The Journal of Laryngology and Otology
January 1995, Vol. **109**, pp. 14–18

Main Articles

Subcutaneous tissue reaction to synthetic auditory ossicle (Apaceram®) in rats

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Abstract

A study was carried out in order to obtain further information about the soft tissue response to thin Apaceram® discs of dense hydroxyapatite (HA) implanted in rats for various periods of time between one day and 10 months. The Apaceram® discs were implanted subcutaneously into the interscapular region of 33 rats. A sham operation was performed on eight rats used as controls. Decalcified histological sections stained with haematoxylin and eosin and Mallory's azan were examined and the different cell types found around the implants were counted. It was found that an acute inflammatory reaction occurred after one day and disappeared at about two weeks after implantation. In the test groups, macrophages and lymphocytes disappeared about one week later, and no inflammatory reaction was observed from one to three months. However, a tissue reaction occurred at six months with the appearance of macrophages and lymphocytes, and decreased gradually at 10 months. Meanwhile, a few foreign body giant cells at the Apaceram®–tissue interface and a thick layer of fibrous connective tissue around the Apaceram® disc were observed at 10 months. No osteogenesis was observed in any specimen. The results obtained so far suggest that Apaceram® is still a useful material for reconstructive surgery, despite the possible appearance of a slight macrophage reaction at six months.

Key words: Hydroxyapatites; Histology; Cell count; Biocompatible materials; Rats

Introduction

Hydroxyapatite (HA), the main inorganic constituent of mature bone (Boedts *et al.*, 1983), has been widely investigated as a calcium phosphate ceramic biomaterial, and is currently used in reconstructive middle ear surgery (Grote, 1990; Goldenberg, 1992). Its high biocompatibility has also been reported by these authors. However, changes in the HA–tissue interface during the early stage of implantation had not been examined in detail. We have therefore investigated the duration of the inflammatory reaction to implanted HA discs and also whether or not a chronic inflammatory reaction remains for a relatively long period after implantation. For relatively long-term implantation, the role of HA in osteogenesis still remains controversial (El Deeb *et al.*, 1990; Jahn, 1992). We decided to implant small, thin, discs of HA subcutaneously in rats and investigate the resulting histological reactions quantitatively by light microscopy within two weeks and up to 10 months after implantation.

Materials and methods

Implant material characteristics

Dense discs (diameter, 4 mm; thickness, 1 mm) of Apaceram® were prepared from commercially available synthetic auditory ossicle (Asahi Optical Co. Ltd., Tokyo), which was shown to contain 99.6 per cent pure HA [$Ca_{10}(PO_4)_6(OH)_2$] by X-ray diffraction analysis. Before implantation, the discs were sterilized at 121°C for 30 minutes.

Animals and operations

Forty-one eight-week-old female SPF Wistar rats were divided into two groups; a short-term group and a long-term group. In the short-term group, specimens of Apaceram® were implanted subcutaneously into the interscapular region of 16 rats as a test group. Eight rats (control group) underwent the same operation but did not receive any implants. Four rats from the test group and two from the control group were sacrificed at 1, 3, 7 or 14 days after implantation.

In the long-term group, Apaceram® discs were implanted in 16 rats using the same procedure as that in the short-term group and the rats were sacrificed at 1, 3, 6 or 10 months after implantation. All surgical procedures were carried out under sterile conditions under general anaesthesia using diethyl ether.

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This paper was presented at the West Japan International Symposium on Ear Surgery in Miyazaki, organized by Miyazaki Medical College, Miyazaki, Japan, on 14 May, 1994.
Accepted for publication: 3 September 1994.

TABLE I
AVERAGE PERCENTAGES OF COMPONENT CELLS IN THE TISSUE SURROUNDING IMPLANTED APACERAM® DISCS

	1 day		3 days		7 days		14 days		1 month	3 months	6 months	10 months
	T	C	T	C	T	C	T	C	T	T	T	T*
Neutrophil	37.1	9.0	–	–	–	–	–	–	–	–	–	–
Lymphocyte	4.3	1.6	2.4	1.7	1.5	–	0.7	–	–	–	4.7	2.8
Macrophage	54.1	85.1	76.9	37.1	20.7	–	2.5	–	–	–	18.9	8.5
FBGC	–	–	–	–	0.1	–	–	–	–	–	–	2.0
Fibroblast	–	–	16.4	47.1	51.3	67.4	50.0	56.3	47.8	23.7	27.4	16.5
Fibrocyte	–	–	1.4	10.9	24.3	27.5	45.1	41.4	50.4	74.8	46.0	68.3
Unidentified	4.5	4.3	2.9	3.2	2.1	5.1	1.7	2.3	1.8	1.5	3.0	1.9

FBGC: foreign body giant cells; T: test group (n = 4); C: control group (n = 2); *: n = 5.

Section preparation and staining

The Apaceram® disc and its surrounding tissue were removed as a single mass and immersed immediately in 10 per cent phosphate-buffered formalin for three days, then decalcified with five per cent formic acid for at least seven days. The implants were dehydrated in an ethanol series, embedded in paraffin, and cut into sections 6 µm thick. The sections were stained with haematoxylin and eosin and Mallory's azan.

Observation methods

The types and distribution of cells around the implants were photographed (colour slides) by light microscopy. The pictures were enlarged with a slide projector to identify and count the different cells. Over 200 cells were counted for each section and the percentages of the various component cells were calculated for each group.

Results

The tissue around the implants had two main structures, a layer of acute inflammatory cells, such as neutrophils, macrophages and lymphocytes, and a surrounding layer of fibrous tissue composed of fibroblasts and fibrocytes, and in some cases capillaries. The average percentages of the component cells are shown in Table I.

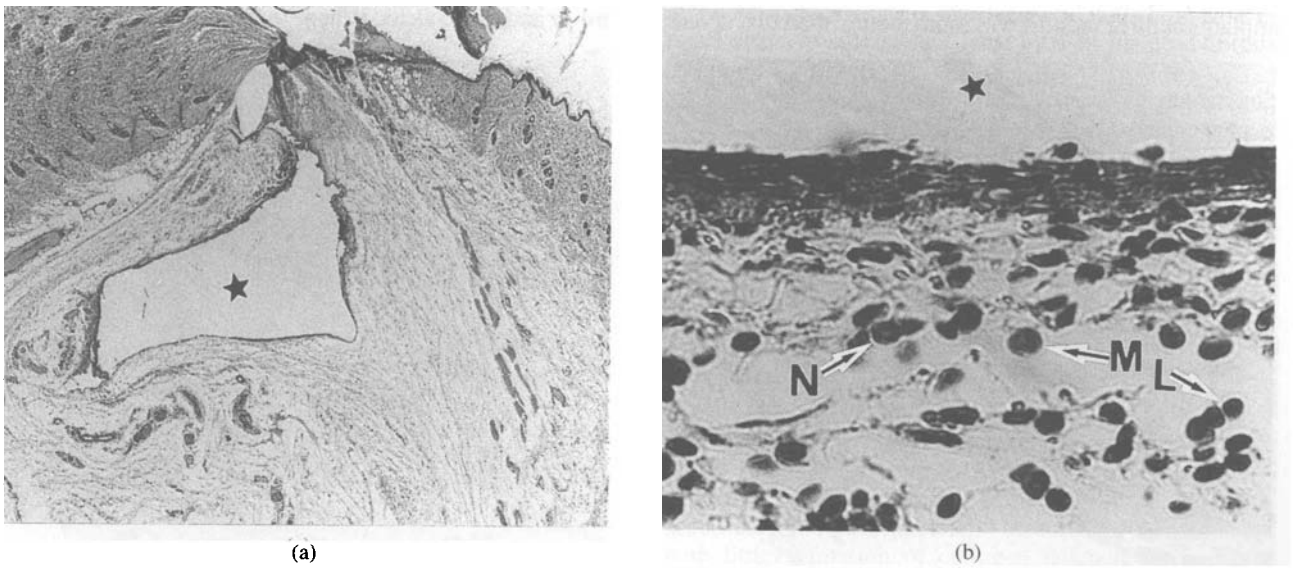
Specimens examined within two weeks

Sections from specimens removed one day after implantation showed an acute inflammatory response around the implants (Figure 1 a, b). Cell counts revealed a predominance of macrophages (54.1 per cent), followed by neutrophils (37.1 per cent) and lymphocytes (4.3 per cent). Neither fibroblast nor fibrocyte proliferation was observed in any of the specimens.

Specimens taken three days after implantation (Figure 2) showed a sharp decrease in neutrophils to almost zero, whereas macrophages had increased to 76.9 per cent. In this group, both fibroblasts and fibrocytes began to be observed.

Sections from specimens removed seven days after implantation (Figure 3) showed a decrease of macrophages to 20.7 per cent. The implants were surrounded by a layer of fibroblasts (51.3 per cent) and fibrocytes (24.3 per cent). Lymphocytes still remained at a low level (1.5 per cent). One specimen in this group revealed foreign body giant cells (0.1 per cent).

Specimens obtained 14 days after implantation (Figure 4) showed that macrophages still remained at a low level (2.5 per cent). The implants were surrounded by a thin layer of fibroblasts (50.0 per cent) and fibrocytes (45.1 per cent), and only a few lymphocytes were still evident (0.7 per cent).



(a) (b)

FIG. 1

(a) Low power photomicrograph one day after implantation showing a thin layer of inflammatory cells around the Apaceram® disc (★), appearing like a cyst. (H & E; × 15). (b) High power photomicrograph of the thin layer shown in (a); neutrophils (N) and macrophages (M) are predominant. L: lymphocyte; ★: Apaceram® disc. (H & E; × 600).

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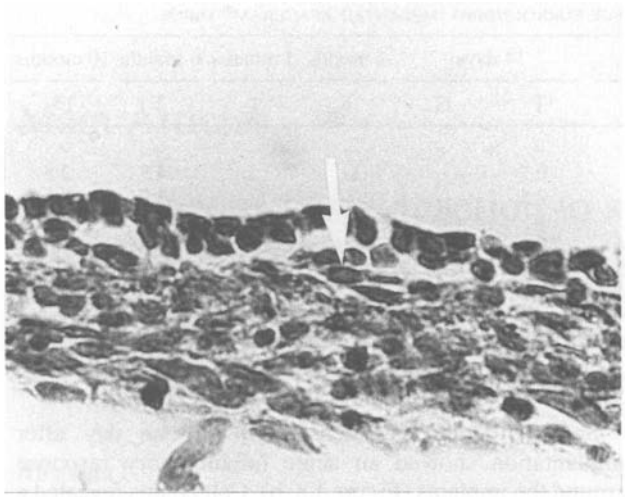

FIG. 2

Three days after implantation, showing a decrease in acute inflammatory cells and appearance of fibroblasts (arrow). (H & E; × 600).

Compared to the test groups, the controls showed the presence of macrophages (85.1 per cent) one day after surgery, and these had completely disappeared by the seventh day after surgery. Neutrophils (9.0 per cent) were evident only one day after surgery. Fibroblasts and fibrocytes increased gradually after three days. The microscopic changes evident in the controls were typical of those normally resulting from surgical intervention.

Specimens examined after one month

At both one and three months after implantation, there was a thin fibrous connective tissue capsule around the implant, with collagen slightly more mature than that found at two weeks. Cell counts showed that fibroblasts and fibrocytes predominated. In comparison with the specimens taken at two weeks, macrophages and lymphocytes had almost disappeared at one and three months after implantation. The collagen that encapsulated the implants at three months was more mature than that at one month. Mallory's azan staining revealed that the Apaceram® disc was encapsulated by a layer of fibroblasts, surrounded by a layer of fibrocytes (Figure 5).

Unexpectedly, in specimens removed at six months after implantation (Figure 6), reappearance of macrophages and lymphocytes was observed. Very fine granular particles were present in the cytoplasm of the macrophages adjacent to Apaceram® discs. In view of the remaining Apaceram® in contact with the macrophages, it seemed likely that these particles were implant-derived material, possibly indicative of biodegradation. In spite of the relative increase in macrophages, a large part of the implant was surrounded by a mature fibrous capsule composed of dense collagen interspersed with fibroblasts and fibrocytes.

At 10 months (Figure 7), a few rather flat foreign body giant cells as well as macrophages and lymphocytes were observed at the Apaceram®-tissue interface, and the Apaceram® discs were surrounded by well matured fibrous connective tissue.

No osteogenesis was found during the entire experimental period.

Discussion

After implantation of a biomaterial, the responses that occur at the interface of the implant and in the surrounding environment are important in determining its biocompatibility. The biological response to an implant can be attributed to acute and chronic inflammatory changes, following the formation of a fibrous capsule, which occur over a period of time (Anderson and Miller, 1984). In the present study, the results obtained in the control group within two weeks were compatible with the normal healing of skin wounds. In the test group (Figure 8 a), the percentages of neutrophils and lymphocytes were four-fold and two-fold greater respectively than those in the control group. The neutrophils quickly disappeared after three days of implantation, whereas the lymphocytes persisted at a lower level until day 14 after implantation. The peak of macrophages appeared two days later than in the controls, and these cells almost disappeared at two weeks

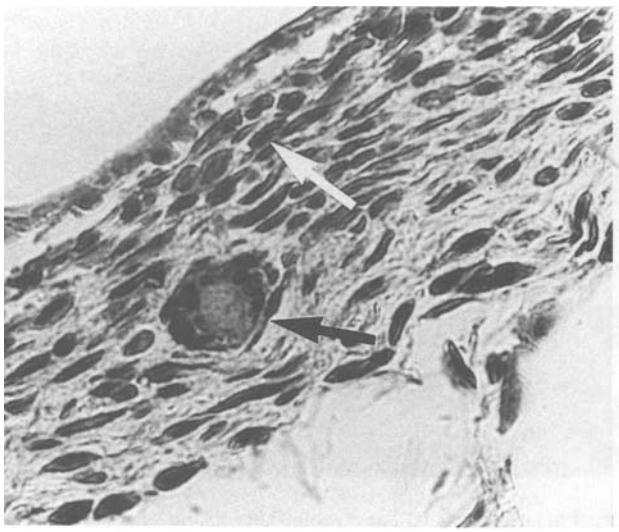

FIG. 3

Seven days after implantation, showing fibroblasts (white arrow) and foreign body giant cells (black arrow). (H & E; × 600).

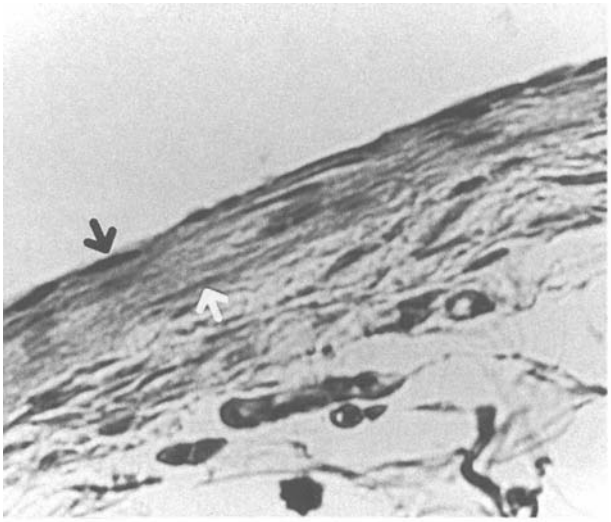

FIG. 4

Fourteen days after implantation, showing a layer of fibroblasts (black arrow) and fibrocytes (white arrow). (H & E; × 600).

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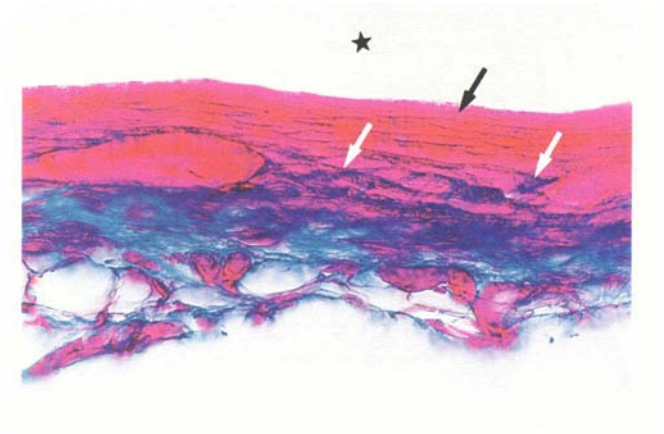

FIG. 5

One month after implantation, showing a thin layer of fibroblasts (black arrow) in contact with an Apaceram® disc (★) and outside it a thin layer of fibrocytes (white arrows). (Mallory's azan; × 600).

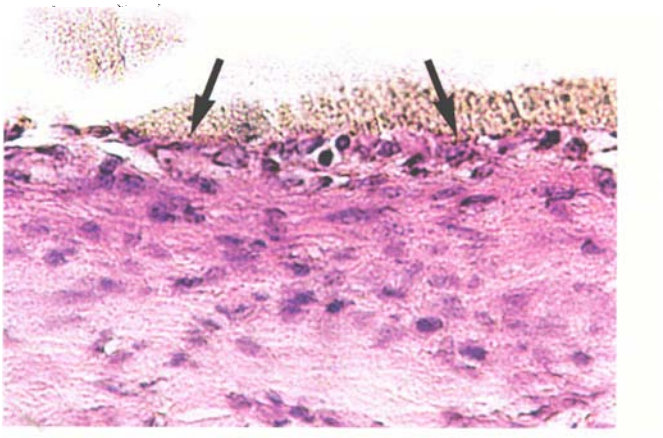

FIG. 6

Six months after implantation, showing fine particles (arrows) in the cytoplasm of macrophages at the Apaceram®–tissue interface, proliferation of fibroblasts, an increased amount of collagen and infiltration by inflammatory cells. (H & E; × 600).

after implantation, which was nearly one week later than in the controls. This must have been due to the presence of the implant, and indicated an attempt by the macrophages to induce healing around the Apaceram® disc. There is no doubt that macrophages play a central role in wound repair and tissue reorganization. They have been shown to have the ability to control fibroblast activity, and thus indirectly the formation of collagen (Leibovich and Ross, 1976; Henke *et al.*, 1993). As normal wound healing is disturbed by the presence of an implanted Apaceram® disc, the soft tissue healing at the implantation site can be considered to be a secondary healing process.

The stability of an implant depends largely on the behaviour of cells at the material–tissue interface. Since macrophages are cells which are particularly relevant to material compatibility, their changes and activities around an implant in the period after implantation may reflect the materials biocompatibility. Macrophage counts at two weeks after implantation were very low, and no macrophages were evident at all at one and three months. Microscopic observation of the specimens at one and three months after implantation also confirmed that the Apaceram® discs were encapsulated by a thin layer of fibrous tissue. This may be considered to be a stable stage for the implant. However, after three months, macrophages appeared to be reactivated. The proportion of macrophages increased gradually up to 18.9 per cent at six months (Figure 8 a), and microscopy revealed that their nuclei were enlarged and the amount of collagen had increased. Meanwhile, lymphocytes and proliferated fibroblasts were observed at the Apaceram®–tissue interface. A possible cause of the increase in macrophages and lymphocytes at six months, was the deposition of an amorphous material adjacent to the interface of the capsule (see Figure 6). It is hypothesized that the inflammatory response might be modulated by the chemical composition and physical properties of the implanted material (Orly *et al.*, 1989). As calcium and phosphate ions are essential cell activity mediators, some changes in their concentrations may produce specific cell responses (Rubin, 1985). However, this occurred only in some areas at the Apaceram®–tissue interface, and the whole Apaceram® disc was still encapsulated by well-matured fibrous tissue at 10 months.

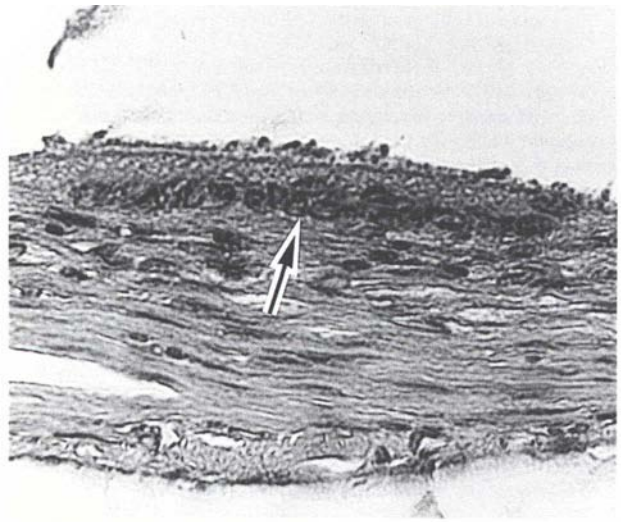

FIG. 7

Ten months after implantation, showing foreign body giant cells (arrow) at the Apaceram®–tissue interface. (H & E; × 600).

With respect to the capsule composed of fibroblasts and fibrocytes shown in Figure 8 b, fibroblasts began to appear on the third day, increased to a maximum on the seventh day, and then decreased gradually for up to 10 months after implantation. In contrast, fibrocytes continued to increase from three days to three months, and then remained at almost the same level for up to 10 months, with slight fluctuation. Fibrocytes usually demonstrate a mature fibrous connective tissue. As the shapes of fibroblasts and fibrocytes depend to some extent on their physical substrate, the outlines of their cell bodies are difficult to discern in some cases by haematoxylin and eosin staining. However, the use of Mallory's azan staining, as used in this study, clearly differentiates fibroblasts from fibrocytes. As shown in Figure 5, fibroblasts were found to accumulate around Apaceram® discs as red-staining cells with little formation of collagen (stained blue), while fibrocytes were stained dark blue showing production of collagen at the two ends of cell body. Therefore, the maturity of the connective tissues around the implanted Apaceram® disc was confirmed histologically.

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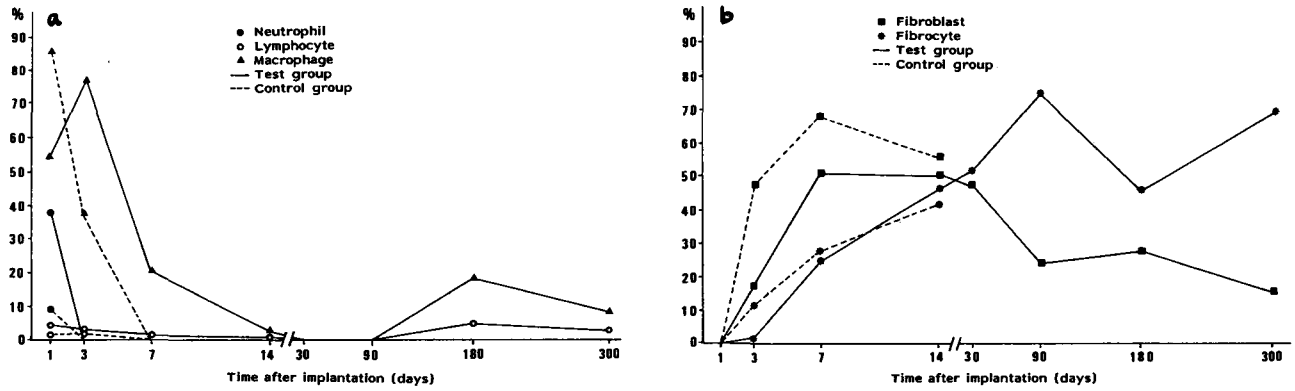

(a) (b)

FIG. 8

(a) Changes in the population of inflammatory cells after implantation. (b) Changes in the population of fibroblasts and fibrocytes after implantation.

Foreign body giant cells (FBGC) were first observed at seven days after implantation, and then reappeared in 10-month specimens accompanied by macrophages and lymphocytes. The formation of FBGC represents a common phenomenon during the course of reaction to foreign bodies. It is thought that FBGC are formed through fusion of activated macrophages (Murch *et al.*, 1982; Hassan *et al.*, 1989). Since FBGC are characterized by decreased phagocytic activity accompanied by lower activity of both acid phosphatase and sodium tetrazolium reductase, their appearance on the surface of implants may represent a barrier-like structure which separates the surrounding tissue from a foreign body which cannot be eliminated or degraded (Smetana, 1987).

Biodegradation of HA has been reported by some authors. Transmission electron microscopy has demonstrated a large number of implant-derived materials in the cytoplasm of macrophages, and these have been further studied by X-ray microanalysis (Grote *et al.*, 1986). A study on biodegradation using a ^{45}Ca tracer confirmed *in vivo* degradation of implanted calcium phosphate ceramics by a biochemical dissolution process (den Hollander *et al.*, 1991). In the present study, we also found very fine particles in the cytoplasm of macrophages at the Apaceram®–tissue interface. This was probably because of a pH shift during inflammation and a phagocyte-induced process (LeGeros *et al.*, 1988). These two factors, either alone or in conjunction, may cause surface dissolution of the Apaceram® disc and thus induce a foreign body response.

None of the animals used in this study showed osteogenesis in the subcutaneous tissues at any stage. This supports observations by other investigators that HA is not an osteoinductive material (El Deeb *et al.*, 1990).

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https://doi.org/10.1017/S0022215100129135 Published online by Cambridge University Press