OSTEOGENESIS IN BONE GRAFTS AFTER SHORT-TERM STORAGE AND TOPICAL ANTIBIOTIC TREATMENT

AN EXPERIMENTAL STUDY IN RATS

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It has been shown in experimental animals that the living cells in a bone autograft can make an important contribution to osteogenesis. However, some common clinical techniques, such as the topical use of antibiotic powders on grafts or on the graft bed, are likely to damage or kill the cells. In this experimental study in rats, bone isografts dusted with chloramphenicol or methicillin powder or with Polybactrin spray before subcutaneous implantation produced little or no new bone over a period of two weeks whereas untreated, control grafts showed abundant osteogenesis, as did grafts pretreated with solutions of antibiotics. The effect of short-term storage of the grafts for 3 to 24 hours in air, saline or culture medium before implantation was also examined. Grafts stored in culture medium generally did as well as, or better than, fresh control grafts whereas immersion in saline inhibited osteogenesis. The importance of these results for clinical bone grafting is discussed.

Quantitative experimental evidence has been presented elsewhere (Gray 1978; Gray and Elves 1979) demonstrating that the living cells within a fresh bone autograft or isograft make an important contribution to osteogenesis within and around the graft. If the graft cells are killed or removed before implantation of bone matrix into an intramuscular or subcutaneous site, little or no new bone is produced in the first two weeks, whereas in an intact, living graft abundant new bone appears. There is also evidence that long-term osteogenesis for four weeks or more, although arising largely by metaplasia of host cells, is partly dependent upon the early phase of osteogenesis derived from the graft (Vainio 1950; Axhausen 1956; Goldberg and Lance 1972; Elves 1974a,b 1975).

To obtain the best clinical result with a bone graft, it is therefore important to prepare the graft and graft bed in a manner that will maintain the cells of the graft in a viable and healthy condition. During a bone grafting operation, there is usually a delay between procurement of the graft and its implantation; the delay ranges from a few minutes to several hours. This raises several practical questions concerning the harmful effects of delay on eventual osteogenesis, how long a graft can be left without being damaged, the optimal environment for the graft, and the deleterious effects of antibiotics on the graft and graft bed. These questions have been examined using experimental bone isografts in which the

osteogenetic performance was assessed qualitatively by histology and quantitatively by ⁸⁵strontium retention histiometry (Elves 1974a).

MATERIALS AND METHODS

Both donors and recipients of the bone isografts were inbred AS2 male rats between two and four months old (average weight 260 grams) at the time of grafting.

The iliac bones were removed from freshly killed donor rats using aseptic procedures; any adherent muscle, the periosteum, and the cartilaginous crest and edges were removed, as was the compact bone from the posterior end of the ilium. The remaining part which was mainly cancellous was split sagittally to give two corticocancellous grafts. The cancellous surfaces were subjected to a gentle jet of sterile 0.9 per cent (weight to volume) saline from a 25SWG needle on a 20 millilitre syringe which removed most of the marrow.

Experimental grafts were incubated before implantation in one of five different media in closed 20 millilitre universal bottles. Three variants of medium 199 (Wellcome) were used, all containing 800 000 units per litre of benzylpenicillin and 500 milligrams per litre of streptomycin sulphate. The first variant was TC199 which was buffered to pH 7.4 with 10 per cent HEPES; the second was TC199+CS which contained in addition 20 per cent heat-inactivated foetal bovine serum (Flow Labs); the third was enriched TC199 which was buffered with sodium bicarbonate and five per cent carbon dioxide in air instead of HEPES, and was enriched by the addition of glucose, several amino acids and vitamins (Gray 1978); the fourth medium was physiological saline (Boots); the fifth was air. Sets of grafts were incubated at 37 degrees Celsius in one or other medium for three, six, 12 or 24 hours to measure the effect upon eventual osteogenesis. In addition similar grafts were incubated for four hours in each of the media at four, 25 and 37 degrees Celsius.

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The effect of antibiotic powder on the bone grafts was examined by implanting freshly prepared grafts dipped in chloramphenicol powder, or methicillin powder, or sprayed with Polybactrin spray. The Polybactrin spray is a mixture of neomycin sulphate, polymyxin B sulphate and bacitracin zinc. Osteogenesis in these grafts was compared with that in untreated fresh grafts and in grafts killed by two cycles of immersion in liquid nitrogen and boiling water before implantation. The effect of antibiotic solution was investigated by comparing grafts incubated for three hours at 37 degrees Celsius in TC199 alone or in TC199 containing various concentrations (10 to 240 micrograms per millilitre) of chloramphenicol.

The grafts were implanted into pockets created between the dermis and the panniculus carnosus muscle of the back of the recipient. As a control every recipient received at least one freshly prepared untreated graft. Twelve days after implantation each recipient was injected intraperitoneally with ^{85}Sr strontium chloride, 80 microcuries per kilogram bodyweight, as described elsewhere (Gray 1978; Gray and Elves 1979). Four days later the animals were killed and the grafts together with the iliac bone of the recipient were removed. These tissues were weighed, fixed in formol saline and measured for ^{85}Sr strontium gamma activity. From the ^{85}Sr strontium activities, a relative index of graft osteogenesis was calculated to compare treated with fresh grafts.

$$\text{Relative index} = \frac{\text{specific activity of test graft (cpm/mg)}}{\text{specific activity of fresh control graft (cpm/mg)}}$$

Student's two-tailed *t*-test was used to test the significance of differences of values of the relative index between different groups of grafts. Probability values less than 0.05 were arbitrarily taken to indicate significant differences. After counting the gamma activity the grafts were put into fresh fixative and were then decalcified and processed in paraffin wax. Representative sections stained with haematoxylin, eosin and alcian blue (Sayers 1973) were examined under the light microscope.

RESULTS

Influence of incubation media. The relative indices of grafts implanted fresh or stored before implantation for periods ranging from 3 to 24 hours in the different media are shown in Table I and Figure 1. It may be seen that grafts stored in air for three hours did not differ from freshly prepared grafts ($P < 0.9$) but that storage for six, 12 or 24 hours in air led to very much lower levels of strontium uptake ($P < 0.001$ for all cases). Saline-incubated grafts similarly gave very low results after six, 12 or 24 hour storage and even three hour incubation produced a smaller but significant drop in osteogenesis ($P < 0.01$).

Grafts stored for three or six hours in TC199, on the

other hand, actually showed an increase in strontium uptake, this increase being statistically significant for the grafts stored for six hours ($P < 0.001$). However, 12 or 24 hour storage in TC199 resulted in a moderate, but not statistically significant, drop in uptake ($P = 0.07$, 0.25 respectively). Storage in TC199+CS for three or six hours resulted in a significant increase in the relative indices ($P < 0.02$ in both cases). The index fell at 12 hours to a value not significantly different from that of the fresh grafts and dropped further at 24 hours, but was still not significantly different from the control value. In the enriched TC199, only the grafts stored for 12 hours were statistically different from the fresh controls ($P < 0.001$). Storage of grafts for four hours at four, 25 or 37 degrees Celsius in each of the five media did not reveal any significant effect of storage temperature upon eventual osteogenesis.

Effect of antibiotic treatment. The effects of sprinkling or spraying one of the three different powders on to groups of fresh grafts immediately before implantation are shown in Table II. The use of any of the antibiotic preparations led to a highly significant drop in the relative index of osteogenesis compared with that of the untreated controls. Furthermore, the indices of the treated grafts were not significantly different from that of the group that had been killed before implantation.

Fig. 1

Relative indices of graft osteogenesis (R.I.) of 16-day-old bone isografts pretreated by incubation at 37 degrees Celsius in five different media for periods between three and 24 hours.

Table I. Effect of graft pre-incubation for various times and in different media upon osteogenesis 16 days later

Incubation time (hours)	Mean relative index $\pm$ SE					
	Air	Saline	TC199	TC199+CS	Enriched TC199	
0	1 (25)*	1 (20)	1 (21)	1 (21)	1 (20)	
3	0.99 $\pm$ 0.11 (11)	0.80 $\pm$ 0.06 (10)	1.20 $\pm$ 0.10 (13)	1.37 $\pm$ 0.13 (13)	0.93 $\pm$ 0.09 (14)	
6	0.21 $\pm$ 0.01 (7)	0.27 $\pm$ 0.04 (6)	1.26 $\pm$ 0.05 (18)	1.28 $\pm$ 0.10 (12)	1.00 $\pm$ 0.09 (14)	
12	0.30 $\pm$ 0.04 (8)	0.24 $\pm$ 0.02 (8)	0.72 $\pm$ 0.13 (7)	1.10 $\pm$ 0.15 (8)	0.79 $\pm$ 0.05 (15)	
24	0.28 $\pm$ 0.03 (8)	0.26 $\pm$ 0.02 (8)	0.77 $\pm$ 0.18 (8)	0.80 $\pm$ 0.14 (8)	0.98 $\pm$ 0.11 (16)	

* Number of experiments given in parentheses.

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Figure 2—Histological section of an untreated bone isograft 16 days after implantation. Abundant new bone (NB) is apparent. (Haematoxylin, eosin and alcian blue, $\times 100$). Figure 3—Histological section of a 16-day-old isograft pretreated with chloramphenicol powder. No new bone is seen among the spicules of dead graft bone (DB). (Haematoxylin, eosin and alcian blue, $\times 100$). Figure 4—Histological section of a 16-day-old bone isograft pretreated with Polybactrin spray. No new bone is seen. (Haematoxylin, eosin and alcian blue, $\times 100$). Figure 5—Histological section of a 16-day-old bone isograft pretreated with methicillin powder. Occasional small islands of new bone (NB) may be seen among the spicules of dead bone (DB), but there is much less new bone than in the untreated grafts shown in Figure 2. (Haematoxylin, eosin and alcian blue, $\times 100$).

The histological sections corroborate these results: comparison of the fresh grafts (Fig. 2) with the grafts treated with antibiotics (Figs 3 to 5) showed that there were copious amounts of new bone in the former, but no new bone in the grafts treated with chloramphenicol

(Fig. 3) and Polybactrin (Fig. 4). Signs of an inflammatory reaction were noticed in the chloramphenicol treated grafts. The fresh grafts produced more new bone than the grafts treated with methicillin (Fig. 5) in which only a few, isolated pockets of new bone were found.

The effects of incubating the grafts for three hours in various concentrations of chloramphenicol solution on eventual osteogenesis are shown in Table III. For concentrations up to 120 micrograms per millilitre, no significant reduction in osteogenesis occurred. However, grafts treated with 240 micrograms per millilitre chloramphenicol solution showed a moderate, and statistically significant, reduction in the formation of new bone compared with the controls.

DISCUSSION

Since a fresh bone autograft is a living tissue in which the cells can make a direct and significant contribution to osteogenesis (Gray and Elves 1979) certain practical conclusions inevitably follow. The graft and its bed

Table II. Effect of pretreatment with antibiotic powder on osteogenesis in bone isografts 16 days after implantation

Pretreatment	Mean relative index ($\pm$ SE)	$P^{\dagger}$	$P^{\S}$
Fresh control	1 (12)*		
Chloramphenicol	0.27 ± 0.014 (12)	<0.001	0.3
Methicillin	0.47 ± 0.07 (11)	<0.001	0.47
Polybactrin spray	0.37 ± 0.05 (12)	<0.001	0.9
Killed grafts	0.38 ± 0.10 (6)	<0.001	

* Number of experiments given in parentheses.

† t -test of treated against untreated control grafts.

§ t -test of killed grafts against grafts treated by antibiotics.

should obviously be prepared in a way designed to give maximal survival and proliferation of the osteogenic cells. The size and shape of the graft is important: if it is cut too small many of the osteogenic cells will be damaged; if it is too large, diffusion of nutrients and the re-establishment of a blood supply to many of the osteogenic cells will be too slow to ensure their survival. Only the smallest dimension of the graft is significant since it determines the rate of diffusion and revascularisation to the innermost graft cells; it also largely determines the proportion of cells damaged during preparation of the graft. Ideally the smallest dimension will be no more than about five millimetres (Bassett 1972) and the graft will be a flat slab or strip of bone with the other dimensions as large as necessary.

If there is a delay between preparation and implantation, steps should be taken to ensure survival of the osteogenic cells. Exposure to moist air for periods up to three hours was not found to be detrimental, whereas immersion in saline for a similar period was harmful. Bassett (1972) suggested that storage of viable grafts for several days in balanced saline containing 10 per cent human serum may be possible. We have shown that tissue culture medium not only preserved the osteogenetic potential of the graft, but under the right conditions could lead to enhanced osteogenesis.

A possible explanation of this effect is that incubation for a few hours will allow the graft to become thoroughly impregnated with the nutritious medium which will sustain it for the first few days after

Another possibility is that the endosteal cells, particularly those in small recesses, may be affected by local accumulation of metabolites or by depletion of oxygen. Tissue culture medium has not yet been approved for clinical use and work is in progress to find an acceptable and effective substitute. At present, the best method of preserving grafts for an hour or two during operation is to keep them in a closed container or covered by a swab moistened with saline, but not immersed in saline.

Various antibiotics are often applied topically to the bone grafts and to the prepared graft bed as a prophylactic measure in procedures such as spinal fusions; many surgeons use the antibiotic in powder form rather than in solution. The three preparations used in this study, chloramphenicol, methicillin and Polybactrin are in common clinical use. It seems likely that the extremely high local concentration around each particle of antibiotic would damage any cell coming close to it either by its pharmacological activity or by osmosis. The osteogenetic cells of the graft and graft bed are surface cells (Ham and Gordon 1952; Bonfiglio 1958; Tonna and Cronkite 1961; Bassett 1961) and would therefore be particularly at risk. Death of these cells would lead to a severe reduction or absence of early osteogenesis and would thereby hinder long-term incorporation of the graft.

The results of pretreatment of experimental grafts with each of the antibiotic powders corroborate this view. It may be significant that chloramphenicol and Polybactrin which have anti-ribosomal activity in mammalian cells appear to be more harmful than methicillin which principally inhibits synthesis of the bacterial cell wall. Although solutions of chloramphenicol of up to 120 micrograms per millilitre (30 times the therapeutic plasma levels) do not inhibit osteogenesis, their use appears unjustified on more general pharmacological grounds. It is now widely accepted that chloramphenicol should never be used for prophylaxis because of its toxic effect on bone marrow (Martindale 1977).

Neomycin sulphate, one of the constituents of Polybactrin, has been used in a controlled trial involving 466 orthopaedic patients (Nachamie, Siffert and Bryer 1968). A 0.1 per cent solution was instilled into a variety of orthopaedic surgical wounds before closure, but there was no effect on healing or upon the rate of infection. However, Davia, Siemsen and Anderson (1970) reported that three of their patients became permanently deaf and developed acute renal failure. One patient also developed muscular weakness and apnoea after irrigation of the orthopaedic wounds with antibiotic solutions. For two of their patients the solution used contained neomycin, polymyxin B sulphate and bacitracin, and for the third patient the solution contained only neomycin (Kelly, Nilo and Berggren 1969). In another experimental and clinical trial (Scherr, Dodd and Buckingham 1972; Scherr and Dodd 1976) the value of frequent

Table III. Effect of pretreatment with chloramphenicol solution on osteogenesis in bone isografts 16 days after implantation

Chloramphenicol concentration ($\mu\text{g/ml}$)	Mean relative index ($\pm\text{SE}$)	Difference from control
0	1 (10)*	
10	0.98 ± 0.19 (8)	NS
20	0.85 ± 0.09 (7)	NS
40	0.94 ± 0.14 (8)	NS
60	0.99 ± 0.04 (10)	NS
80	0.86 ± 0.12 (10)	NS
100	0.96 ± 0.16 (9)	NS
120	0.98 ± 0.05 (10)	NS
240	0.73 ± 0.06 (10)	$P < 0.005$

NS, not significant.

* Number of experiments given in parentheses.

implantation until the blood supply is re-established. It is perhaps surprising that incubation for 12 and 24 hours do not have equally beneficial effects, but it is possible that prolonged incubation may lead to loosening and loss of some of the osteogenic cells or to their dedifferentiation away from an osteogenetic role.

irrigation of the surgical wound with a solution of polymyxin B sulphate and bacitracin was reported. It was concluded that thorough irrigation with saline containing 25 to 50 units per millilitre of bacitracin and 25 to 50 micrograms per millilitre polymyxin B sulphate was beneficial in reducing the rate of infection, but that the use of neomycin was not justified because of the risk of neurotoxic complications.

In conclusion it appears that there is good evidence for the inclusion of antibiotics in solutions used to irrigate orthopaedic wounds, and our results suggest that this will not inhibit osteogenesis. However, certain antibiotics such as neomycin and chloramphenicol seem to be unjustified because of possible toxicity. The use of any antibiotic in powder form is extremely detrimental to osteogenesis and is not in any way advantageous.

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