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# THE EFFECT OF SUBSTRATUM ROUGHNESS ON OSTEOCLAST-LIKE CELLS *IN VITRO*

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## Abstract

Calcium phosphate powders were used to produce three groups of experimental substrata for the culture of primary rat bone marrow cells in conditions which permitted the survival and function of osteoclasts. Each of the three experimental groups were subdivided by differences in substratum surface roughness and following a culture period of 7 to 11 days, the culture units were stained for tartrate-resistant acid phosphatase activity. In all samples both small, sometimes mononuclear, and large multinucleate cells stained positive for tartrate-resistant acid phosphatase activity and the numbers and types of cells were quantified and statistically analyzed. Following histochemical staining the samples were dehydrated and gold coated for examination by scanning electron microscopy. Cells were found to create distinct resorption lacunae in most substrata, but not on the dense, high temperature sintered hydroxyapatite, and cells responsible for this activity were confirmed as exhibiting positive tartrate resistant acid phosphatase activity. Statistical analyses showed that both the total number of tartrate-resistant acid phosphatase positive cells and the number of multinucleate tartrate-resistant acid phosphatase positive cells was greater on the rougher than the smoother surfaces.

**Key Words**: Osteoclasts, synthetic hydroxyapatite, cell culture, resorption.

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## Introduction

The surface roughness of materials has long been recognized as an important factor in the generation of cell and tissue responses to implants. Particular attention has been paid to the effects on cells of the monocyte and macrophage lineages which are the first cells to colonize the surfaces of implants, and of obvious importance in the scavenging of particulate material from and enzymatic digestion of the material surface (Gross *et al.*, 1991). Furthermore, increased cell adhesion to rougher, rather than smoother, surfaces was reported by Behling and Spector (1986).

We report herein the results of three separate experiments in which bone marrow cell populations, known to contain cells of osteoclast lineage, were cultured on various calcium phosphate preparations of different roughness. Since the expression of the tartrate-resistant enzyme acid phosphatase (TRAP) is indicative of osteoclasts, although not uniquely so (Andersson *et al.*, 1992), we used this standard histochemical assay as a marker for the presence of osteoclast-like cells.

The purpose of the experiments was to demonstrate the effect of change in substratum roughness on the appearance and nuclearity of TRAP-positive cells.

## Materials and Methods

### Cell isolation

Bone marrow cells (RBMC) were obtained from the femora of young adult male Wistar rats (approximate weight 120 gms) according to the method described by Maniatopoulos *et al.* (1988) and modified as previously described (Davies *et al.*, 1991). Briefly, femora were excised and washed four times in alpha-minimal essential medium ($\alpha$-MEM) that contained 1 mg/ml penicillin G, 500 $\mu$g/ml gentamicin and 3 $\mu$g/ml fungizone. Epiphyses were removed and the marrow from each diaphysis was flushed out with 15 ml supplemented $\alpha$-MEM containing 1/10th of the concentration of antibiotics (see above), 15% fetal bovine serum, freshly added 50 $\mu$g/ml ascorbic acid and 10 mM Na-ß-glycerophosphate. Marrow cells of both diaphyses were collected in sterile 30

ml tubes and resuspended with a 30 ml pipette. This cell suspension was used for the following experiments.

**Experiment 1**

**Hydroxyapatite**: The hydroxyapatite (HAP) used in this experiment was supplied by Taihei Kagaku Sangyo Co. Ltd., Osaka. It was synthesized by mixing 10 l of 1 M $Ca(OH)_2$ and 6 l of 1 M $H_3PO_4$ solutions at 80°C (comprising wt%: CaO, 53.60; $P_2O_7$, 40.59; $H_2O$, 5.70+), dried at 200°C (HAP200) (Sakae *et al.*, 1989) and pulverized.

**Preparation of pressed discs**: Pressed discs were produced by placing 40 mg of HAP200 powder into 13 mm diameter commercially pure titanium formers with a central depression of 10 mm and compressed at 625 MN/m$^2$ using a model 129 KBr die (Barnes Engineering Co., Stamford, CT). Different surface finishes were produced by applying a rough surface on the formers with either 600 or 320 grit silicon carbide (Sicarb) sandpaper, which had a grain size of 26 $\mu$m and 46 $\mu$m respectively. One former was left untreated and represented therefore a "smooth" surface (see Figure 1). The compressed HA had an average grain size of 0.1 $\mu$m.

**Preparation of sintered discs**: HAP200 powder was heated to 900°C and ground in a mortar. This powder was placed in 400 mg aliquots into stainless steel dies (Model 129 KBr Die) and compressed at 150 MN/m$^2$ under vacuum. The resulting discs were dried for 12 hours at 70°C and sintered for 30 minutes at 1130°C. Sintered discs had an average grain size of 0.5 $\mu$m. They were polished to a thickness of 300 $\mu$m and the surface was either left untreated, or unidirectionally roughened with 600 or 320 grit Sicarb sandpaper (Figure 2). All sintered discs were ultrasonically cleaned for 3 minutes in double distilled water and placed in 24-well trays. After sterilization by $^{60}$Co gamma-irradiation (2.5 MRad), both pressed and sintered discs were preincubated in 2.0 ml of supplemented medium for 24 hours at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ prior to cell culture.

**Cell culture**: Discs were inoculated with 150 $\mu$l of cell suspension and after 18 hours 1.5 ml of supplemented medium was added. Cultures were maintained for 8 days and refed 3 times a week in a humidified atmosphere of 95% air and 5% $CO_2$.

**Experiment 2**

**Hydroxyapatite**: HAP200 powder was prepared as described in experiment 1.

**Preparation of pressed discs**: HAP200 powder was compressed at 250, 750 or 1125 MN/m$^2$ according to the method described in experiment 1. Surface porosities were 18.1 $\pm$ 7.1, 6.1 $\pm$ 5.2, and 3.8 $\pm$ 2.3 $\mu$m respectively and were calculated as described and illustrated previously (Ogura *et al.*, 1991). The compressed HA had an average grain size of 0.1 $\mu$m. All discs were placed in 24-well plates and then sterilized and preincubated as described in experiment 1.

**Figure 1.** Surfaces created on the pressed discs using different tops. (**a**): smooth, (**b**): 600 grit, and (**c**): 320 grit finished. Field widths = 44.6 $\mu$m.

**Figure 2.** Surfaces produced on the sintered HAP discs. (**a**): original "smooth" sintered surface, (**b**): 600 grit finished, and 320 grit finished. Field widths = 44.6 $\mu$m.

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**Cell culture**: Aliquots of 1.5 ml cell suspension were placed on the discs. A further 0.5 ml of supplemented medium was added after the first 24 hours and the cultures were maintained for 7 days with one further medium change.

**Experiment 3**

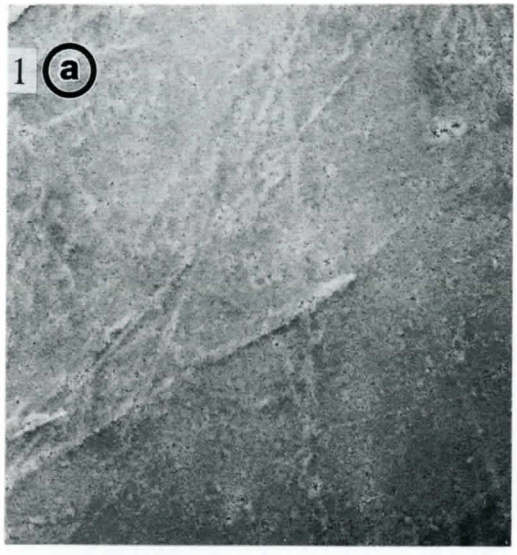
**Hydroxyapatite**: Approximately 45 g of spray dried hydroxyapatite powder (CAM-Implants bv, Leiden, The Netherlands) was placed into a stainless steel die (115 x 17 x 18 mm) which was lubricated with stearic acid. Blocks of unsintered hydroxyapatite were produced by compressing the powder under vacuum for 10 seconds at 100 MN/m$^2$. The blocks were subsequently pushed out and sintered for 6 hours in air at 1250°C. This temperature was reached at a rate of 100°C/h. The sintered HA blocks (HA/1250) had a microporosity of 1.6% and an average grain size of 3.1 $\mu$m.

**Surface roughening**: Sections with a thickness of approximately 1 mm were produced with a diamond saw and they were made to fit 24-well trays by reducing the square size to approximately 10 x 10 mm with 800 grit Sicarb sandpaper. Different surface finishes were prepared by unidirectional roughening with either 4000, 800 or 320 grit Sicarb sandpaper (grain size 5 $\mu$m, 21 $\mu$m and 46 $\mu$m respectively). Smoother surfaces were also produced by polishing with 1 $\mu$m diamond paste (see Figure 3). The samples were ultrasonically cleaned for two minutes in double distilled water, placed in a 24-well tray and sterilized by $^{60}$Co gamma-irradiation (2.5 MRad).

**Cell culture**: A 150 $\mu$l drop of cell suspension was placed on each sample and the cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. After 1 day, 1 ml of culture medium was added and the cultures were subsequently refed three times a week and maintained for 11 days. As a control, samples were incubated in culture medium in the absence of cells.

**Light microscopy**

Cultures were washed three times in culture medium without serum and subsequently fixed for 2 hours at 4°C in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4). Tartrate-resistant acid phosphatase activity was detected by a modification of the azo dye method of Barka (1960), in which 3.9 mg/ml tartaric acid was added to the incubation solution, prior to filtration.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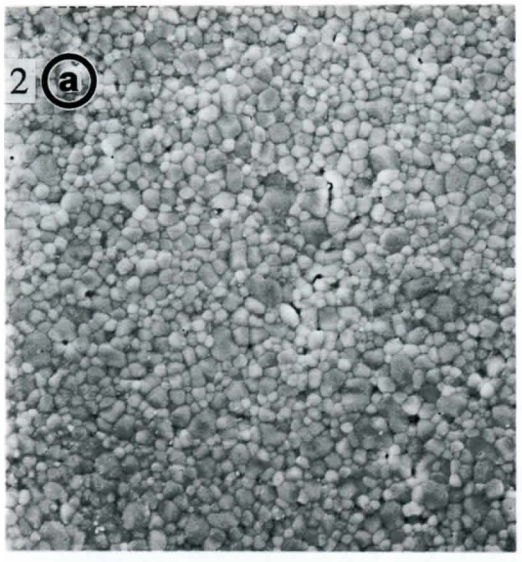
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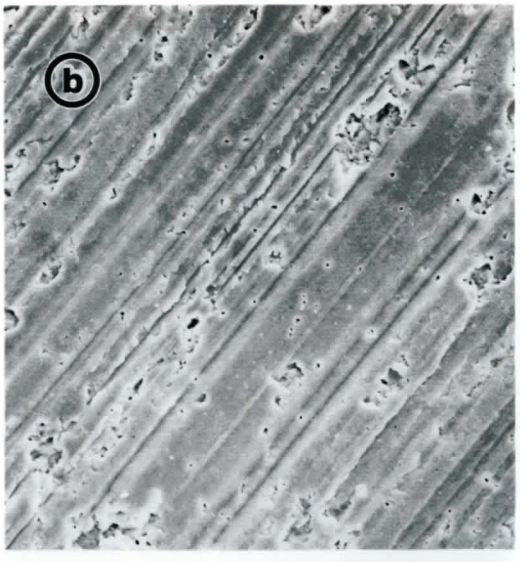
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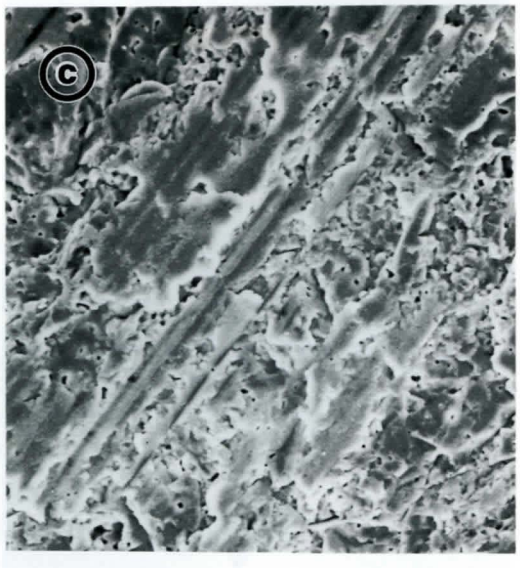
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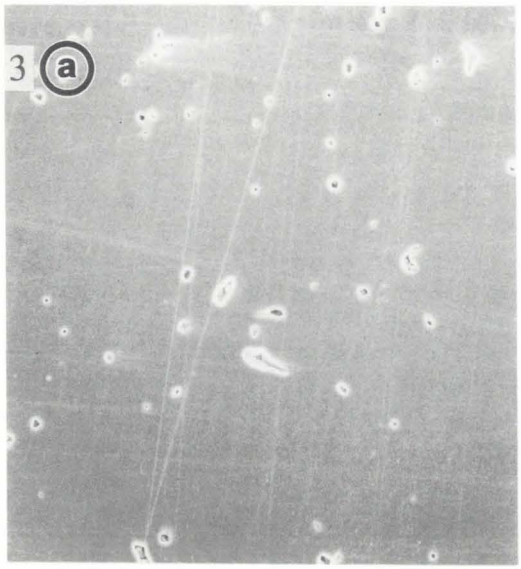
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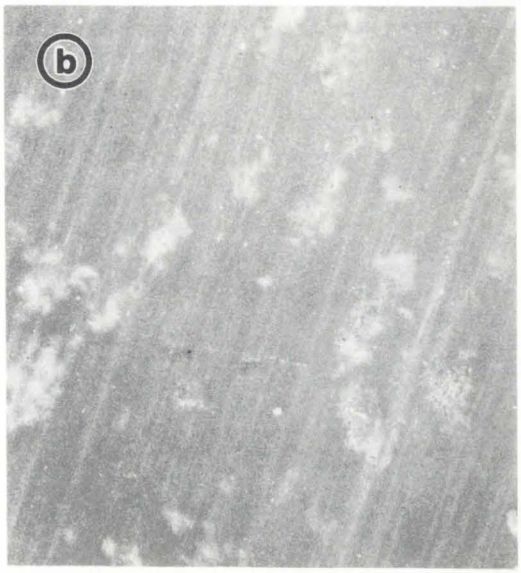
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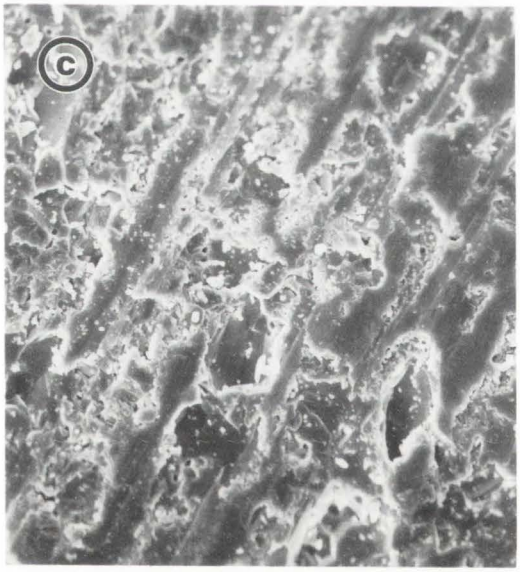
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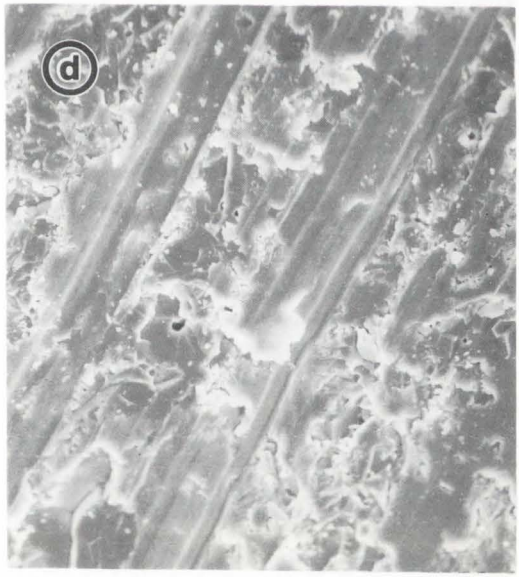
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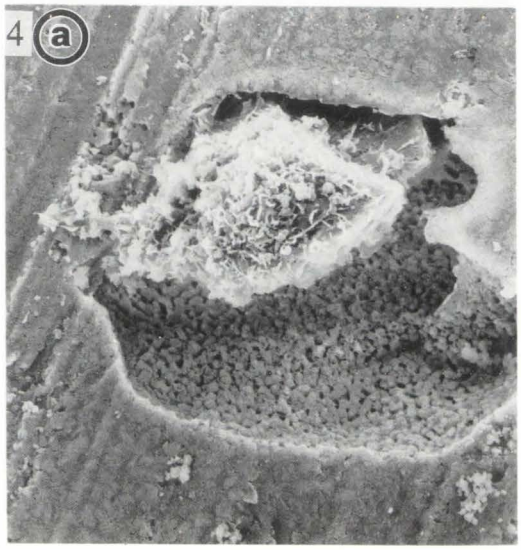
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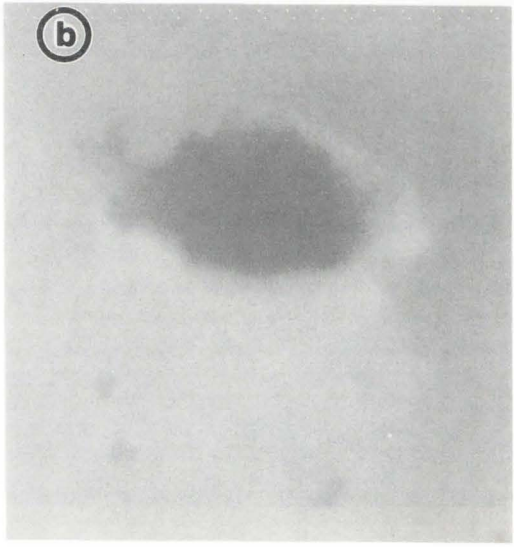
b

**Figure 3**. The disc surfaces created by 1 $\mu$m diamond paste (**a**), and 4000 grit (**b**), 800 grit (**c**), and 320 grit (**d**) silicon carbide sandpaper. Field widths = 44.6 $\mu$m.

**Figure 4**. Confirmation of the TRAP(+) character of a resorbing cell. The resorbing osteoclast-like cell on 600 grit finished sintered HA in experiment 2 examined by scanning electron microscopy (**a**), was confirmed to be TRAP(+) (**b**). Field width = 24 $\mu$m.

### Scanning electron microscopy

Cultures were fixed according to the light microscopical procedures, dehydrated through a graded ethanol series and critical point dried from $CO_2$ (Ladd Research Industries Inc., Burlington, VT). Cell multilayers were removed with compressed air and the specimens were sputter coated with gold (Polaron Instruments Inc., Doylestown, PA) and examined in a Hitachi (model 2500) scanning electron microscope operating at an accelerating voltage of 10 kV.

### Statistical analysis

Statistical analyses were carried out in the following manner for experiment 1: The total number of cells, the number of TRAP(+) cells and the number of TRAP(+) multinucleated cells were counted on each disc and the results were expressed as disc mean $\pm$ standard deviation (S.D.) for the three different types of surfaces. One-way ANOVA was used for statistical analysis. For experiment 2, the number of multinucleated TRAP(+) cells were counted in twenty fields, using a magnification of 250x and the "Student-t" test for unpaired data was used to estimate significance. For experiment 3, the number of TRAP(+) cells was counted on the whole disc and statistical analysis was performed using the "Student-t" test.

## Results and Discussion

The three experiments reported were performed by different operators. However, each experiment was completed by one operator and thus subjective observations were consistent within each group.

Figures 1, 2 and 3 show scanning electron micrographs of the materials used in the various experiments. Figure 1 shows the surfaces of the pressed discs of three distinctly different finishes. The smoothest surface still bears both some random machining marks from the surface of the titanium former as well as evident microporosity. However, in the roughened surfaces the topography created by the different formers becomes predominant although microporosity is still present. In the latter, the difference between the 600 and the 320 grit finished surfaces are also evident. In Figure 2, which shows the surfaces produced on the sintered hydroxyapatite discs, the "smooth" surface evidently comprises the individual grains of the sintered apatite and attendant microporosity. No orientational effects are seen in this

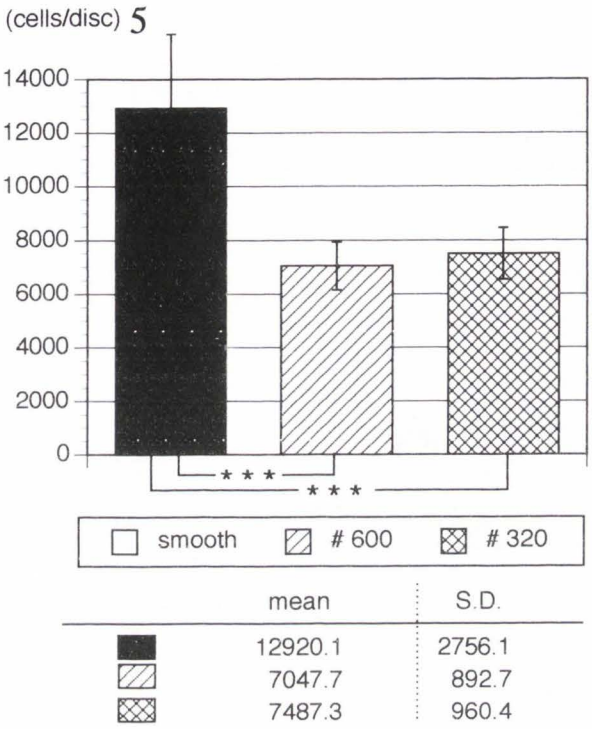


**Figure 5**. Number of total cells in experiment 1 (***P < 0.001).

preparation which can be easily compared to those illustrated in Figures 2b,c where the same type of surface has been roughened using 600 grit (b) and 320 grit (c) finishing Sicarb paper. Finally, in Figure 3, the four prepared surfaces of experiment 3 are illustrated, the smoothest (a) being that produced by 1 $\mu$m diamond paste. The subsequent photographs show the surfaces produced by finishing the sintered hydroxyapatite using 4000, 800 and 320 grit silicon carbide paper. A comparison of Figures 2b,c with Figures 3c,d demonstrate the similarity of the final surfaces in these two sintered materials, although the grain sizes are 0.5 and 3.1 mm respectively. However, a distinct difference due to the attendant microporosity is seen in the surface of the pressed discs (Figure 1c) after equivalent surface finishing.

### TRAP staining and scanning electron microscopy

As described above, superficial cells in the culture system were removed using a compressed air jet to expose the cells immediately adjacent to the underlying substratum. Figure 4a provides an example of a single small osteoclast-like cell resorbing the surface of a 600 grit finished HA sample in Experiment 1. The surface roughness of the substratum is easily seen and can be compared with that shown in Figure 2b. It is also evident that the surface of the resorption pit, in Figure 4a, displays a distinctly different morphology to either the original surface of the substratum, or those areas of the substratum where the smear layer has been removed to

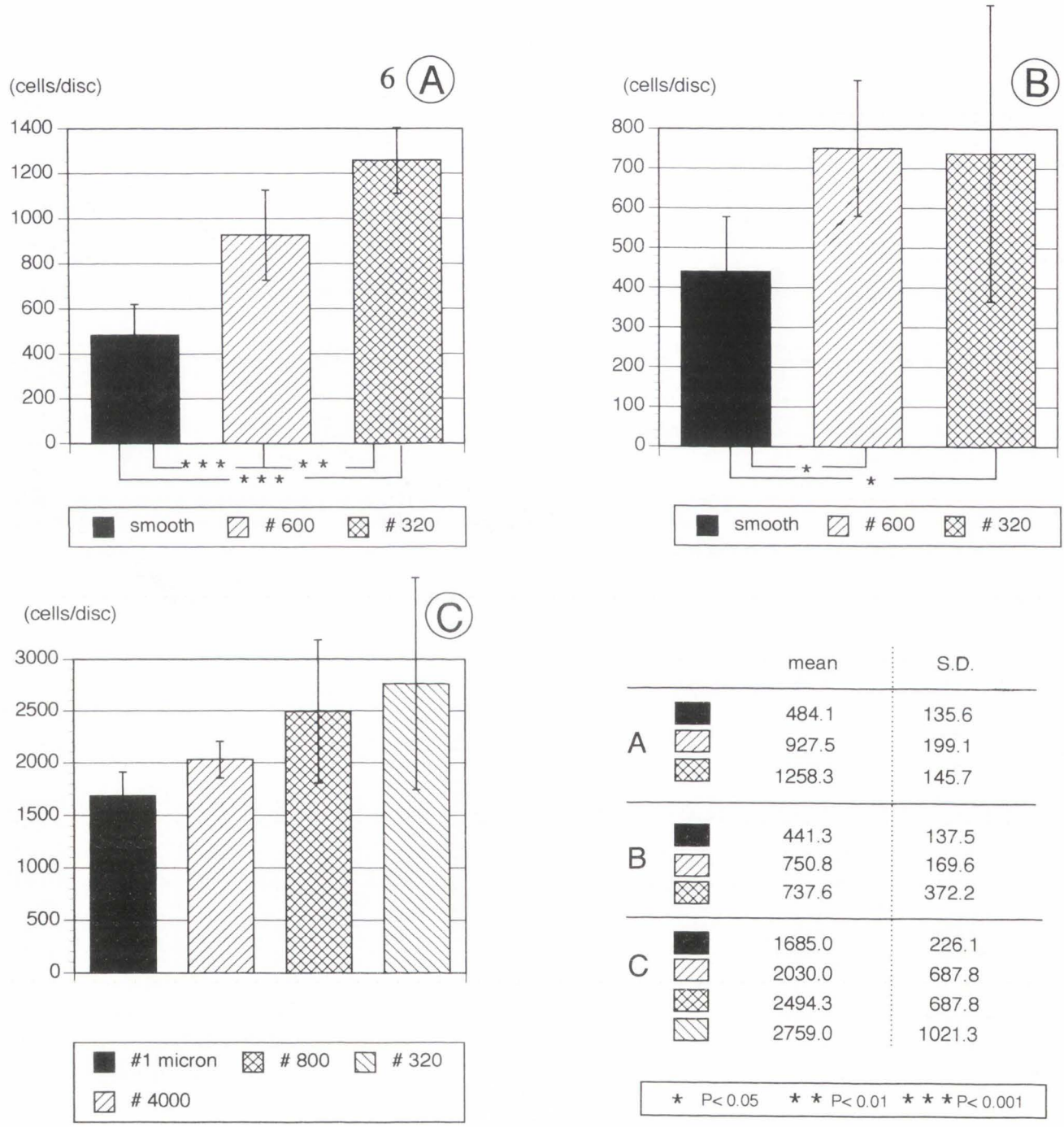


**Figure 6.** Number of TRAP(+) cells: (**a**) on the pressed discs in experiment 1; (**b**) on the sintered discs in experiment 1; and (**c**) experiment 3.

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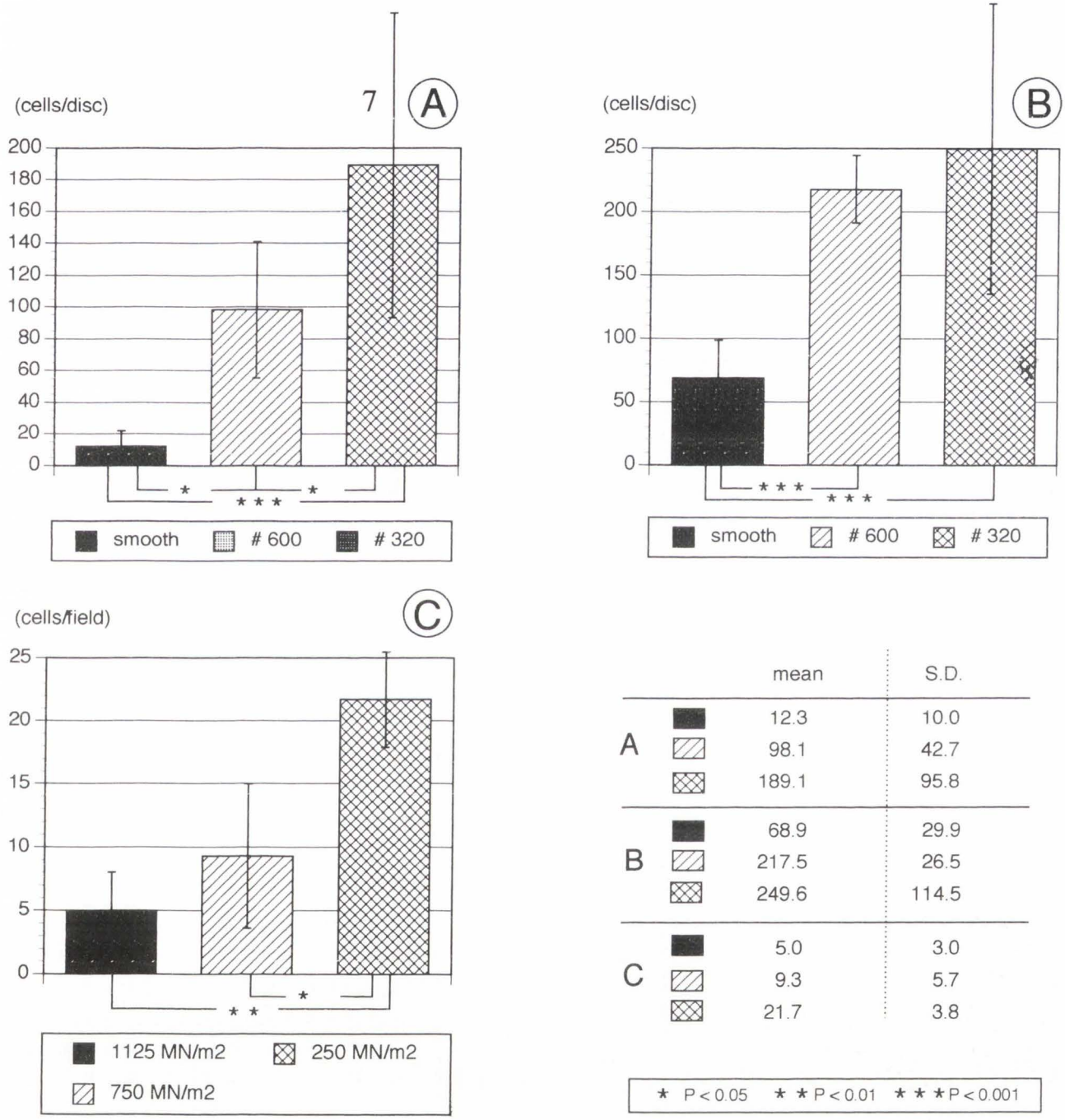
expose the underlying crystalline structure. This change in surface morphology, due to the resorptive activity of the cell, would illustrate that the surface of the substratum has been changed chemically as a result of such activity. The cell is partly hidden by a shelf of ceramic in the top part of the figure indicating that the cell is burrowing beneath the surface of the ceramic. The smoothed edges of this resorption lacunae are also evidence of cellular activity rather than simply the phagocytic removal of granular ceramic material. Such cells, which were imaged using scanning electron microscopy, were then re-examined with a light microscope for their staining for tartrate resistant acid phosphatase. Thus, Figure 4(b) shows the same cell as that in Figure 4(a)




| | | mean | S.D. |
|---|---|---|---|
| A | ■ | 12.3 | 10.0 |
| | ▨ | 98.1 | 42.7 |
| | ▩ | 189.1 | 95.8 |
| B | ■ | 68.9 | 29.9 |
| | ▨ | 217.5 | 26.5 |
| | ▩ | 249.6 | 114.5 |
| C | ■ | 5.0 | 3.0 |
| | ▨ | 9.3 | 5.7 |
| | ▩ | 21.7 | 3.8 |

* P < 0.05 * * P < 0.01 * * * P < 0.001

**Figure 7.** Number of multinucleated TRAP(+) cells: (a) on the pressed discs in experiment 1; (b) on the sintered discs in experiment 1; and (c) experiment 2.

viewed by transmitted light microscopy in which the resorptive cell is darkly stained due to a positive tartrate resistant acid phosphatase reaction. It should be emphasized that the opacity of this photomicrograph is due, not only to light being transmitted through bulk ceramic, but also the fact that following histochemical staining, the sample had been coated with gold to render it suitable for examination in the scanning electron microscope. TRAP(+) osteoclast-like cells were only seen scavenging the hydroxyapatite surfaces of debris in experiment 3, whereas no resorption lacunae were observed. This discrepancy with the results of experiments 1 and 2 may be due to the difference in grain size and microporosity, which would influence the susceptibility for dissolution of the different materials.

### Statistical analysis

**Experiment 1**: On the sintered discs, the number of total cells on the smooth surfaces was significantly higher than those on either 600 grit or 320 grit surfaces, but no significant difference was found between the number of total cells on 600 grit and 320 grit (Figure 5).

The number of TRAP(+) cells and multinucleated TRAP(+) cells on the pressed discs, was significantly different between each group: smooth, 600 grit, and 320 grit (Figure 6a and 7a).

On the sintered discs, as on the pressed discs, the smooth surface had significantly lower numbers of TRAP(+) cells and multinucleated TRAP(+) cells than the rougher surfaces (Figure 6b and 7b).

Also, we calculated the ratio between total cells and TRAP(+) cells, and between TRAP(+) cells and multinucleated TRAP(+) cells. The percentage of TRAP(+) cells to total cells on sintered discs was 3.34 $\pm$ 0.85% on the smooth, 9.64 $\pm$ 2.12% on the 600 grit, and 8.90 $\pm$ 3.33% on the 320 grit. The percentage of multinucleated TRAP(+) cells to TRAP(+) cells on pressed discs was 2.28 $\pm$ 2.25% on the smooth, 10.20 $\pm$ 4.12% on the 600 grit, and 15.58 $\pm$ 7.22% on the 320 grit. On sintered discs, the numbers were 17.02 $\pm$ 2.51% on the smooth, 30.20 $\pm$ 5.62% on the 600 grit, and 33.02 $\pm$ 5.15% on the 320 grit. All showed significant differences between the smooth surface and the others.

The number of resorption lacunae, or pits, on the smooth surfaces appeared to be significantly higher than on the roughest surfaces. However, it was very difficult to distinguish between resorption lacunae and artifacts on the rough surfaces.

### Experiment 2

The number of TRAP(+) multinucleated cells on the most porous surfaces were significantly higher than those on more compact surfaces. In contrast, the number of resorption lacunae on the compact surfaces were higher than on the porous surfaces, but there were no significant differences between groups (Figure 7c).

### Experiment 3

No significant differences were observed between each group; however, a similar tendency was shown as in experiment 2: rougher surfaces showed a higher number of TRAP(+) cells than smoother surfaces (Figure 6c).

The same trend was recognized in all experiments. That is, rougher surfaces had a higher number of TRAP(+) cells and multinucleated TRAP(+) cells than smooth surfaces, and in most instances the differences were significant. Furthermore, the TRAP(+) cells on the rougher surfaces had several stages of maturation which were represented by a staining gradient from slightly brownish to a true red, whereas mature red staining TRAP(+) cells were predominant on the smooth surfaces.

In these experiments we have deliberately chosen to employ rat bone marrow cells as primary cultures without artificially increasing the osteoclast density. While the latter procedure has been successfully employed in studies where specific aspects of osteoclast function are being examined (de Vernejoul *et al.*, 1988), we have preferred to maintain the proportions of the heterogeneous marrow population as closely as possible to that which would be found in the bony implantation bed.

## Conclusions

The results of this study show that osteoclasts are capable of resorbing certain synthetic calcium phosphates *in vitro* and suggest that substratum surface roughness stimulates the differentiation to TRAP(+) cells and induces mononucleated TRAP(+) cells to fuse to form multinucleated TRAP(+) cells.

## Acknowledgements

We are grateful to R. Chernecky for assistance with the scanning electron microscopy, and to the Medical Research Council (Canada - PG#11439), Netherlands Organization for Scientific Research (NWO) and the Ontario Centre for Materials Research (OCMR) for financial assistance.

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### Discussion with Reviewers

**S. Jones**: Can you be sure that the machined or polished surfaces (with a smear layer) have the same chemistry as those left untreated?
**C. Müller-Mai**: The experiments presented here were designed to show influences of physical implant surface properties on the attraction, activity, and nuclearity of osteoclast-like cells *in vitro*. How do the authors exclude the possibility of chemical influences since the specimens of each experiment are treated differently (e.g., by using Sicarb sandpaper or diamond paste for surface preparation or by pressing discs in Ti-formers)? Did the authors try to exclude the possibility of impurities, e.g., by applying surface analytical methods?
**Authors**: Not only were the physical implant surface properties shown to affect the osteoclasts, but also the preparative procedures employed. Thus, physicochemistry (crystallinity, grain size and microporosity) of the various types of hydroxyapatite were shown to influence the resorptive capability of the osteoclasts. Following roughening with either Sicarb sandpaper or diamond paste, the materials were ultrasonically cleaned in double distilled water for 2-3 minutes to remove grains that may have detached from the sandpaper. The smear layer is, therefore, thought to represent remains of hydroxyapatite grains, and thus to have the same chemistry. However, without surface compositional analysis, which we did not undertake on these specimens, it is not possible to provide a definitive answer to this question.

**S. Jones and C. Müller Mai**: How many discs were used in each experiment?
**Authors**: In experiments 1 and 2, 10 discs were used for each parameter tested. In experiment 3, 4 discs were used for each parameter.

**S. Jones**: Was the cell suspension used for one whole experiment derived from one rat?
**Authors**: Yes.

**S. Jones**: Was the detection of uninuclear and/or multinuclear TRAP(+) cells more difficult on some textures than on others? How thick were the discs?
**Authors**: In experiment 1 and 2, we could use a light microscope in either transmitted or incident light configuration and it was easy to recognize the number of nuclei on both the sintered and pressed discs. The thickness of the sintered discs of experiment 1 was 300 $\mu$m, but we were unable to measure the thickness of the pressed discs. In experiment 3, we used a reflected light microscope for the evaluation of TRAP(+) cells because the disc was cut approximately 1 mm thick.

**S. Jones**: How did the figures for TRAP(+) cells compare with those on plastic/glass surfaces? Did your discs entirely fill the floor of the 24 well plates?
**Authors**: We did not count the number of TRAP(+) cells on plastic or glass surfaces. We removed the discs from the 24 well plates to a special container and then counted the number of TRAP(+) cells. The diameter of the well of the 24 well plates was approximately 15 mm. The diameters of the discs for experiment 1 were sintered discs 11 mm and pressed discs 13 mm. In experiment 2, 13 mm discs were used and 10 x 10 mm squares were used in experiment 3.

**S. Jones**: You report that "the number of resorption lacunae, or pits, on the smooth surface appeared to be significantly higher than on the roughest surface". How many pits were identified and associated with a TRAP(+) cell?
**C. Müller Mai**: The authors used a compressed air jet to remove superficial cells. How can the authors exclude the possibility that osteoclast-like cells were removed? Is it possible that such cells were fixed more sufficiently, e.g., in depressions of implants with rougher surfaces as compared to smoother surfaces? This would explain the higher numbers on rough surfaces. How many empty lacunae were detected on smooth versus rough surfaces in the different experiments?
**S.C. Miller**: Is it possible that the roughness of the surface had nothing to do with the differentiation and expression of cells, rather only their adhesion properties and thus their ability to resist washing prior to the analyses?
**Authors**: Following fixation and TRAP staining, the number of TRAP(+) cells were counted employing light microscopy. Cells were **subsequently** removed with compressed air, which indeed may also have removed TRAP(+) cells. Therefore, the higher numbers of TRAP(+) cells on rough surfaces still stands, as they were counted prior to the compressed air treatment, although the association with TRAP(+) cells and resorption lacunae cannot be made, since the former may have been removed by compressed air to visualize the resorption lacunae. Thus, this type of correlation was not possible.

**C. Müller Mai**: Did the authors measure the rugosities of the final discs according to standard procedures? What is the height of individual elevations on the different implants? Are there differences comparing the form of individual grains from the different implant types?
**Authors**: Surface roughness measurements were only performed on the materials in experiment 3. For the 4000, 800, 320 grit Sicarb sandpaper and the 1 $\mu$m diamond paste treated materials, the roughnesses (Ra) were respectively 0.1, 0.2, 0.7, and 1.1 $\mu$m.

**C. Müller Mai**: The number of pits on the smooth surfaces was higher but the number of resorbing cells was lower. This would mean that rough surfaces attract more but rather non-active osteoclast-like cells. It seems, that the detection is much more difficult so that a systematic fault in counting such pits cannot be excluded. Can the authors comment on that?
**Authors**: On the rough surfaces, as we have discussed in the text, no conclusive data can be given for the number of pits, since it was difficult to distinguish between resorption lacunae and artifacts.

**C. Müller Mai**: What is the pore size of the hydroxyapatite specimens in experiments 1 and 3?
**Authors**: Although the pore sizes in all materials were in the submicron region, we did not measure the actual pore size. From Figure 4a, pores up to approximately 250 nm can be visualized.

**S.C. Miller**: Assuming the "coupling" of osteoclasts with osteoblasts in the response to implants, might it be assumed that implants with a greater surface rugosity might elicit a greater bone remodeling response?
**Authors**: This is certainly an interesting assumption, although one should first assess osteoblastic response to surface roughness, and the response of inflammatory cells, which can produce substances such as prostaglandins and interleukins, that directly affect osteoblastic and osteoclastic activities. Indeed, we have recently demonstrated that some calcium phosphate preparations are resorbed by osteoclasts, *in vitro*, only following the elaboration of bone matrix, by osteoblasts, on their surfaces [JD de Bruijn, YP Bovell, JE Davies, CA van Blitterswijk: "Osteoclastic resorption of calcium phosphates is potentiated in post-osteogenic culture conditions" submitted to J. Biomed. Mat. Res.; March 1993]. Those results provide a strong indication of the importance of coupling between not only these two cell types but also the extracellular matrix of bone.

**S.C. Miller**: Do you have any evidence of differences in pit formation by the osteoclast-like cells on the different surfaces?
**Authors**: Pits were only formed in the hydroxyapatite used in experiments 1 and 2. They were not observed in the hydroxyapatite used in experiment 3, which is most probably due to its high crystallinity, density and large grain size that makes it less susceptible to resorption. No striking morphological differences were seen in pit formation in experiments 1 and 2.

**P. Osdoby**: One theory of bone remodeling suggests that osteoblasts or other cells in the bone environment prepare the bone surface for osteoclast resorption by releasing collagenolytic enzymes and perhaps other proteases to remove osteoid and perhaps begin the digestion of the mineralized collagen. Do you believe that the roughening of the surface has any relationship to this possible mechanism? In this context if you selectively roughened one area of polished cortical bone slab would you expect the increase in osteoclast-like cells to be restricted to this area or throughout the slab when compared to a totally smooth slab?
**Authors**: This is an interesting question. The roughness of a preconditioned bone surface could well be a factor which influences the attachment of osteoclasts, although other signals (e.g., from matrix proteins) may also play an important role in the activity of osteoclasts on such surfaces. By roughening a bone surface, one would incur not only purely topographical changes, but also change the distributions of both organic and inorganic components at the surface. Chemical variations could also be created from contaminants which would depend upon the method of roughening. Thus, we would not be surprised if a correlation between changes in cell distribution and topographical variations in the underlying bone slice could be demonstrated. However, we are unable to speculate on the relative importance of these variables which do not complicate the interpretation of the present results based on only synthetic substrata.