long-term normal marrow cultures, and capable of eroding huge single-scoop lacunae (Jones et al. 1986), are probably witness to the success of the preosteoclast production in vitro. We do not exclude the possibility that fusion of osteoclasts, or preosteoclasts with osteoclasts, may

have occurred during the culture period use in this study – we would expect some fusion. The cumulative frequency curves for the distribution of the nuclei in the osteoclasts after 6 h in culture and after 24 h were very similar, there being some cells at 24 h larger than the largest at 6 h. The smallest osteoclasts, those with two or three nuclei, may also have been slightly under-represented in the pit sample, because a tiny pit, or light etching without cavitation, below an osteoclast went undetected. However, this did not shift the cumulative frequency curves to a significant amount.

*Sizes of resorption pits*

One might expect that the correlation between pit size and osteoclast size which we found would be seen to be higher if the period of time to make each individual resorption pit were known. The choice of 24 h for the culture period was in order to allow time for the majority of the osteoclasts to make pits, but not several pits. Obviously some osteoclasts would have started later than others, and some would have made more than one pit, and not all would have continued resorption until the end of the experiment. Undoubtedly, there were also a few misfits, where an osteoclast was wrongly identified as being responsible for the adjacent pit. Additionally, the rate at which an osteoclast resorbs can vary during the making of one pit (Jones and Boyde 1988), and may even vary at two pits being made simultaneously by one osteoclast (Kanehisa and Heersche 1988). We cannot exclude at present the possibility that oligo-nuclear cells may begin to resorb earlier in vitro than larger osteoclasts.

We were not able to find any enhancement of resortive voracity in the cells with the addition of extra nuclei beyond that due to increase in nuclear number and hence size. Rather, we found a trend for the volume of mineralized tissue destroyed by an osteoclast per nucleus to decrease as the number of nuclei per cell increased. This result is consistent with the data of Zheng and colleagues (1991). These authors reported that oligo-nuclear rat osteoclasts showed greater nuclear uptake of tritiated uridine than cells with many nuclei and that the nuclei of the latter cells showed greater variation in their nuclear labelling. They concluded that the nuclei of oligo-nuclear osteoclasts have higher rates of RNA synthesis than those of osteoclasts with many nuclei. Our finding that oligo-nuclear cells resorb more dentine per nucleus than cells with higher numbers of nuclei could therefore show a direct result of a reduced synthetic potential for proteases and enzymes involved with proton secretion per nucleus of the larger cells. The plan area of a pit is dictated to a large extent by the size of the cell – its contact area; in contrast the depth of resorption strongly reflects the working efficiency of the cell.

Measuring the work done by an osteoclast is a challenge that has occupied us since 1979 (Boyde and Jones 1979; Boyde et al. 1983). The methods we have used have changed as newer technologies have become available (Boyde and Jones 1992) but the need for a volumetric measurement has not (Boyde and Jones 1991). On the assumption that no proton is wasted, and that we know the composition of the resorbed tissue, it allows us to determine how many protons are produced by an osteoclast in unit time – an indulgence in sub-molecular biology. Although we have again shown that the correlation between the area and the volume of a pit is high under standard culture conditions of short duration, it is a changing relationship with time (Delaisse et al. 1987), and changes under different experimental conditions (Taylor et al. 1990). To think otherwise would be to err.

*Acknowledgements.* This work has been supported by the MRC, SERC and The Wellcome Trust. We are grateful for the skilled assistance of Maureen Arora, and thank Mr. Komiya of Lasertec Corporation for kindly lending us the equipment to measure the resorption pits.

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