JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 9 (1998) 535–541

Studies of the healing of bone grafts, and the incorporation of titanium implants in grafted bone: an experimental animal model

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An insufficient quality and amount of bone often necessitate the clinical use of implants together with bone transplants. The present study describes an experimental animal model for the study of implants in bone grafts. Circular defects were made bilaterally in the tibia of 36 rabbits. The defects received either autologous cortical bone (control), demineralized bone matrix (DBM), plasma-augmented DBM or were left empty (without bone graft). In all defects a titanium implant was centrally placed and anchored in the opposite cortex. Evaluation with light microscopic morphometry showed that the insertion of a threaded titanium implant centrally in a cortical defect was followed by a spontaneous healing of the defect after 6 mon. After 6 wk, all implants in cortical grafts were well integrated with a significantly higher bone-to-implant contact than in the DBM and plasma-augmented groups. After 6 mon, all experimental groups had a mean bone area within the threads ranging between 69% and 80% and a mean bone-to-implant contact between 31% and 42%. The results from the present study indicate that the model allows comparative studies on the early formation, resorption and remodelling of bone around implants after modification of implant, graft and host properties.

1. Introduction

Lack of adequate bone volume and density of the jaws, particularly common in the posterior maxilla, may prevent the clinical use of implants. In these situations, bone grafting and subsequent rehabilitation with implants may be the only alternative to dentures [1]. In addition, tumour surgery, previous excisions and local pre- and postoperative irradiation, provide a non-optimal host site for healing of implants and grafts and accordingly raise further problems which must be coped with [2]. Several issues of importance for the use of grafts and implants have previously been discussed [3–5]. These include factors related to surgery and control of trauma (e.g. the atraumatic handling of the graft, minimal preparation time, optimal drills and avoidance of excessive heat), integration of the implant in the graft bone (including time allowed for healing, available graft and host bone volume, density and cortical thickness) and the integrity of the soft issue (such as optimal healing and shielding of the graft area).

It is known that iliac onlay bone grafts in the maxilla will exhibit bone resorption [6]. Further, the loss of implant is more frequent in iliac grafts compared to non-graft cases [7, 8]. Because resorption is a major problem in conventional cortical bone grafts, it is of importance to find methods for bone augmentation with osteoinductive potential. Promising results have been presented with regard to osteogenic capacity using different kinds of proteins [9–11]. Therefore, it is important to evaluate such therapeutic measures in connection with the insertion of implants. Despite the routine clinical use of a number of both inlays and onlay grafts, there is a lack of information

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with regard to the structure and function of the bone–implant interface in grafted tissue. Furthermore, it is not known if the process of bone formation around implants inserted in grafted bone occurs in the similar manner as described for titanium implants in normal cortical bone of rabbits [12, 13] and humans [14]. It is therefore difficult to determine the pathophysiology and mechanism(s) of failure for implants inserted in grafted bone.

Several of the issues related to implant success/failure may be studied in experimental and clinical models. The aims of the presented study were:

1. to develop an animal model with the possibilities to create a well-defined, reproducible defect that allows the incorporation of different implant and graft materials;
2. to characterize the interaction between titanium implants and different types of grafted bone with light microscopic (LM) morphometry, as well as the incorporation of these grafts in the host issue.

2. Materials and methods

2.1. Titanium implants

Titanium implants were threaded and manufactured by machining of a commercially pure (99.7%) titanium rod. A central implant (2.0 mm in diameter and 10.0 mm length) and two peripheral markers (2.0 mm diameter and 1.5 mm length) were used at each implantation site. All implants were ultrasonically cleaned in trichlorethylene (10 min), acetone (10 min), and ethanol (99.5%) for 3×10 min, and thereafter air dried. Immediately before surgery, the implants were placed in polymer sterilizing bags and steam sterilized.

2.2. Preparation of demineralized bone matrix

The femur and tibial diaphysis of three New Zealand white rabbits were used to prepare freeze-dried demineralized bone matrix (DBM) [15]. After complete removal of attached soft tissue, the bone was washed in sterile deionized water, and the bone segments were extracted in a 1 : 1 chloroform and methanol solution (30 ml/g bone) for 90 min and left to dry overnight at 30 °C. The specimens were cut in 10 mm pieces and milled in a bone mill in liquid hydrogen (Spex freezer mill, Spex Industries Inc., Metuchen, NJ USA), after which the material was sieved to a particle size between 60 and 300 μm. The bone powder was demineralized in 0.6 M HCl for 3 h (50 ml/g bone matrix). The pH was adjusted to 6.0 by rinsing in serial changes of sterilic deionized water and lyophilized. Three batches of DBM were prepared on three different occasions. The preparations were stored at − 70 °C.

2.3. Preparation of plasma

Two batches of fresh rabbit blood, anticoagulated with trisodium citrate dihydrate was obtained from the Swedish Institute for Infectious Disease Control, Stockholm. The blood cells were separated from plasma by centrifugation (6000 r.p.m. for 15 min). The batches were kept at − 70 °C.

2.4. Sterility and endotoxin content

To confirm sterility, bacteriological samples (plasma and lyophilized DBM) were taken and incubated (aerobic and anaerobic) before storage at − 70 °C. The endotoxin content was determined using the chromogenic *Limulus* amoebocyte lysate test [16]. The plasma was found to contain 0.11 ng ml^{-1} at 6 wk and 0.03 ng ml^{-1} at 6 mon and the endotoxin level in the DBM was $\leqslant$ 0.01 ng mg^{-1} at both times.

2.5. Animals

A total of 36 adult New Zealand White rabbits of both sexes, weighing 3–4 kg, were used. The animals were studied for 6 wk and 6 mon, respectively, kept in separate cages and fed a standard diet and tap water *ad libitum*. The experiments were approved by the Local Ethics Committee, Göteborg University.

2.6. Surgery

The animals were anaesthetized by intramuscular (i.m.) injections of a combination of phentanyl and fluanizone (Hypnorm Vet.®, Janssen Farmaceutica, Denmark) (1 mg/kg body weight (b.wt) and intrapertoneal (i.p.) injections of diazepam (Apozepam®, Apothekarnes lab. A.S. Oslo, Norway) (2.5 mg/kg b.wt). Lidocaine (5% Xylocain®, Astra AB Södertälje, Sweden) was infiltrated subcutaneously to obtain local anaesthesia. A careful surgical technique was applied under aseptic conditions, and all hard tissue preparation was performed under generous irrigation with sterile saline (NaCl 9 mg ml^{-1}; ACO, Sweden) using a dental handpiece at low speed.

The proximal tibiae were used for the bone grafting procedure. After an incision through the skin and periosteum, a flap was raised to expose the bone. The facilitation and standardization of drilling, creation of defects and preparation of transplants were enabled by the use of a drill guide (Fig. 1a). The guide was placed on the surface of the tibial metaphysis. Two holes (diameter 1.8 mm) were drilled through the upper cortex via the peripheral holes (Fig. 1b) in the drill guide and the two peripheral markers were inserted. This procedure allowed the drill guide to be secured in position. Thereafter, a circular defect was made in the tibiae with a trephine drill (Fig. 1c) inserted through the middle hole in the drill guide. After insertion of a centre piece (with a 2 mm diameter internal hole) (Fig. 1d) into the middle hole of the drill guide, a hole was drilled (diameter 1.8 mm) and prethreaded through the centre piece, the bone marrow and into the opposite cortex, where a 10 mm titanium implant was anchored.

In 12 animals, a cortical graft was harvested from one tibia (defect without bone graft, Fig. 2a), and transferred to the other tibia, (cortical graft, Fig. 2b).

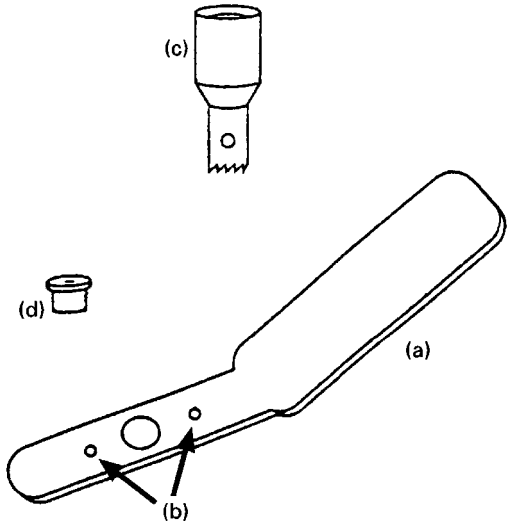


Figure 1 Schematic illustration of (a) titanium drill-guide, (b) holes for peripheral titanium markers, (c) trephine drill, (d) centre piece.

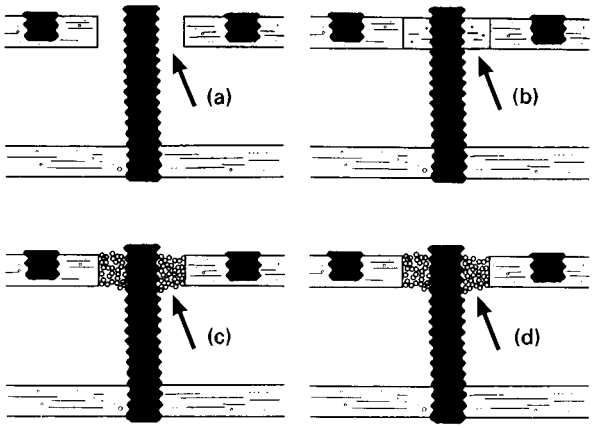


Figure 2 Schematic illustration of (a) defect without bone graft, (b) cortical bone graft (control) (c) defect grafted with demineralized bone matrix (DBM) and, (d) defect grafted with plasma-augmented DBM.

The cortical graft was prepared with the unicortically and centrally placed implant with the aid of the centre piece, before drilling with the trephine drill. Thereafter, the graft with implant was transferred to the recipient defect where the opposite cortex had been prethreaded (Figs 2b and 3). The defects were 4 mm diameter on the recipient site (cortical graft) and 5 mm on the donor site (the cortical graft diameter corresponded to the inner diameter of the 5 mm trephine drill). In the defects without bone graft, the implants were inserted and firmly anchored in the opposite cortex. In 24 animals, the defects were 5 mm diameter on both legs, of which half were grafted with DBM while the other half received plasma-augmented DBM (Fig. 2c, d). As described earlier, a central implant was anchored in the opposite cortex. The DBM (10 mg per defect) was saturated in either sterile saline or plasma, and packed in a modified syringe (inner diameter 5 mm) just before extrusion around the implant. Despite of some bleeding from the marrow cavity, the bone matrix particles (with or without plasma augmentation) appeared to fill and remain in the defect (Fig. 4). Immediately after

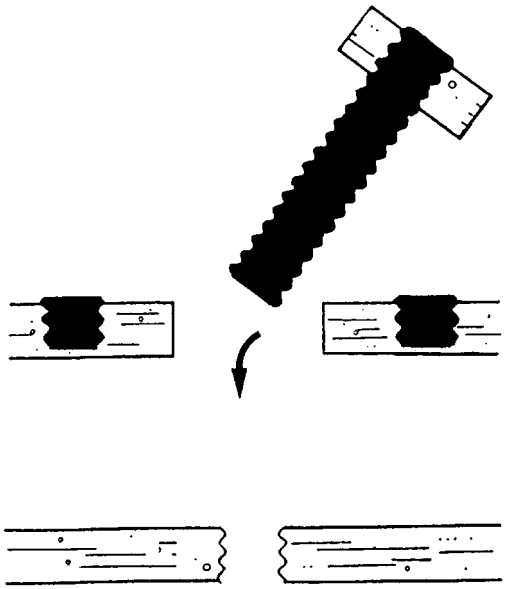


Figure 3 Schematic drawing of a cortical graft with implant, being transferred to the recipient defect.

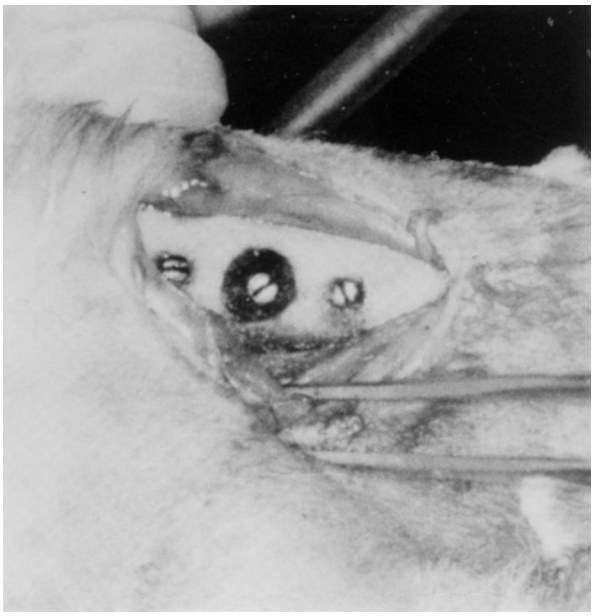


Figure 4 Photograph of a tibial defect grafted with DBM.

the grafting procedure, the periosteum was repositioned and sutured to prevent bleeding and scattering of the DBM particles.

All wounds were closed in thee layers: the periosteum and muscle fascia with resorbable polyglactic sutures and the skin with polyamide sutures. Postoperatively, antibiotics (0.1 ml/kg b.wt., Intencillin, LEO®, Pharmacia, Sweden) and analgetics (0.05 mg/kg b.wt., Temgesic®, Rechkitt and Coleman, USA) were administered as single i.m. injections daily for 3 d.

2.7. Animal sacrifice

The animals were killed after 6 wk and 6 mon, respectively, with an overdose of barbiturate (Mebumal®, ACO Läkemedel AB, Solna, Sweden) and fixed by perfusion with 2.5% glutaraldehyde in 0.05 M sodium

cacodylate buffer, pH 7.4. The implants and the surrounding bone tissue were removed *en bloc*, further immersed in glutaraldehyde for 24 h, and post-fixed in 1% osmium tetroxide for 2 h. After dehydration in ethanol, the undecalcified specimens were embedded in plastic resin (LR White, The London Resin Co Ltd, Hampshire, UK).

The specimens were divided longitudinally by sawing (Exact cutting and grinding equipment, Exact Apparatebau, Norderstedt, Germany) and ground sections of 15–20 μm [17] prepared and stained with 1% toluidine blue.

2.8. Light microscopy and morphometry

Light microscopic morphometry was performed on the ground sections using a Leitz Microvid equipment connected with a Microvid computer. The specimens were evaluated with regard to bone area and bone-to-implant contact in the threads of the central implant.

2.9. Statistics

The Kruskal–Wallis test was used for statistical analysis.

3. Results

3.1. Morphology

3.1.1. 6 wk

All cortical grafts were easily positioned, and after 6 wk, well incorporated with the host tissue (Fig. 5a). In general, the interface between host and graft was not discernible. However, signs of remodelling were evident (Fig. 6). The newly formed bone appeared to have its origin from the periosteum. In some instances the bone covered the central screw as well as the indicator screws. The other three groups had a more similar morphological appearance. The area between the host tissue and the central implant was filled with irregular, woven bone. Remodelling with resorption and new bone formation were characteristic features for the group without bone graft, and for the groups with or without plasma-augmented DBM (Fig. 5b–d). Bone trabeculae reached and partly filled the implant threads from the adjacent cortical bone. At this time stage individual animals had an appearent different ability to mount an efficient bone response. Thus, an interindividual variation with regard to the presence of woven versus lamellar bone was evident.

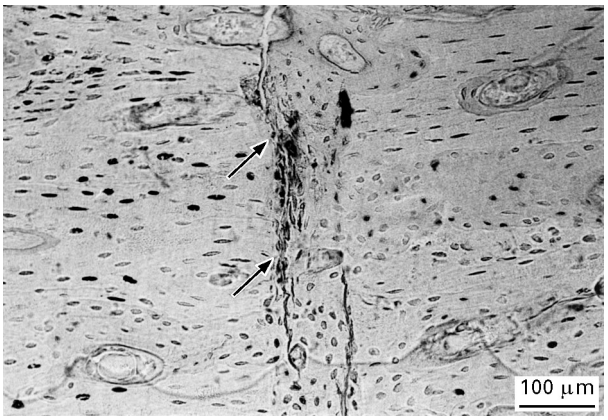


Figure 6 Border between host cortex and cortical graft, 6 wk after surgery.

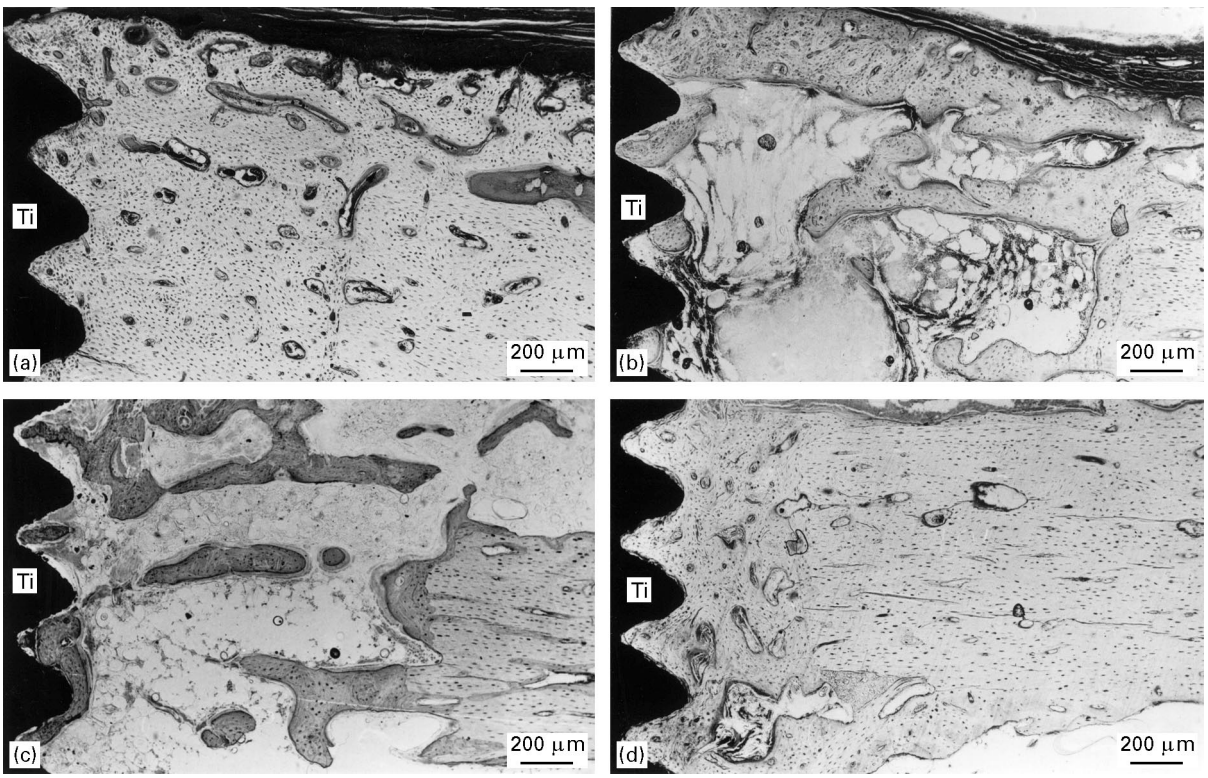


Figure 5 Light micrographs from ground sections showing (a) cortical bone graft, (b) defect without bone graft, (c) defect grafted with demineralized bone matrix (DBM), (d) defect grafted with plasmaaugmented DBM; 6 wk.

3.1.2. 6 mon

In the cortical graft group, an intimate contact had been established between the host and graft and between the graft and implant. All groups showed comparable amounts of bone area and bone-to-implant contact in the threads (Fig. 7a, b). Both the indicator screws and the central implant were well integrated in the bone. The newly build trabecular bone seen at 6 wk, were substituted by more lamellar bone.

3.2. Morphometry

3.2.1. 6 wk

In the cortical graft group all implants were well integrated and the mean bone area within threads (75%) and bone-to-implant contact (46%) (Fig. 8a, b) were considerably higher than in the other groups. The least amount of bone within the threads and in contact with the implants was detected in the DBM group (21% and 6%, respectively). The amount of bone within threads and bone-to-implant contact in the cortical graft group was significantly higher than in the DBM and plasma-augmented DBM groups.

3.2.2. 6 mon

After 6 mon all experimental groups had a mean bone area within threads ranging between 69% and 80% and a mean bone-to-implant contact between 31% and 42% (Fig. 8a, b). After 6 mon, the bone-to-implant contact and amount of bone within threads appeared to be higher in the DBM and plasma-augmented DBM groups in comparison with data obtained after 6 wk. In contrast, implants in the cortical graft group seemed to have a slightly lower bone contact after 6 mon.

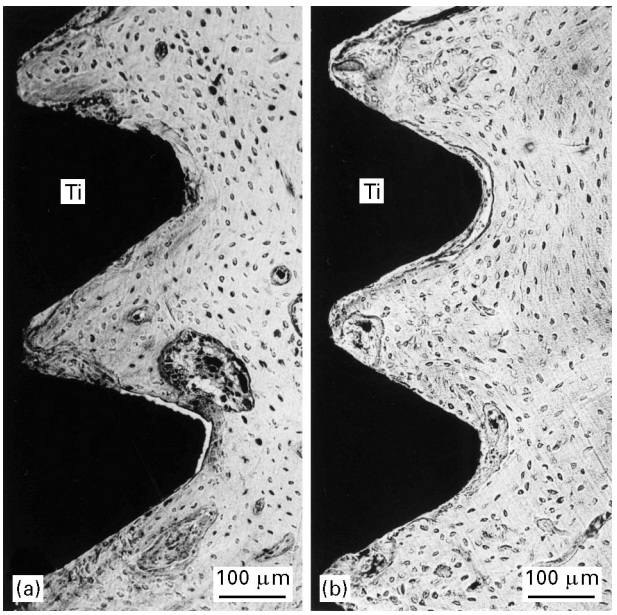
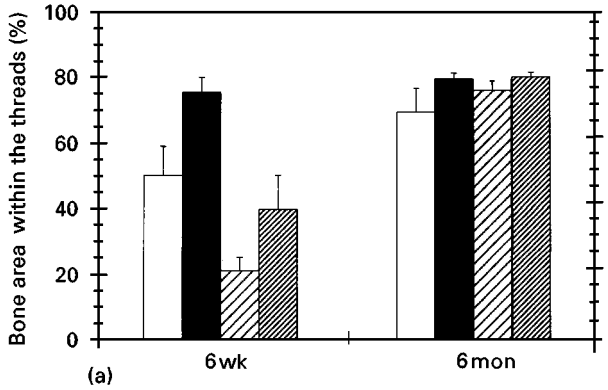


Figure 7 Light micrographs showing (a) control defect, (b) defect grafted with plasma-augumented DBM, 6 mon.



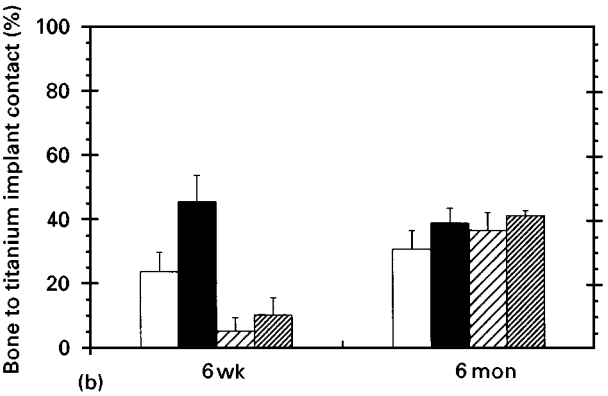


Figure 8 Morphometry. (a) Bone area within the titanium implants threads after 6 wk and 6 mon (mean + S.E.M.%). (b) Bone-to-titanium implant contact (%) after 6 wk and 6 mon (mean + S.E.M.%). (□) defect without bone graft, (■) cortical graft, (▧) DBM, (▨) DBM + plasma.

4. Discussion

In the present study emphasis was put on the changes of the amount of bone (evaluated by light microscopic morphometery) close to the implant (bone area within threads) and the proportion of bone in contact with the implant surface (degree of bone-to-implant contact). The results show that the amount of bone in defects without bone grafts increases from day 0 (5 mm diameter defect, no bone within threads) to about 50% bone within threads after 6 wk and, subsequently, to about 70% after 6 mon. Previous morphometric studies on the amount of bone within threads of larger diameter titanium implants inserted after drilling and pre-treading in normal rabbit tibial cortical bone, have revealed values (about 40% at 6–7 wk and between 35% and 55% at 3 mon between 41% and 62% after 6 wk and at 3 mon between 35% and 56% [18, 19]. Therefore, assuming that the amount of bone within threads after 6 mon (in this study ranging between 70% and 80% in all experimental groups) is representative of a steady-state stage which is close to uneventful and final healing, it may be concluded from the present observations that the defects heal spontaneously. However, before any definite conclusions can be drawn with regard to late-term implant-close morphological differences between empty defects and different graft materials, additional observation periods have to be examined.

Taking the clinical perspective into consideration, the spontaneous closure of a defect with bone

represents a draw-back with the present model. On the other hand, although seemingly more ideal bone-stimulatory conditions prevail in this experimental model, comparisons of the amount of bone and the structure of the interface between implants and normal bone and different graft treatments are urgently required. One possibility to decrease the chance of spontaneous closure would be to increase the size of the defect. This approach was undertaken during the course of the experimental work by a considerable effort to create larger size defects in another osseous site (rabbit iliac bone) and in the tibal bone. However, due to an unacceptable increased risk for fractures, this approach was abandoned.

A major observation in the present study was the large differences in the kinetics of bone formation between the various graft treatments. The highest amount of bone within threads and the highest bone-to-implant contact after 6 wk was found in the group subjected to treatment of defects with implants in autologous cortical transplants. Fresh autografts provide osteogenic cells, osteoinductive capacity and mechanical support [20].

A recent review [21] of 12 clinical studies (between 1990 and 1996) reporting on failures of osseointegrated dental implants inserted in bone grafts and loaded between 0 and 10 y, revealed a failure rate of 14.6% (220/1505 implants). The great majority (98%) of these implants were located in the maxilla and a majority (2/3) of the failed implants could be considered as early failures (unstable implants which were removed before bridge insertion). Most likely, there are multiple factors which increase the risk for failure in transplanted bone. However, the precise mechanisms are not understood [22]. Disadvantages in using autogenic bone for the grafting procedure include the postoperative host morbidity from a second surgical site, limited amount of available autogenic bone and, as previously stated, the tendency for resorption of grafts [1, 6]. Interestingly, although a slight reduction in bone-to-implant contact was observed between 6 wk and 6 mon, the amount of bone within threads of implants inserted in cortical transplants was similar after 6 wk and 6 mon. These findings suggest that during the present experimental conditons, an excessive bone resorption and imbalance in bone remodelling around implants can be avoided. These results are encouraging in the light of dental clinical observations of graft resorption and implant loosening. It is possible that factors inherent to the implant material and design, the good fit of the graft, sufficient stability of the graft by the anchorage of the implant in the opposite cortex and the proper closure of the area with the periosteum, all provide favourable conditions for the expression of graft osteoinductive properties which unfortunately are less likely to be attained in clinical practice.

Considerable pioneering efforts have been made in order to explore alternative bone-graft substitutes. In 1965, Urist described the osteoinductive potential of demineralized bone [29]. Demineralized bone is currently used in many different clinical areas, including oral and maxillofacial surgery [24, 25] and orthopaedics [26]. The exact mechanisms of osteoinduction are not fully understood, but proteins and inductive molecules, present in the matrix of the implanted bone [27, 28] are implicated as stimuli for the differentiation of mesenchymal cells into chondroblasts. The sequence of events – analogous to normal endochondral ossification [29] – includes a formation of cartilage, followed by calcification and a subsequent replacement with bone. Extraskeletal bone formation by the implantation of demineralized bone matrix (DBM) has been described in rodents [23], but there are contradictory results in primates, with some reports even failing to show bone induction by DBM alone in monkeys and humans [30].

The present study does not support the alleged osteoconductivity of demineralized bone matrix. There are several possible factors which could have played a role in this result. The role of particle size for bone induction is not entirely clear. In the present study the particle size varied between 60 and 300 μm. In a histomorphometrical study on the osteogenic effects of demineralized bone (ranging in particle size between < 75 and > 450 μm), Glowacki *et al.* [31] showed that smaller particles, presumably offering a larger surface area, were more effective. Syftestad and Urist [32] found that a particle size of 125–250 μm was more bone inductive than 44–75 μm and 500–1000 μm. The latter finding is supported by results from subcutanous implantation of DBM showing a local differentiation of endochondral bone with a particle size of 74–420 μm but not with fine DBM (size 44–75 μm) [33]. One explanation of why matrix particles of a given size result in more bone is, that it is necessary to allow for mesenchymal cell anchorage, proliferation and differentiation and that small particles might limit the available space for cell migration. Further, another possibility is that differences even in these sizes of particles (much larger than cells) might be important for the recognition and handling by cells belonging to the phagocyte system. It is also likely that the size of particles affects the possibility of maintaining the matrix at the surgical site. Smaller particles might allow a too rapid diffusion of osteoconductive proteins away from the site of implantation. This aspect is less well dealt with in the literature. However, a development is required of delivery and carrier substrates for osteoinductive proteins and cells during the phase of bone formation close to the implant.

A small, positive effect on bone regeneration was detected after the addition of plasma to DBM. This could be due to osteoinductive properties of one or several of the plasma proteins. Another possibility is that the plasma was promoting an immobilization of DBM particles in the defect. Although the effect of plasma augmentation could be judged as very moderate, further studies are needed to evaluate the effects of different proteins with proposed bone-stimulatory effects [9–11]. The present model would allow an evaluation of the rate of bone formation in conjunction with implants.

Another factor to be considered is the possibility that the biological and synthetic materials are

contaminated with microorganisms and their products during the preparatory work *in vitro*. The endotoxin contents in the DBM and plasma were determined using a Limulus assay. The level of LPS in the DBM was less than 10 pg ml^{-1} at both times, which is below the sensitivity limit for the LAL test used [16, 34]. The LPS was found to be 0.11 ng mg^{-1} in the plasma that was used at 6 wk, while the other specimens showed values below the sensitivity limit. Previously published data [35–37] show that it requires over 0.1 μg LPS ml^{-1} to achieve significant bone resorption. On the basis of these studies, it is less likely that an LPS content of 0.11 ng ml^{-1} would have any positive or negative influence on the bone, although we cannot exclude the possibility. In the present study an experimental model for the study of implants in bone grafts was introduced. The results from the present study show that the insertion of a threaded titanium implant centrally in a cortical defect was followed by a spontaneous healing of the defect after 6 mon. The simultaneous transplantation of an autologous cortical graft resulted after 6 wk in a higher amount of bone within the threads and a higher bone-to-implant contact than in control defects and defects which had recieved demineralized bone and plasma-augmented demineralized bone. The results from the study indicate that the model offers a possibility to examine, in a standardized manner, the sequences of early bone formation, bone resorption and remodelling of grafted bone, surrounding and in contact with, an implanted biomaterial.

Acknowledgements

Financial support from the Swedish Medical Research Council (grant 9495), Hjalmar Svensson Research Foundation, the Sigge Persson and Alice Nybergs foundation. The Royal Society of Arts and Sciences in Göteborg, University of Göteborg, is gratefully acknowledged.

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Received 14 January
and accepted 25 September 1997