CROSS-SPECIES GRAFTS OF DEPROTEINISED BONE

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Over the years many substances other than the patient's own bone have been used for grafting. There are many excellent reviews of the older work where full bibliographies of the large number of published reports can be found (Chase and Herndon 1955; Dick 1946; Groves 1917; Jacob, Gowing and Dunphy 1959). More recently it has been suggested that the usefulness of cross-species bone as a graft material could be increased greatly by removing its tissue proteins beforehand because the latter may provoke an antigenic response in the host and thus complicate the reaction towards heterografts.

The proteins of bone are located both in the bone tissue proper, as collagen and polysaccharide, and in the intertrabecular spaces, as marrow, vessels, nerves and so on. Their removal ("deproteinisation") can be effected by destructive reagents such as ethylenediamine or hydrogen peroxide; enzymatic proteolysis has also been employed. Protein can also be burned out by incinerating bone, but this seems to weaken it excessively. When successfully deproteinised, samples consist for all practical purposes simply of the mineral skeleton of the bone. They have a low nitrogen content and do not char on heating.

Animal experiments show that deproteinised heterogenous bone grafts when used for filling defects and for bridging gaps give at least as good a result as fresh homogenous or even autogenous material. It has also been suggested that the graft material not only serves as an excellent scaffold for the deposition of new bone by the host, but, in addition, has genuine osteogenic properties; that is, the graft induces the host's tissues to form new bone in sites where otherwise none would have been formed.

Treated animal bone would have much to recommend it clinically; large supplies could be prepared and stored against an emergency; grafts of various shapes and sizes could be prepared beforehand very much more easily than they could from fresh bone; sterilisation is easy and certain because the material withstands autoclaving; and the patient does not have to suffer the second incision and inconvenience attached to autografting. In view of these advantages it seemed worth while to test by animal experiments the biological findings of others. Such previous work had been carried out in dogs and rats. A different species, the sheep, was used in the experiments described below (Campbell, Brower, MacFadden, Payne and Doherty 1953; Chalmers and Sissons 1959; Curtiss and Herndon 1956; Goff 1944; Losee and Hurley 1956*a* and *b*, Hurley and Losee 1957; Losee and Boyne 1957; Maatz and Bauermeister 1957; Maatz, Lentz and Graf 1954; Orell 1937; Ray and Holloway 1957).

MATERIALS AND METHODS

The bone samples—Heterogenous bone for deproteinising was obtained from calves between two and six weeks old. A few samples of banked human bone were also used. Spongy cancellous bone tissue was removed from beneath the articular cartilages of the long bones and cut into small cubes weighing two to three grammes. These were treated in three different ways: *a*) deproteinised in hydrogen peroxide as described by Maatz and Bauermeister (1957); *b*) in ethylenediamine (Losee and Hurley 1956 *a* and *b*, Hurley and Losee 1957); and *c*) by a combination of the two methods, as follows. The pieces were first exposed to a large volume of 40 per cent hydrogen peroxide at 38 degrees Centigrade for twenty-four hours, after which the reagent was renewed and allowed to act again for twenty-four hours at the same temperature. The pieces were then washed under a stream of running water to dislodge any adhering

remnants of soft tissue. At this stage they appeared to be quite free of intertrabecular tissue; however, they charred readily on heating and retained most of their original physical strength which indicated that the collagen fibre framework was in all probability still more or less intact. After defatting in ether for twenty-four hours, the pieces were next extracted for twenty-four hours in a Soxhlet apparatus with ethylenediamine, washed in a stream of distilled water, dried and finally extracted for twenty-four hours in absolute ethanol to remove last traces of ethylenediamine. Such pieces, chalky white in colour, did not char on heating. They were stored in ethanol or ether until required for use, when the solvent was evaporated off. They were then autoclaved at fifteen pounds pressure for twenty minutes.

To obtain fresh frozen homogenous bone, the femora and tibiae were removed aseptically from a sheep; the bones were stored in the bone bank for twenty-one days at -37 degrees Centigrade before being used to provide cancellous homografts.

Operative technique—Anaesthesia was induced in the sheep by intravenous pentobarbitone sodium. A McGill tube was next inserted and anaesthesia continued with closed-circuit cyclopropane and oxygen (Gregory 1947). No anaesthetic difficulties were met.

In the experiments the upper parts of the tibia or ulna (group one) and the lower parts of the femora (group two) were used to receive the grafts. Holes, seven-thirty-seconds of an inch diameter and about three-eighths of an inch deep, were drilled through the compact bone into the cancellous bone. All wounds healed rapidly by first intention and the sheep were running normally in a few days. As described below, the number of holes and their treatment differed in the two groups of experiments which were carried out.

GROUP ONE

Details of experiments—In this group of seven sheep an attempt was made to ascertain *a*) whether a drilled hole would repair more rapidly if left empty or filled with autogenous or treated heterogenous material; *b*) whether heterogenous bone deproteinised with hydrogen peroxide or ethylenediamine would give the better result; and *c*) whether deproteinised material would induce bone to form by metaplasia.

Three or four defects were drilled in the upper end of either the ulna or the tibia in each of the sheep and a plug of autogenous bone was removed with a burr of the same diameter. One hole was left empty, whilst others were filled by inserting plugs of the test material. The sheep were killed after seventeen days. The graft sites were recovered, fixed in 10 per cent formol for ten days, decalcified and embedded in polyester wax (Steedman 1957). Serial sections were stained with haematoxylin and Gomori's chromotrope/aniline blue mixture.

Results—It had been hoped that the microscopic anatomy of the bone surrounding the drill holes would be reasonably comparable in each case. However, wide variations in the trabecular density occurred. In some cases, in which the compact bone was thick, the bone density was high, so that the rate of healing would be expected to be slow; in others, in which the bone around the hole was much more cancellous, the healing rate would be expected to be faster. Thus, direct comparison of all holes could not be made indiscriminately; variations of this sort had always to be kept in mind when repair processes were being studied, particularly if quantitative assessments were involved.

1. *No graft*—After seventeen days healing had not progressed very far. The defects were filled with granulation tissue and a little new bone had been laid down in the depths, and to a less extent around the sides of the hole. The appearances agreed with the usual findings that the endosteal osteoblasts had proliferated, migrated inwards, and deposited new bone.

2. *Autografts*—Results were disappointing in that the samples had obviously suffered mechanical injury whilst being removed with the burr. The bone trabeculae were tightly compressed and their osteocytes dead. The grafts were very shallow, and lay, not as a plug extending in depth into the defect, but as a lid across the top. In some sheep this lid was more or less anchored in place by new trabeculae, whilst in others the attachment was not as firm. Events in the

FIG. 1

The appearance of a heterograft, prepared by extraction with hydrogen peroxide, at seventeen days. A low-power view of whole specimen. The hole contains reaction tissue and graft trabeculae. New bone is growing in from edges and from the bottom. ($\times 50$.)

the sectioned trabeculae. In places large aggregations of plasma cells and lymphocytes had gathered near the graft material, suggesting sites of antigen-antibody reaction. There were large foreign body giant cells against the graft substance, and areas suggestive of liquefaction (Figs. 3 and 4). These observations suggest very strongly that deproteinisation

depths did not seem to have been influenced significantly.

3. Treated heterografts—Appreciably more bone had been formed. Towards the bottom of the hole, the trabeculae of the graft material had been covered over by new bone produced by the host and incorporated into newly formed trabeculae. Around the sides a similar process had often occurred (Figs. 1 and 2). In this way bone formation in the filled holes had spread farther towards the centre of the graft.

The trabeculae of the grafts were easy to recognise. Both their histological appearance and the reaction they had provoked differed according to the treatment they had received. After hydrogen peroxide extraction the trabeculae looked opaque and stained intensively with acid dye. Since the tissue had been decalcified—thus removing bone salts—before sectioning, and since in theory the protein had also been removed from the graft, there should have been little or no solid residue left in

FIG. 2

A heterograft, prepared by ethylenediamine extraction, at seventeen days. The area shown is near the bottom of the graft bed; graft trabeculae (paler-stained) have been covered by newly deposited bone (darker). ($\times 300$.)

FIG. 3

A heterograft, prepared by extraction with hydrogen peroxide, at seventeen days. The tissue around the graft material (G) is densely infiltrated with plasma cells and lymphocytes. ($\times 300$.)

FIG. 4

A heterograft, prepared by extraction with hydrogen peroxide, at seventeen days. It comprises bone (darker-stained) and cartilage (paler). Foreign-body giant cells (right-hand edge, top and middle) are present. Abundant thick collagen bundles are accumulating. ($\times 300$.)

had been far from complete. In contrast, after ethylenediamine treatment the graft trabeculae were much more transparent and paler-stained (Fig. 2). They seemed to have provoked no abnormal tissue response; cells resembling osteoclasts had gathered here and there (Hancox 1949, 1956). Plasma cell-lymphocyte infiltration was not commonly seen and it seemed that deproteinisation had been much more complete. In both cases the reaction tissue filling in the spaces between the graft trabeculae was quite different from that seen in the empty holes. It contained many bundles of collagen fibres (Fig. 4). These were not present in sites occupied by the plasma cell-lymphocyte aggregations mentioned above (Fig. 3).

In several instances small islets of newly formed bone were seen in the interior of the graft, more or less at the centre, and either actually upon the implanted trabeculae or else extremely close to them (Figs. 1 and 5). This occurred with both hydrogen peroxide and ethylenediamine treated material. The question then arose whether the osteoblasts responsible

FIG. 5

A high-power view of the centre of the area enclosed within the circle on Figure 1. New bone (B) has been deposited on or near the graft material (G). ($\times 450$.)

for this bone had been produced locally, as a metaplasia from indifferent connective tissue cells caused by an osteogenic stimulus emanating from the graft, or whether they had simply migrated inwards, although over quite a long distance, from the undamaged bone around the hole. By the use of serial sections it was possible to demonstrate that the apparently isolated bone islets could be traced back in direct continuity to newly formed bone at the edge of the defect. In other words, tongues of new bone had grown out from the sides of the hole towards the centre of the graft along its trabeculae. This ruled out any question of the direct osteogenic effect reported by Maatz and Bauermeister (1957), although there is no explanation why osteoblasts migrated so far into the graft, but did not migrate in empty holes. Perhaps the explanation lies with the collagen bundles formed between the graft trabeculae. These could serve as a ladder along which osteoblasts could grow, in the same way that tissue culture cells grow along artificial fibres. It is, however, difficult to explain why the collagen bundles form in the first place.

Graft material was also implanted intramuscularly in two sheep. In contrast to the findings of Maatz and Bauermeister (1957) no bone was formed.

GROUP TWO

Details of experiments—In this group of nine sheep a simple comparison was made between the merits, as graft material, of fresh frozen homogenous bone stored in the bone bank and treated heterogenous bone. Two holes were drilled in the left femoral condyle. One was filled with banked homogenous bone and the other with heterogenous bone deproteinised by the combined hydrogen peroxide and ethylenediamine technique. In this group the graft sites were recovered after thirty-six days, and fixed, decalcified, embedded and stained as before.

Results—As with group one, experimental conditions proved to have been rather variable. By chance most of the homogenous bone grafts had been inserted in rather dense compact bone, whereas the deproteinised grafts were mostly surrounded by cancellous bone which would tend to heal more quickly. However, by the time thirty-six days had expired healing was advanced in both and qualitative assessment of the histological result was simple.

FIG. 6

FIG. 7

Figure 6—A heterograft, prepared by the double extraction method described in the text, at thirty-seven days. Low-power view of whole specimen. The drill hole is still recognisable. Graft trabeculae (pale) lie buried in new bone which appears as a dark line around them. ($\times 50$.) Figure 7—The same host as Figure 6, showing a banked homograft, but at this magnification it is not possible to distinguish the newly formed bone from implanted material. ($\times 50$.)

In general with both types of graft most of the implanted trabeculae were more or less buried in a covering of newly deposited bone (Figs. 6 to 11) though there was some variation in the depth and completeness of the covering. In the grafts with advanced healing (four out of nine) fatty, and even actively haemopoietic, marrow had replaced the reaction tissue between graft trabeculae (Figs. 8 and 10). In those with less advanced healing, fatty marrow was seen spreading in from the sides. The reaction tissue in the central zone no longer possessed the collagen fibre bundles seen after seventeen days in the first group of experimental animals. As cartilage frequently occurs in callus it is perhaps rather surprising that it was seen around graft material in one sheep only (Fig. 11). It is possible that this is a species peculiarity on the part of the host. It happened that this was the single sheep in which treated human bone was employed.

FIG. 8

A heterograft, prepared by the double extraction method, at thirty-seven days. The pale-stained core of implanted material is covered by newly deposited bone (darker-stained). Haemopoiesis proceeds in the surrounding marrow. ($\times 300$.)

FIG. 9

A banked homograft, at thirty-seven days. The bar of implant material lies in undifferentiated tissue. Osteoclasts are seen along its upper edge. No new bone has formed. ($\times 300$.)

FIG. 10

A banked homograft, at thirty-seven days. The graft material (G) is shown with its empty lacunae as a central core buried in newly formed bone (B). ($\times 300$.)

FIG. 11

A heterograft of human cancellous bone prepared by the double extraction method. The pale-stained implant is covered by a thick layer of new bone seen on the right and above; below and to the left, cartilage can be seen. ($\times 300$.)

In some implants the trabeculae of the graft protruded above the top of the drill hole; although surrounded by reaction tissue similar to that seen in the central zone of the graft, new bone was lacking, whereas it was plentiful in the implant material immediately below the top level of the hole. If an osteogenic stimulus from the graft had caused a local metaplasia of osteoblasts, new bone should have formed equally in both of these sites. This favours the view that endosteal osteoblasts had migrated into the graft.

There were some differences in the local effects of the two materials. With the banked homografts there were occasional large gatherings of plasma cells and lymphocytes, suggesting a lingering antigenic response. Their dead trabeculae were often seen lying in undifferentiated reaction tissue, with no covering of new bone (Fig. 9); it was as if they were tolerated by the host's tissues, but had not stimulated osteogenic cells and fibres to grow into them. This might have been caused at an earlier stage by plasma cell and lymphocyte accumulations interfering with migration of osteoblasts. On the whole, there seemed to be rather less new bone around the homografts than the treated heterografts, but this is a subjective impression which could not be verified objectively in our material. It is certainly safe to say that thirty-six days after grafting as much new bone had formed in the holes filled with treated heterografts as with homografts.

DISCUSSION

In these experiments there was no evidence that the grafts caused bone to form by metaplasia. Implants in muscle failed to form bone; in the drill holes the new bone grew in from the edges. The study of bone induction is notoriously difficult, and one of the problems is the variation with different species. It may well be that the sheep is more refractory than the dog; this may be why we failed to confirm the findings of Maatz and Bauermeister (1957).

From our results, it is apparent that the sheep tolerates deproteinised heterogenous bone as well as the dog, monkey and rat (Campbell *et al.* 1953, Losee and Hurley 1956*a* and *b*, Hurley and Losee 1957, Maatz and Bauermeister 1957, Chalmers and Sissons 1959, Ray and Holloway 1957).

The heterogenous grafts did not give rise to any unfavourable tissue reactions, and the trabeculae made a suitable framework for the deposition of new bone by host cells. It is difficult to imagine that the human body would behave very differently towards such graft material, and indeed treated animal bone has already been used in patients (Orell 1937, Goff 1944, Maatz *et al.* 1954, Maatz and Bauermeister 1957).

The present experiments show only what happens when the material, shaped to a solid plug, is placed in a hole in bone. Much more work is needed to determine its usefulness under other conditions. Powdered material (Ray and Holloway 1957) might offer advantages over cubes for filling cavities and other bone defects. Grafts having a denser and more compact nature, with a greater tensile strength, might be more suitable for some clinical purposes where mechanical stability is needed—such as in the management of non-union of fractures. The superior texture of the treated human bank bone was observed during the grafting; it was less crumbly and less friable than the treated calf bone, yet easily shaped by a scalpel to the right size. This suggests that the calf bone was immature and that bone from older animals might be stronger.

SUMMARY

1. Cancellous bone cubes from calf and man were deproteinised with hydrogen peroxide and with ethylenediamine.
2. Long bones were removed aseptically from sheep, stored in the bone bank and used for cancellous homografts.
3. Holes were drilled in the upper part of the tibia or ulna or in the lower part of the femur of sheep. Some were left empty; others were filled with plugs of the deproteinised heterogenous bone, with autografts, or with homografts.
4. Histological appearances were studied after seventeen and thirty-six days.

5. At seventeen days repair was more advanced in the plugged holes; the biological result was better with the ethylenediamine-treated than with the peroxide-treated material. After thirty-six days repair was at an advanced stage. As much new bone had been deposited on the trabeculae of the deproteinised heterografts as on those of the homografts.
6. There was no evidence of metaplastic bone formation; new bone seemed to form from endosteal osteoblasts.
7. Certain clinical implications are briefly discussed.

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