HS

A HISTOLOGICAL SURVEY
of
BONE HOMOGRAFTS
in
YOUNG CHICKS

by

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INTRODUCTION AND REVIEW of the LITERATURE

A - The History of Bone Grafting

Since Duhamel first placed silver wire subperiosteally in 1739 and reported in 1742 that the bone he found covering them some weeks later was of periosteal origin the controversy over the source of osteogenesis raged for over 150 years. In 1763 von Haller put forward the theory that the periosteum was solely a support for the arteries which then produced bone by exudation.

By 1867 Ollier had performed extensive experiments and reviewed the literature, both of which led him to the conclusion that transplanted periosteum and bone could survive and become osteogenic. This was repudiated by Barth in 1893, who after working with trephined skulls and replacing the bone, claimed that all the bone and periosteum died.

Axhausen's findings on material from both animal and human sources between 1907 and 1911 are considered to be the classical works in this field. Having been confirmed by later investigators (e.g. - Hammack and Enneking, 1960), his views are still widely accepted. He believed that the transplanted bone dies but that the periosteum survives, proliferates and forms new bone.

The first twenty years of the twentieth century saw a marked increase clinically in the use of autogenous bone as a free graft with one author alone (Albee, 1919) reporting 1600 successful cases.

The success of these bone autografts stimulated interest in homogenous bone as a likely and, hopefully, larger source of graft material. A great deal of experimentation on methods of storage and much clinical research produced much contradictory information until a simple method of storing bone was evolved (Bush, Garber, 1948).

In the twenty years since the development of this method of storing bone homogenous bone grafting has become a much more useful and common clinical technique. It has become so widespread that as a result there is such an increased demand for new sources of bone that heterologous bone was being considered (Karges, et al., 1963; Kingma, Hampe, 1964).

As both clinical and experimental work proceeded it became increasingly apparent that the basic factors influencing both the acceptance of foreign tissues and production of new bone by the host were poorly, if at all, understood (Bonfiglio et al., 1955; Chase, Herndon, 1955; Siffert, 1955; Curtiss et al., 1956; Voisin, Maurer, 1956-57; Simonsen, 1957).

B - Transplantation Immunity

Since the appearance of Burnet's Clonal Selection Theory and its application to acquired immunity (Burnet, 1959) in which recognition of self (Burnet, 1961; Burwell, 1963a) with all its implications in the phenomena of rejection and tolerance (Billingham et al., 1955; Terasaki et al., 1958; Medewar, 1961; Stark et al., 1961; Holborow, 1967; Lambert, Frank, 1967) was included, many advances of the field of transplantation have been made. Medewar and his associates, as well as many others, have attempted to correlate the results of transplantation of various tissues and organs with other factors such as sex (Eichwald et al., 1958), blood groups (Schierman, Nordskog, 1961; Craig, McDermid, 1963), degree of consanguinity (Polley et al., 1960), age (Billingham et al., 1955; Polley et al., 1960), etc. which could be measured before transplantation and so predict the outcome. In this way it was also hoped that further information could be gained about the site and nature of histocompatibility antigens (Silvers et al., 1967; Van Rood et al., 1967; McDonald, 1966).

Attempts were then made to correlate the varying results obtained from bone transplantation (Hutchison, 1952; Siffert, 1955; Chalmers, 1959; Kingma, Hampe, 1964)

with the results from other tissue and organ transplantation (Polley et al., 1960; Stark et al., 1961; Van Rood et al., 1967) in view of this new information. As a result rapid advances in the knowledge of the immunological properties of bone and their bases have been made (Burwell, 1962, 1963b, 1964, 1966; Burwell, Gowland, 1961a, 1961b, 1962; Enneking, 1962; Burwell et al., 1963; Sabet, Ray, 1964; Chalmers, 1967).

C - Induction and Modulation

As work progressed it became apparent that bone, a tissue already in a precarious state due to its blood supply (Trueta, 1963), does not, unlike other tissue and organ transplants, establish a blood supply rapidly enough to ensure that more than a few surface cells (Hutchison, 1952; Bonfiglio, 1958; Zeiss et al., 1960) and the periosteum, when present (Hammack, Enneking, 1960), survive. This then presented the problem of distinguishing between the new bone formed by the proliferation of surviving graft cells (Ray, Sabet, 1963), especially from the periosteum, and that formed by induction from the host (Buring, Urist, 1967a; Dubuc, Urist, 1967). At present the question is still incompletely resolved.

This introduction of induction and the associated concept of modulation as terms applicable to adult organisms as well as embryos, opened up another dimension in the search for the sources of bone formation.

As originally used induction was an embryological term describing the "process by which one group of cells stimulates a second group to differentiate" (Balinsky, 1965). Specialized tissues and organs appeared to depend on the influence of preceding and adjacent tissues and structures for their development (Amprino, 1953; Barth, 1953). Inducing agents have been shown, in some instances in embryos, to be diffusible substances of nucleotide structure (Hommes et al., 1962; Lash et al., 1962; Cooper, 1965). However, in the case of bone induction in the mature animal, the inducing agent appears to be relatively insoluble and diffuses only over very limited distances, therefore requiring fairly close cell contact (Urist et al., 1967).

It has also been shown that as the embryo differentiates, the more specialized tissues react more specifically to a narrower range of inducing agents (Barth, 1953; Balinsky, 1965). Definitive areas become self-differentiating after an initial stimulus (Lacroix, 1956; Benoit, 1960b) but will, in their turn, act as inducers only in

a relatively limited area (Benoit, 1960b). This demonstrates the fact that reacting areas must undergo some prehistologic change which will commit them to a predetermined course if the proper (and specific) stimulus is forthcoming (Benoit, 1960a). This problem becomes increasingly complex with the additional information that the various reacting areas show differing reactivity to the same inducing agents at different stages in their development (Barth, 1953; Cooper, 1965). It has also been shown that dissociated embryonic cells will reform, at least partially, the organ from which they are taken, but only if the different cell types are present in the proper ratio (Moscona, 1956; Weiss, Taylor, 1960).

The application of this concept of induction to differentiated adult tissues has pointed up the great differences as well as the definite similarities between them and the orderly progression of differentiation in embryonic induction. Prehistological changes can be demonstrated (Siffert, Barash, 1961) in bone repair. The responses of adult tissues, however, can be elicited by a wide range of relatively non-specific inducing agents (Huggins et al., 1936; Moss, 1958; Benoit, 1960a; Makin, 1962) and are more limited in scope leading to a tissue differentiation (histogenesis) rather than an organogenesis (Weiss, Taylor, 1960).

Modulation refers to the process by which specialized forms and functions of cells can, under certain circumstances, either revert to their original less specialized state or transform into another specialized state (Urist, McLean, 1953; Weiss, 1953; Holtzer et al., 1960). The changes in the cell population of the bones of the laying hen (Bloom et al., 1958) or in the bones of mammals under the influence of parathyroid hormone (Heller et al., 1950) are thought to be good examples.

The whole adult organism is in a state of "dynamic equilibrium" (Barth, 1953) and bone shares in this (McLean, Urist, 1961). It has been hypothesized recently that this "morphostasis" is the primary function of lymphoid tissue and that immunity and/or recognition of "self" is a secondary but axiomatic corollary of this (Burwell, 1963a).

With the development of radioisotopes as tracer substances it has been found that a startling amount of renewal occurs in many tissues (Amano et al., 1959; Messier, Leblond, 1960). Even in bone, generally considered a rather permanent fixture, a high rate of turnover of both cells and matrix has been demonstrated in trabecular bone (Leblond et al., 1950; Kember, 1960; Tonna, 1961). Since this is the case, there must be a

source of unspecialized cells with the capacity to form this highly specialized tissue when presented with the proper stimulus. Which cells? and what stimuli? have been the basis of many investigations, some still in progress (Amprino, 1953; Tonna 1960; Tonna, Cronkite, 1961; Young, 1962c; Buring, Urist, 1967a). Once the undifferentiated cells of bone have become specialized they still appear able to undergo modulation between widely differing forms with rapidity under a variety of conditions (Bloom et al., 1958; Heller et al., 1950).

The crystal-collagen relationships of bone matrix have been well demonstrated (Robinson, 1952a; Watson, Robinson, 1953; Robinson, Watson, 1954-55) and have led to attempts to relate them to the electrical currents that bone produces under stress (Bassett, Becker, 1962). Bone, as is well known, remodels under stress (Bassett, Becker, 1962) to conform to mechanical requirements. The possibility that the cellular modulation believed to be responsible for this remodelling is affected by the weak electric currents produced by stress as some aspects of embryonic development (Sedar, 1956) and amphibian regeneration (Becker, 1961) are influenced by weak electric fields has been considered (Bassett, Becker, 1962).

D - Types of Bone and Cartilage Transplant

Bone may be considered as a living transplant or, if treated so as to kill the cells, as an implant (Freiburg, Ray, 1964). As a transplant it may be used as an "organ" and transferred in toto with its cartilage and marrow undisturbed. Whole bones of various ages to various ectopic sites (Sabet et al., 1961; Chalmers, Ray, 1962) and massive bone grafts with attached joint cartilage (Herndon, Chase, 1954; Entin et al., 1962) have been used in this fashion.

Cancellous bone, with marrow and washed free of marrow (Burwell, 1964, 1966), has been investigated for its immunological properties. It has also been studied to discover if any cells survive (Arora, Laskin, 1964) and if not, if it induces new bone (Urist, McLean, 1952; Bridges, Pritchard, 1958; Williams, 1960; Ray, Sabet, 1963) or merely serves as a framework for invading host cells (osteoconduction - Chalmers, 1967).

Dead bone killed and treated in many ways has been extensively studied to discover if it is immunologically active (Burwell et al., 1963; Buring, Urist, 1967a) and if it retains its inducing activity (Urist, McLean, 1952; Buring, Urist, 1967a; Dubuc, Urist, 1967).

Bone marrow has been studied alone (Burwell, 1964),

combined with bone matrix (Burwell, 1964) or as part of a whole bone (Herndon, Chase, 1954). It has been considered as a source of the osteoprogenitor cells in osteogenesis (Burwell, 1964) and as a factor in eliciting the immune response of the host (Burwell, 1963b).

Cartilage has been extensively investigated. Many in vitro studies have been carried out to elucidate its mode of metabolism (Laskin, Sarnat, 1953), and conditions under which it differentiates (Holtzer, Detweiler, 1953; Bassett, Herrmann, 1961; Hall, 1967) or dedifferentiates (Holtzer et al., 1960). In vivo its role in endochondral bone formation (Lacroix, 1956), its activity as an inducer of osteogenesis (especially in fracture callus) (Lacroix, 1951; Bonfiglio, 1958; Urist et al., 1965) and its apparent ability to resist rejection when transplanted (Peer, 1958; Peacock et al., 1960) have all been subjects of much interest to investigators. The immunological properties of whole cartilage (Khvorostukhin, 1958; Peacock et al., 1960) and some of its chemical components (Humphrey, 1943; Boake, Muir, 1955; Quinn, Cerroni, 1957; Quinn, Singh, 1957) have been studied and the results are equivocal.

E - Aims of the Present Study

Migicovsky and Nielson (1952) noted that when they

used the intact animal (chick) as a culture medium in their studies on the action of Vitamin D, that the implanted bones became encapsulated and surrounded by a partly calcified tissue.

This study was undertaken to elucidate the nature and source of this calcified tissue.

The preliminary series were designed to show whether subcutaneous or intraperitoneal transplantation provided a more favorable site for its development.

The age of the donor was to be varied to see if this had any effect on the amount and type of new tissue produced. All the host birds were to be two weeks of age; at this time chickens are considered to be immunologically competent (Billingham et al., 1955) so that new growth still taking place more than two weeks after implantation could be attributed to host tolerance naturally present and not tolerance induced by a graft before the host was immunologically competent (Nisbet et al., 1960; Medewar, 1961; Stark et al., 1961) if this new tissue was to prove to be of donor origin.

The tibiae were to be left <u>in situ</u> for varying periods from three days to four weeks to distinguish the source and follow the maturation of the new tissue produced. It was also hoped that if this tissue were rejected, some picture or the process, if and when it

appeared, could be formed.

A large series of implanted tibiae were to be left *in situ* for three weeks since rejection has usually begun by two weeks (Polley et al., 1960) and would be well established by twenty-one days. It was hoped to demonstrate whether or not the same success rate for whole bone grafts, shown by growth of the transplant, would be found as has been found in skin grafting in chickens (Craig and McDermid, 1963). If this were indeed the case, some of the same factors that influence skin grafting might be demonstrable statistically (Schierman and Nordskog, 1961; Craig and McDermid, 1963).

II - METHODS and MATERIALS

A - Experimental Animals

White Rock cockerels, ages one day to two weeks, (supplied by The Fisher Orchards, Burlington, Ontario) and of an unknown degree of inbreeding were used as both hosts and donors in all the experiments.

The first 180 birds were obtained during the summer and kept on AOAC rachitogenic diet supplemented with 1000 units of Vitamin D_3 /kgm. of feed and water *ad libitum*, following the method of Migicovsky and Nielson

(1952). This group of birds never appeared to be healthy from the time they were received and there was a high mortality rate of approximately one-third among the implanted birds.

The 285 chicks used in Parts 4 and 5 of the experiments were obtained in the fall. They appeared to be healthy. They were kept on a Standard Green Diet and water ad libitum throughout the experimental period. Only one bird was lost during an accident while transferring cages.

B - Experimental Methods

1 - Subcutaneous versus Intra-abdominal Whole Bone Transplants

Migicovsky and Nielson (1952) used both subcutaneous and intraperitoneal sites in their study. This preliminary series was done in order to decide which was the more favorable site for the increase in size of the implant noted in the original study.

One day old whole tibias were implanted subcutaneously or intraperitoneally into two-week old hosts and left for periods varying from one to four weeks (Table I).

Table I - Summary of Transplantation Sites in Part 1

Weeks in situ	Number of Specimens			
	Subcutaneous		Intra-abdominal	
	Implanted	Survived	Implanted	Survived
1	5	4	5	5
2	6	6	5	4
3	5	5	6	3
4	4	4	4	3
Total	20	19	20	15

2 - Ca^{45} as a Tracer of the Calcification of the Transplant

Twenty days after a whole day-old tibia had been placed subcutaneously in each of five hosts were given 1 μc Ca^{45} by intravenous injection. The host was sacrificed and the transplanted bone removed 24 hours later.

3 - Bone Fragments

As a result of the variation in amount of growth observed at different sites within the same donor bone in Part 1 fragments from three regions of day-old chick tibiae were used as transplants in an attempt to ascertain which region made the major contribution. The three regions studied were the cartilage of the

head of the bone (Zone A - Bélanger, 1959a), the zone of hypertrophied cartilage (Zone C), and the bone of the mid-diaphysis. Slices of these areas were implanted subcutaneously in two week old hosts and left in situ for three weeks.

Table II - Summary of the Types of Grafts in Part 3

		Type of Graft		
		Zone A (cartilage)	Zone C (cartilage)	Diaphysis (bone)
Number of Hosts	Implanted	10	10	10
	Survived	8	8	4

4 - Variation in Donor Age and Length of Time in situ

These transplants were arranged to observe both the effects of the age of the donor on the success of the graft and also the progress with time of any new bone and cartilage formation. Therefore, two main groups - A - with donors one-day old and - B - with donors one-week old were further divided into five subgroups of ten host birds each, according to the length of time the graft was to be left in situ. These time intervals

ranged from three days to four weeks: 1 - three days; 2 - one week; 3 - two weeks; 4 - three weeks; 5 - four weeks. This then gave two series of five groups of ten birds each: A_1, A_2, A_3, A_4, A_5 (donors one-day old) and B_1, B_2, B_3, B_4, B_5 (donors one-week old) (Table III).

In addition two birds one-day old (A_o) and two one-week old (B_o) were sacrificed and their tibiae fixed and processed without being implanted in order to obtain normals for comparison.

Table III - Summary of Donors in Series A and B - Part 4

Days in situ	Sub-group	Number of Specimens Series A	Series B
0	0 (control)	2	2
3	1	10	10
7	2	10	10
14	3	10	10
21	4	10	10
28	5	10	10

5 - Series of Whole Bone Subcutaneous Transplants for Statistical Analysis of Rejection

This series consisted of 90 subcutaneous transplants of one-day old donor tibiae into two-week old hosts. These were all left in situ for three weeks. One host died and so 89 specimens were finally obtained. In 28 of the donors records were kept of which hosts had received grafts from the same donor so that the growth of identical grafts could be observed and compared in different hosts.

C - Surgical Techniques

1 - Preparation of the Grafts

a - Whole Bones

For both the intra-abdominal and subcutaneous grafts the procedure of preparing the implant was the same. The donors, of various ages, were sacrificed and both tibiae removed from each. These bones were then carefully cleaned of muscle and soft tissue taking care not to damage the articular surfaces. The fibula was then removed. The fibrous periosteum remained intact except for a small area at the upper end of the tibia where the fibula had been strongly attached. The tibiae were then placed in a beaker of distilled water at room temperature until used - usually about ten to fifteen minutes.

b - Bone Fragments

For this section of the experiments, the whole bones were prepared as noted above. Cross-sections 1 mm. thick were cut with a razor blade through the cartilage cap, the metaphyseal region and the mid-shaft of each bone. Each section was implanted in a separate host.

2 - Anaesthesia

The abdominal transplants were carried out under nembutal anaesthesia. About ten minutes preoperatively, each host bird received 15 mgm. of nembutal intravenously in a wing vein.

No anaesthesia was used for the subcutaneous implants as it was felt that it was an unnecessary risk, since several birds were lost in attempting to establish a dosage of anaesthetic and since the subcutaneous implants did not appear to cause the birds undue suffering.

3 - Subcutaneous Transplants

For the subcutaneous graft, the two-week old host bird was held in the assistant's hand with the right leg drawn forwards and upwards to expose the lower ribs. A small incision was made by lifting a fold of skin just

posterior to the distal rib and cutting across this fold with scissors. Next a pocket was formed in the loose subcutaneous tissues by slipping a small artery forceps anteriorly just under the skin and then opening its jaws to enlarge the pocket. If a whole bone was being used as the graft, the donor tibia was then slid into this space with its proximal and anteriorly. If a bone fragment was used, the graft was placed flat against the ribs. Finally the skin was closed with two or three cotton sutures. As long as the incision was placed posterior to the ribs and the correct plane of cleavage in the tissues was found, no bleeding was encountered. Even when bleeding did occur it was minimal and required no special measures to stop it.

4 - Intra-abdominal Transplants

The procedure for the intraperitoneal transplants was to hold the anaesthetized host in the assistant's hand with the leg drawn forwards and upwards to expose the abdomen. A small incision was made about three-quarters of an inch caudal to the distal rib in the same way as for the subcutaneous transplant. When the abdominal muscles were exposed they were split along the muscle fibers as much as possible and retracted

to expose the peritoneum. This was then incised and the whole tibia inserted gently into the peritoneal cavity attempting not to damage the internal structures. The wound was then closed in layers with sutures in the peritoneum and skin. The muscles fell back into place without sutures.

All the above procedures were carried out under clean but not aseptic technique. No gross infection was noted.

D - Histological Techniques

1 - Decalcified Sections

a - Fixation, Decalcification and Embedding

At predetermined intervals, groups of host birds were sacrificed; the implants removed with any adherent soft tissue and placed immediately in A.F.A. (acetic acid: formaldehyde: ethanol - 1: 4: 15 parts) to fix for twenty-four hours. They were then decalcified in 10% (saturated) EDTA (disodium salt of ethylenediamine-tetra-acetic acid, Sequestrene, Geigy) in distilled water (pH ≈ 5.5) for ten days according to the constant replacement method of Bélanger et al., 1965. At the end of this period, the bones were washed in running water for thirty minutes and then sectioned longitudinally with a razor blade leaving three-quarters of the bone to be embedded and one-quarter to be discarded. Specimens too

long to fit the plastic cases used during impregnation were cut in half. If any resistance to the razor blade when cutting was encountered, the specimen was rewrapped in cheesecloth and returned to the EDTA solution for another three to four days. The specimens were then routinely embedded in paraffin for sectioning.

b - Staining (Dilute Wright's Stain)

Sections seven micra (7μ) thick were cut longitudinally from each specimen and stained with dilute Wright's stain. This gives good metachromatic staining of normal cartilage matrix as well as good differentiation of bone marrow cells and connective tissue infiltrations. It also distinguishes the slightly basophilic areas of osteocytic osteolysis (Bélanger et al., 1968) from the rest of the bone which stains pink. Mast cell granules can also be distinctively stained by this method (Bélanger et al., 1957).

Dilute Wright stain is prepared by mixing

100 c.c. Wright stain

and 100 c.c. distilled water

then allowing the mixture to stand, stored in a dark glass bottle for five to six days before use.

To use the stain, the sections are first brought down to water in the usual way. They are then stained for

thirty (30) minutes in the dilute Wright solution which has been filtered before using. The sections are next rinsed quickly in distilled water, 95% alcohol and absolute alcohol in turn. Following these rinses, they are left one minute in a second change of absolute alcohol. Finally they are left for two minutes each, in a third change of absolute alcohol and three consecutive changes of xylol before being mounted.

c - Alpharadiography

Alpharadiography was used on selected sections from the first part of the experiment to determine if the density of the cartilage at the bone ends varied with its changing staining properties. The method used was a modification of that of Bélanger, 1959b.

All the following procedures are carried out under Safelight (Kodak).

The slides already coated with N.T.A. emulsion (Kodak) when purchased, are coated with a 1% solution of celloidin (Mallencroft) and allowed to dry overnight. Before use, they can be numbered with a diamond pencil in the usual manner. They are then dipped under the five micra (5μ) paraffin sections which have been prepared. Each prepared section is floated on distilled water in

an individual numbered preparation dish. The section is gently floated over the slide and accurately centred taking care not to scratch the emulsion. The slide is then firmly blotted with three layers of fine filter paper (Whatman filter paper, No. 50) to remove the excess water and render the section flat and adherent. The slides are then placed in a light-tight box to dry at least overnight.

Before exposure, the paraffin is removed from the sections by immersion, first in toluol and then in benzene for approximately one minute each. The slides are placed, for exposure, a fixed distance (2.4 cm.) from a source of alpha particles of known strength (Polonium^{210}, 3 millicuries) and size (1 x 3 cm.). The specimens are exposed for three hours.

After exposure, the section and the celloidin are both removed by immersion in acetone. This is followed by immersion in absolute alcohol. The slides are then gradually brought down to water before developing.

During fixing and developing the fluids used are kept at a temperature of about 68^{o} F. by means of a bath of running tap water. The slides to be processed are placed in a staining boat and immersed in the developer (D-19, Kodak) which has been diluted with an equal amount of distilled water for 10 minutes. During

the first few minutes they are gently agitated by hand. They are then rinsed (by being gently agitated) in distilled water for approximately one minute. Next they are placed in the fixer (Kodak) for fifteen minutes. (The normal lights may be turned on during the last ten minutes of this last process and left on for the rest of the procedure). Finally, the slides are washed in running tap water for twenty minutes, then fan-dried for thirty minutes before microscopic inspection.

They are allowed to dry overnight before being mounted. Before being mounted, they are immersed in toluol for three minutes; the coverslip is then fixed in place with Permount (Fisher Scientific Company).

d - Histochemistry: Stains for Mucopolysaccharides

Acid Mucopolysaccharides:

i - Thionine-eosin

Maximov's Alcoholic Thionine (McManus, Mowry, 1960)

Eosin - 0.1% in 95% alcohol

Stain with alcoholic thionine for 7 minutes.

Rinse with distilled water until the background is clear.

Place in 95% alcohol for 2 minutes.

Stain with 0.1% eosin for 2 minutes.

Follow with three changes of absolute alcohol for 2 minutes each.

Finish with three changes of xylol for 2 minutes each - then mount.

Neutral Mucopolysaccharides and Glycoproteins:

ii - Schiff's Reaction (P.A.S.)

Solution I

0.5% periodic acid diluted in distilled water

Solution II - Schiff's Reagent

Dissolve 1 gm. basic Fuchsin in 200 c.c. boiling distilled water.

Cool to about 50° C.

Add 1 c.c. of concentrated hydrochloric acid and 2 gms. of sodium bisulphite.

Shake the flask, stopper it tightly and let it stand at room temperature for 24 hrs.

Add 0.5 gm. of absorbent charcoal, shake and filter the mixture.

Keep the mixture refrigerated.

Solution III

2% solution of sodium bisulphite.

Stain

Stain with Solution I for 10 minutes.

Wash in running water for 10 minutes.

Place in Solution II for 12 minutes. (10 - 15 minutes)

Rinse in distilled water.

Place in Solution III for 1 minute. (1 - 2 minutes)

Wash for 5 minutes. (5 - 10 minutes).

Counterstain - Delafield's Maematoxylin and Orange G

Stain with filtered Delafield's Haematoxylin for 6 minutes.
Differentiate in acid alcohol.
Place in distilled water containing a pinch of Lithium Carbonate for 1 minute.
Wash in running water for 10 minutes.
Rinse in 95% alcohol.
Stain with Orange G for 1½ minutes.
Rinse quickly in 95% alcohol and two changes of absolute alcohol.
Place in a third change of absolute alcohol for 2 minutes,
Finally place in three successive changes of xylol for 2 minutes each - then mount.

2 - Calcified Sections

a - Stained Radioautographs

The implants from the hosts which had received 1 µcurie of Ca^{45} 24 hours before sacrifice were fixed in AFA and embedded in celloidin without prior decalcification. Sections 5 µ thick were cut and the method of Bélanger (1961) was followed for processing and staining autoradiographs. The sections were exposed for two weeks before developing.

b - Microincineration

Five hosts each received a subcutaneous graft of a one-day old tibia three weeks before sacrifice.

Fresh frozen sections 5 μ thick were cut on a Jung freezing-microtome, fixed to slides and ashed (Glick, 1949) before being covered with a cover slip. The edges of the cover slip were then carefully sealed.

III - OBSERVATIONS

A - Subcutaneous versus Intra-abdominal Transplants

1 - One Week in situ

a - Subcutaneous Transplants

After one week in situ these tibiae had increased in size mainly around their ends so that they were "dumb-bell" shaped. They were adherent to the overlying skin.

Histologically new bone and cartilage could be seen around the metaphyseal area of the bone. The bone appeared to be surrounded by a layer of mesenchymal tissue.

b - Intra-abdominal Transplants

These bones were adherent to various abdominal

organs, e.g. liver, intestine, etc. or to the abdominal wall. They were larger in diameter than the subcutaneous group.

Under the microscope, large numbers of young trabeculae could be seen surrounding the shaft. This bone gave way to cartilage at the metaphysis and this formed a cap over the ends of the implanted bone.

2 - Two Weeks in situ

a - Subcutaneous Transplants

Grossly these transplants had increased mainly in diameter and were roughly cylindrical in shape.

The sections showed large numbers of young bone trabeculae with a moderate number of osteoclasts around them. Some small round cells could be seen in the tissue between the trabeculae.

b - Intra-abdominal Transplants

In this site the transplants had continued to grow chiefly in circumference.

Microscopically there was increased cartilage formation outside the new trabeculae. At the metaphyseal level the new cartilage appeared hypertrophied. No small round cells were noted.

3 - Three Weeks *in situ*

a - Subcutaneous Transplants

These grafts had remained relatively stable in size when compared to those taken the previous week.

Microscopically the main change was an increase in the number of small round cells which under high power, were seen to be small lymphocytes. The trabeculae had lost their covering of osteoblasts.

b - Intra-abdominal Transplants

In this group the bones had continued to increase in girth.

The sections showed a deeper layer of cartilage around the new bone. The new cartilage at the metaphysis was definitely hypertrophied with the loss of some of its cells. The bone trabeculae were still lined with osteoblasts though a moderate number of small lymphocytes had appeared around them.

4 - Four weeks *in situ*

a - Subcutaneous Transplants

All except one bone had markedly increased from their original size, mainly in circumference. One bone was smaller than at the time of transplantation.

Histologically the large grafts showed very large numbers of trabeculae lined with osteoblasts. The ends of the grafts were covered with cartilage. One graft showed no new bone or cartilage formation. The original bone was acellular.

b - Intra-abdominal Transplants

Grossly this group of grafts resembled the subcutaneous grafts of the same age.

Microscopically the new bone and cartilage were both acellular. There was no lymphocytic infiltration.

B - Calcified Sections

1 - Ca^{45} Radioautographs

When the stained radioautographs were examined under the microscope, a heavy deposit of silver grains could be seen overlying the new bone trabeculae which were oriented mainly at right angles to the original shaft. The bone of the shaft itself showed no radioactivity at all.

The new cartilage which could be distinguished by its staining properties, did not show any overlying grains indicative of Ca^{45} deposition in it. The cartilage in these sections did not appear to be hypertrophied.

2 - Microincineration

The sections showed mineral ash in the region of the original shaft of the bone as well as in the calcified cartilage. The numerous new trabeculae which had formed around the transplant during its three weeks in situ, were also shown to be calcified.

C - Bone Fragments

1 - Zone A Cartilage

The cartilage fragments still retained most of their metachromasia after three weeks. Approximately half were beginning to lose their metachromatic staining at the edges. The chondrocytes in all the grafts were viable. The cartilage was surrounded by a small amount of loose connective tissue inside a thin capsule of dense connective tissue. Blood vessels were not prominent in the connective tissue. There was a light to moderate infiltration of the loose connective tissue with small lymphocytes and scattered plasma cells. No growth was evident in any of these fragments.

2 - Zone C Cartilage (hypertrophied cartilage)

Half of the specimens in this group had formed new bone and/or cartilage. When the transplanted cartilage had contained calcified areas, the new bone was found deposited in close apposition to them. There were no

osteoblasts lining the new trabeculae. A moderate to heavy lymphocytic infiltration was found between the trabeculae and under the dense connective tissue capsule which surrounded all the grafts. The occasional small marrow cavities were infiltrated by these same types of cells as well.

The other grafts showed loss of metachromasia in the cartilage and apparently no attempt at bone or cartilage formation. They also were surrounded by lymphocytes, usually in large numbers.

3 - Diaphyseal Bone

Small amounts of new cartilage were found in close contact with three of the four transplants in this group. The other transplant showed a few cellular trabeculae which were surrounded by large numbers of osteoclasts. A few osteocytes survived within the lacunae of the original trabeculae. Around the bone fragments and extending into the marrow cavity, a heavy infiltration of small lymphocytes and plasma cells was noted.

D - Variation of Donor Age and Length of Time in situ Subcutaneously (Series A and B)

A_o - control - one-day old chick tibia - unimplanted

Light Microscopy

The periosteum covering the tibia of the day-old chick consists of two definite layers: an outer fibrous one which merges with the flattened cartilage cells covering the articular surfaces and an inner osteoblastic layer. This latter layer is closely applied to the bone and is one to two cells thick over the shaft. The osteoblasts are flattened and oval with a basophilic cytoplasm. They are arranged with their long axis parallel to the long axis of the bone. In the region where the flare of the metaphysis begins, this layer becomes five to eight cells thick. It stops abruptly at the level of the epiphysis. Also at the flare of the metaphysis, the woven bone of the shaft gives way to hypertrophied cartilage whose peripheral zone can be shown to be calcified (Fig. 1).

The epiphysis itself consists of cellular cartilage with darkly metachromatic staining matrix containing numerous cavities with thin-walled blood vessels and a few connective tissue cells within them. In some sections the distal cartilage shows an area of hypertrophy which coincides with the area of endochondral ossification seen in older specimens. The articular surfaces are covered by a layer of flattened cells which have a

very small amount of metachromatic matrix between them so that this area appears less darkly stained. These cells merge with the epiphyseal cartilage through a narrow band of transitional forms (Fell, 1925).

At both ends of the bone just below the epiphyseal cartilage, there is a narrow zone of grossly flattened cartilage cells (Fig. 1). This area merges with a wider region in which the cells are at first discoidal and then as they progress toward the shaft, polyhedral; all are lined up in definite longitudinal rows. This region in turn merges with a large area of hypertrophied cartilage which extends as a tongue deep inside the trabecular bone of the diaphysis (Fig. 1). The matrix retains its metachromasia though it is slightly paler in the hypertrophied zone.

The diaphysis consists of a tube of woven bone with most of the trabeculae oriented in a general longitudinal direction. The trabeculae are covered with a layer of flattened osteoblasts except at the junction with the metaphysis where the osteoblasts are plump. The osteocytes appear healthy though some of the lacunae have distinctly metachromatic borders in areas where the bone matrix is slightly basophilic. At the region where the flare of the metaphysis begins, a small

amount of endochondral bone being laid down over cores of calcified cartilage can be found at the periphery just beneath the periosteum. On the more central trabeculae towards the ends of the marrow cavity, some osteoclasts can be found.

The marrow cavity fills the diaphysis. The marrow is very cellular and islands of haematopoietic cells are numerous. It extends around the diaphyseal end of the zone of hypertrophied cartilage and between it and the narrow zone of endochondral ossification. It also extends for some distance into the longitudinal channels containing thin-walled blood vessels which run through the hypertrophied cartilage (Fig. 1). A few of these channels and their contained vessels extend into the epiphyseal cartilage.

B_o - control - one-week old chick tibia - unimplanted

Light Microscopy

In the tibia of the week-old chick, the periosteum still consists of two definite layers. Again the fibrous layer merges with the flattened cartilage cells over the articular surfaces. The osteoblastic layer is very cellular in the metaphyseal region but tapers off to only one cell thick over the diaphysis (Fig. 2).

The epiphysis still consists of cellular cartilage with a deeply metachromatic matrix. The numerous cavities containing blood vessels are now noted to be branching.

An area of hypertrophied cartilage with a small amount of osteoid laid down to form spicules can be found in some sections of the distal epiphysis. The flattened cells of the articular surface still retain a narrow band of metachromasia between them.

The cartilage at either end of the bone is now distinctly divided into three zones (Bélanger, 1959a). The change from the epiphyseal cartilage (Zone A) to the narrow zone of flattened cells (Zone B) is sudden. There is then another abrupt change to a zone of hypertrophied cartilage cells (Zone C). Finally, a clearly demarcated border between the hypertrophied calcified cartilage and the zone of endochondral bone formation can be seen (Fig. 2). This endochondral ossification now extends from the beginning of the flare of the metaphysis to include approximately two-thirds to three-quarters of the zone of hypertrophied cartilage originally seen in the one day old chick tibia (Fig. 1).

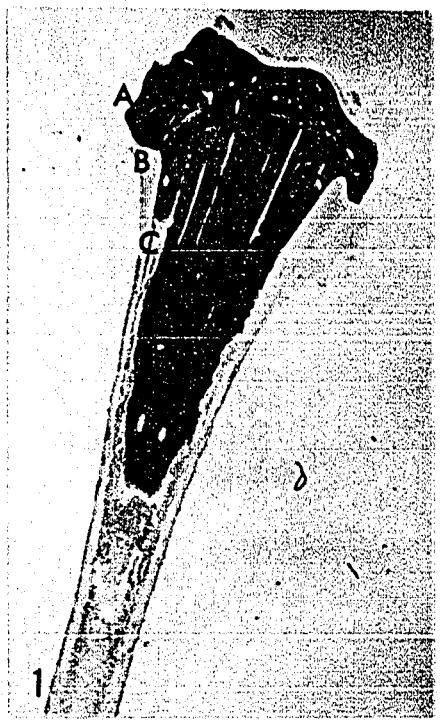
The diaphysis is now composed of more compact longitudinal lamellae of bone. Some osteocytes in lamellae close to the marrow cavity have metachromatic borders and the surrounding matrix is somewhat basophilic. The trabeculae at either end of the marrow cavity are covered by flattened cells except those subperiostally

Fig. 1 - Section through a one-day old chick tibia - unimplanted control (A_o). Note the three zones of the cartilage; A - cartilage of the head, B - zone of flattened cartilage cells, C - tongue of hypertrophied cartilage extending down inside the shaft. The marrow is noticeably cellular. Dilute Wright stain. 4½x.

Fig. 2 - Section through a one-week old chick tibia - unimplanted control (B_o). The three zones of cartilage are still present but the hypertrophied zone is now narrower. Islands of haematopoietic cells can be seen in the marrow. Dilute Wright stain. 4½x.

Fig. 3 - A_1 - one day old donor - implanted three days. Note the patchy loss of metachromasia in the head. An adhesion with blood vessels can be seen (arrow). Dilute Wright stain. 4½x.

Fig. 4 - B_1 - one-week old donor - implanted three days. Note patchy loss of metachromasia in the head, and in the line between Zones A and B. The marrow appears acellular. Dilute Wright stain. 4½x.



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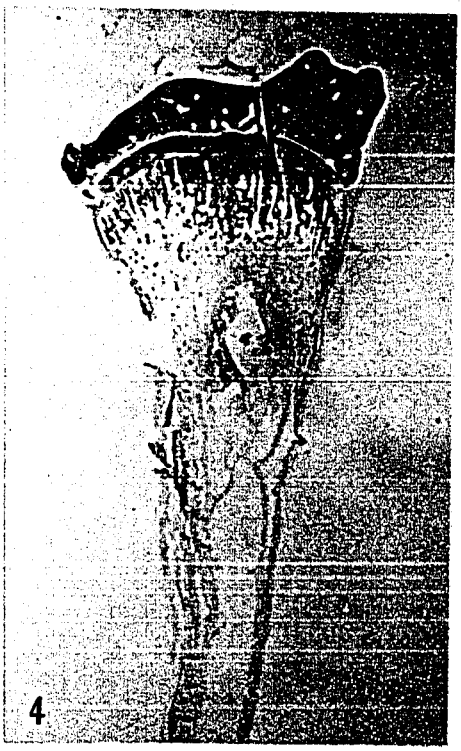
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in the region of the metaphysis which are lined by plump osteoblasts.

The marrow fills the diaphysis and extends between the spicules of forming endochondral bone. It contains islands of actively haematopoietic cells in recognizable adipose tissue. There are more longitudinal channels in the hypertrophied cartilage and more of these extend into the epiphysis than in the one-day old bone.

A_1 - one-day old chick tibia - implanted three days

Gross observations

After each chick was sacrificed the skin was split along the keel and peeled back to reveal the implant adherent to the skin, lying in an envelope of areolar connective tissue. The areolar tissue was transparent except over the anterior aspect which was hyperaemic where it overlay the proximal end of the implant (tibia). Careful dissection revealed the bone to be lying in a pocket in the loose connective tissue with the anterior and adherent to the skin of the host. Tiny blood vessels appeared to run through this adhesion and enter the bone below the cartilage cap.

The large vein from the runner feathers forms a subcutaneous network in this area. It appeared engorged when compared with that of the opposite side.

Light Microscopy

After implantation in the subcutaneous tissues for three days, the tibia retains its original architecture. The osteocytes and chondrocytes appear healthy (Fig. 3) except in the hypertrophied cartilage where they have completely disappeared (Fig. 6). Most of the cells of the bone marrow are dead or dying apart from some mature cell types which remain at the periphery. Most of the nuclei are smudged and indistinct though the cells retain their outlines and even their specific granulation in the case of those of the polymorphonuclear leucocyte series.

The periosteum is still identifiable; the fibrous layer remains unchanged. The osteoblastic layer appears thicker over the metaphysis. The cells are paler and more rounded now in this area (Fig. 8). The nuclei of these cells are large and oval, often with two nucleoli visible. This layer now has the appearance of mesenchyme in some areas around the metaphysis.

The epiphyseal cartilage in nine of the ten specimens is still metachromatic though paler in patches than previously (Fig. 3). The layer of flattened cells over the articular cartilage appears to have increased in depth and has lost its metachromasia. Scattered mitotic

figures can be found in the outermost layer with an immediately subjacent zone which is undergoing necrosis. Beneath this, immediately adjacent to the metachromatic epiphyseal cartilage, is a narrow zone of cartilage which has lost its metachromatic staining properties and is very pale (Fig. 5). This loss of staining accounts for most of the apparent increase in the depth of this zone.

In one specimen, the cartilage has lost its metachromasia except for the area of calcified cartilage in the metaphysis and some peropheral epiphyseal cartilage (Fig. 16).

No new cartilage or bone can be seen in any specimen.

Alpharadiography

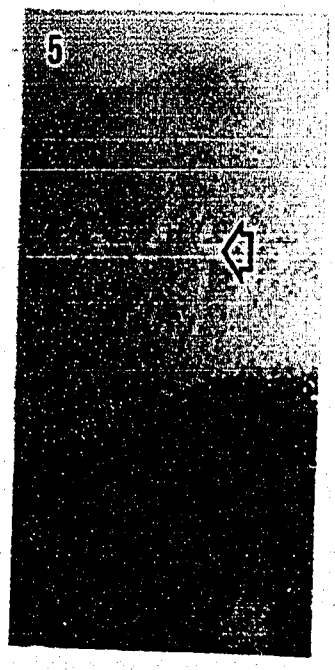
Exposure of a decalcified section to a source of alpha rays showed the cartilage of both the head of the bone and the hypertrophied cartilage or the metaphyseal area to be of uniformly low density, except for a narrow band of increased density at the edges of the channels containing the blood vessels.

The trabeculae of the shaft show decreased density in the areas corresponding to the areas of basophilia seen in the stained sections.

Fig. 5 - A_1 - one-day old donor - implanted three days. Section through the edge of the articular cartilage. The metachromasia is absent from the surface layers. Note the zone of beginning necrosis (arrow). Dilute Wright stain. 110x.

Fig. 6 - A_1 - one-day old donor - implanted three days. Zone B cartilage is seen at the top of the picture. Note the absence of cells in the lacunae of the hypertrophied zone (Zone C). Dilute Wright stain. 110x.

Fig. 7 - A_4 - one-day old donor - implanted twenty-one days. Old bone trabeculae without osteocytes can be seen at the bottom of the picture. The dark area (arrow) is amorphous eosinophilic debris. This debris is surrounded by a layer of vacuolated multinucleated cells Dilute Wright stain. 220x.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Histochemistry to Demonstrate Mucopolysaccharides in the Cartilage.

Thionine-eosin

With this stain the cartilage of the head is deeply metachromatic except for a narrow border at the articular surface. The hypertrophied cartilage also stains metachromatically. Some metachromasia can also be demonstrated around some osteocyte lacunae in some of the trabeculae of the shaft.

PA-Schiff reaction.

The main areas which reacted positively in this case were limited borders around the channels which originally contained blood vessels in the epiphyseal cartilage and the small zone of cartilage in the metaphysis which had calcified.

B1 - one-week old chick tibia - implanted three days

Gross Observations

When the skin was reflected in each case the implant was found to be adherent to the skin and lying in a boggy, yellow envelope of tissue. When this envelope was cut clear, colourless fluid seeped out of the cut surfaces. Two of the bones were lying free within their envelopes; the rest were attached by at least one end (usually the proximal), to the surrounding tissue. There was marked

hyperaemia of the enveloping tissues which was especially marked anteriorly over the proximal end of the implant.

Light Microscopy

Three days after implantation, the week-old tibia retains its original architecture. The osteocytes and chondrocytes are healthy except in the hypertrophied region where they have disappeared. No osteoblasts can be found on the trabeculae.

Many of the cells of the marrow are dead or dying though their outlines are still present. In a few sections a very light scattering of small lymphocytes can be seen in isolated areas.

The two layers of the periosteum appear unchanged. The osteoblastic layer was thick over the metaphysis in the control specimens and remains so after three days in the subcutaneous tissues.

The epiphyseal cartilage at one end of each of three tibiae has lost its metachromasia except for a small rim near the articular surface and in the region of calcified cartilage (Fig. 17). In the other epiphyseal regions, there is a patchy decrease in the metachromatic staining apart from two narrow bands of complete loss between the three zones of the cartilage which makes them even more distinct (Fig. 14). The layer of flattened cells over the articular surface has lost its metachromasia

as have the transitional cell types beneath it and a narrow band of subjacent cartilage. Scattered mitoses can be seen in the outermost layer. Only a few small areas of necrosis can be found in the transitional zone.

No new bone or cartilage formation can be seen.

A_2 - one-day old chick tibia - implanted seven days

Gross Observations

The whole envelope of the now translucent tissue surrounding the implant was hyperaemic. Eight of the bones showed some visible growth; three of these only at the proximal end. The tibiae were more adherent to the tissue around them, especially at the proximal end, than those of the preceding group. The two implants which showed no growth could be bent with moderate ease. This apparent decalcification seemed to have occurred mainly in the shaft as the metaphyses were still firm to the touch.

Light Microscopy

Seven days after implantation all the cartilage except the mineralized portion has lost its metachromatic staining apart from small areas of irregular growth at the edge of the head in five of the ten specimens (Fig. 19). The chondrocytes of all but the newly forming

Fig. 8 - A_1 - one-day old donor - implanted three days. From left to right: hypertrophied cartilage (Zone C), marrow with cell outlines still visible, spicules with calcified cartilage cores, trabeculae with viable osteocytes, osteoblastic layer of periosteum which has increased in depth (arrow) and finally the fibrous periosteum. Dilute Wright stain. 110x.

Fig. 9 - A_2 - one-day old donor - implanted seven days. From left to right: trabeculae with osteocytes in the lacunae, newly forming cartilage, fibrous periosteum which is less well defined than in Fig. 8.
Dilute Wright stain. 110x.

Fig. 10 - A_4 - one-day old donor - implanted twenty-one days. The new cartilage seen forming in Fig. 9 is now hypertrophied and some of the lacunae have disrupted walls. Large dark granules indicative of calcification can be seen in some areas (arrow).
Dilute Wright stain. 220x.

Fig. 11 - A_5 - one-day old donor - implanted twenty-eight day The cartilage lacunae are now being invaded by fibrous tissue and small round cells. Occasional chondroclasts can be seen (arrow).
Dilute Wright stain. 220x.


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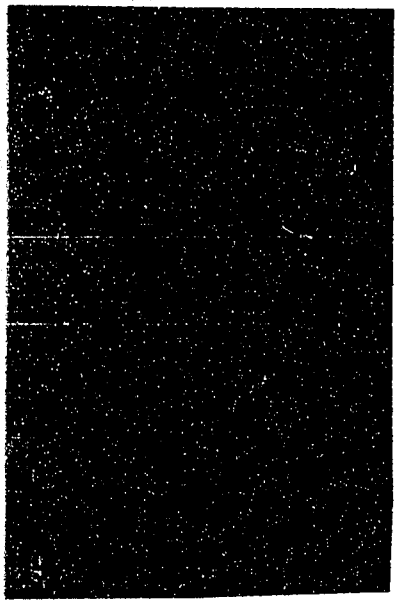

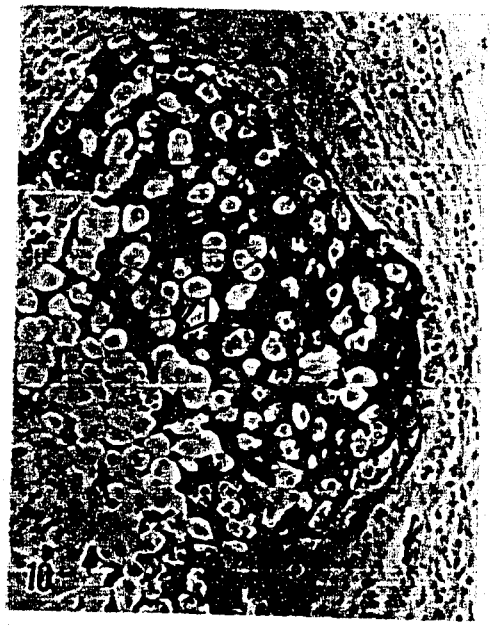
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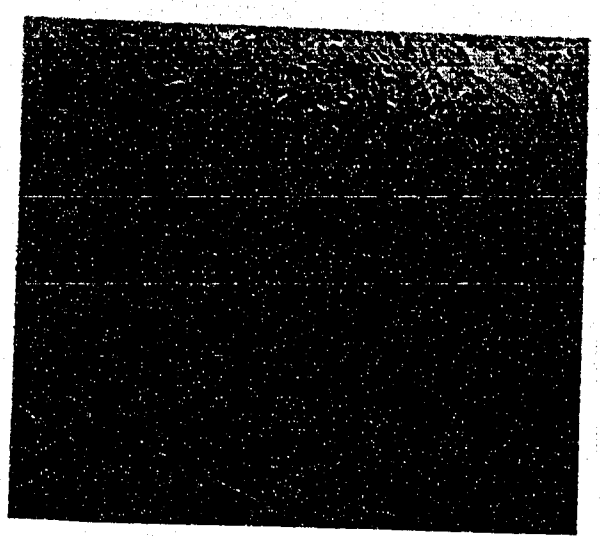
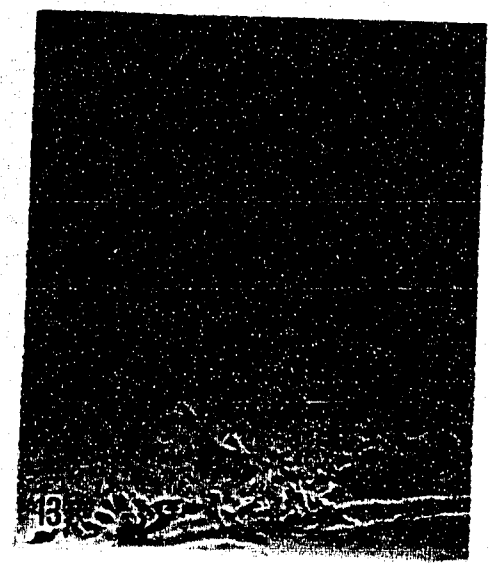
Fig. 12 - A_2 - one-day old donor - implanted seven days. Pale cellular trabeculae covered with plump osteoblasts can be seen forming in the tissue above the old trabeculae in the bottom left-hand corner. Blood vessels can be seen among the new trabeculae (arrow). Dilute Wright stain. 110x.

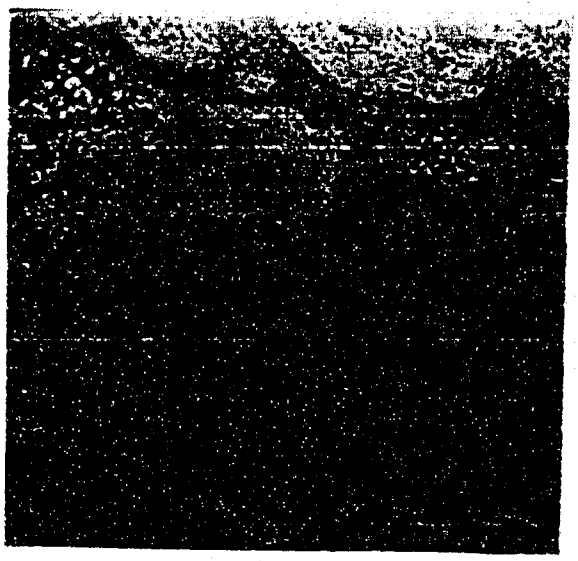
Fig. 13 - A_3 - one-day old donor - implanted fourteen days. New trabeculae can be seen without a covering layer of osteoblasts. Occasional osteoclasts can also be noted (arrow) on the new bone. The old trabeculae can be seen at the bottom with no osteocytes in their lacunae and frequent osteoclasts on their outside surface. Dilute Wright stain. 110x.

Fig. 14 - A_4 - one-day old donor - implanted twenty-one days. The new trabeculae now show areas of osteolysis in the centre of many of the trabeculae (arrow). The vascular connective tissue between the trabeculae is infiltrated with a few small round cells. Dilute Wright stain. 110x.

Fig. 15 - A_5 - one-day old donor - implanted twenty-eight days. The infiltrate around the trabeculae is now heavier than in Fig. 14. Large osteoclasts are present (arrow). Dilute Wright stain. 220x.







areas are pyknotic. The matrix of the epiphyses of the other five bones are now pale or medium pink in colour.

The periosteum is less well demarcated. Its fibrous layer appears to consist of younger, less dense collagenous fibrous tissue which forms a continuous capsule over both ends of the bone. Peripherally it merges with the surrounding loose connective tissue. Between this layer and the bone there is a wide area of very young cellular tissue. In some areas pale stellate cells such as those seen in primitive mesenchyme are seen; in others the cells are more basophilic and polyhedral and appear to be condensing. Many mitotic figures can be seen in both these regions. Areas of very immature bone trabeculae covered by one or even two layers of plump osteoblasts (Fig. 12) can be found in seven of the ten samples with adjacent sites of formation of very cellular cartilage with a deeply metachromatic matrix (Fig. 9). Between these areas, transitional forms which blend these areas gradually with one another, can be distinguished. Many thin-walled vessels can be found in this layer.

The osteocytes of the original trabeculae are still generally healthy (Fig. 9). Some are becoming pyknotic. There are no osteoblasts lining the trabeculae. Occasional osteoclasts can be found. Small areas of granular or

amorphous eosinophilic debris with basophilic areas resembling pyknotic nuclei are seen along the diaphysis and metaphysis. They are usually surrounded by small multinucleated cells.

The marrow is acellular and only the outlines of cell remnants can be distinguished.

In the connective tissue of the capsule, poorly defined accumulations of cells can occasionally be detected around small blood vessels (Fig. 20). They are composed of a few small lymphocytes with a large number of other cells with a much larger, paler nucleus containing a large, distinctly eosinophilic nucleolus.

Alpharadiography

The cartilage which stains a pale pink with the Wright stain is of uniform low density on the alpharadiograph (Fig. 24).

The bone trabeculae again show decreased density in the areas which stained basophilic in the histological preparations.

Histochemistry to Demonstrate Mucopolysaccharides in the Cartilage

Thionine-eosin

This stain shows some metachromasia in the vascular channels and in the small areas of mineralized cartilage. Most of the original bone trabeculae now show some mild

Fig. 16 - A_1 - one day old donor - implanted three days. This specimen shows loss of metachromasia except from the zone of calcified cartilage and a small patch at the edge of the articular surface. Dilute Wright stain. 4½x.

Fig. 17 - B_1 - one week old donor - implanted three days. This section shows loss of the metachromatic staining from the distal part of Zone A and all of Zone B cartilage. Zone C and a rim at the articular surface have retained their staining properties. Dilute Wright stain. 4½x.

Fig. 18 - B_2 - one week old donor - implanted seven days. New patches of cartilage can be seen at the metaphyseal level (solid arrow). Irregular areas of metachromatic cartilage can also be seen at the border of the former articular surface. Dilute Wright stain. 4½x.

Fig. 19 - A_2 - one day old donor implanted seven days. Areas of very young cartilage in the metaphyseal area (see Fig. 9) can be seen. One patch of metachromatic cartilage remains at the articular surface (solid arrow). Dilute Wright stain. 4½x.

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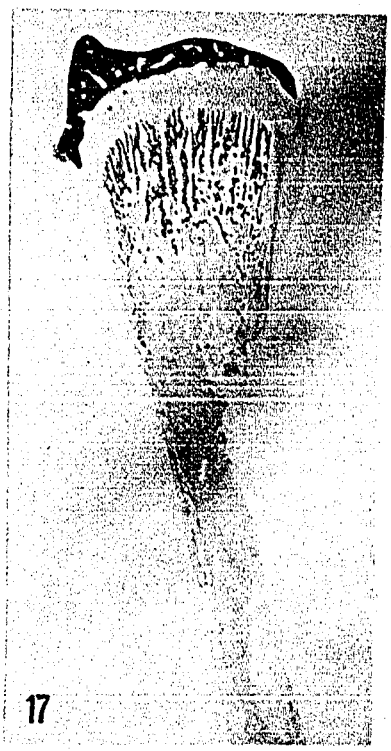

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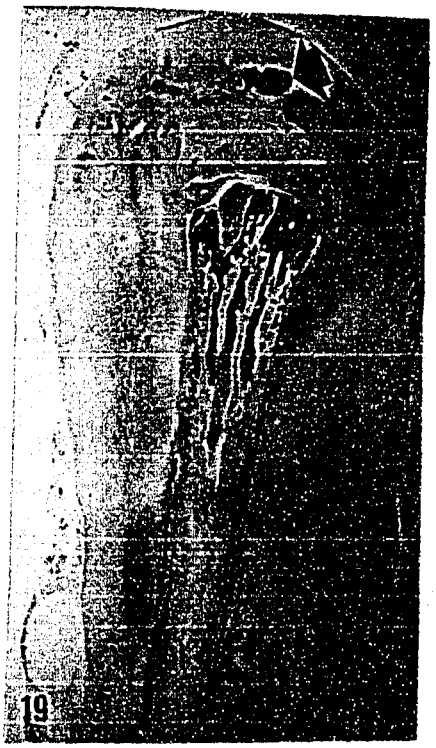

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metachromasia. The edges of the areas of amorphous debris along the shaft are deeply stained.

PA-Schiff

This stain gives a positive magenta colour in the channels of the blood vessels in the cartilage of the head. The middle layer of the patches of amorphous debris is also strongly positive. The mineralized cartilage is deeply stained as well as the centres of some new trabeculae.

B_2 - one week old chick tibia - implanted seven days.

Gross Observations

The envelope surrounding five of the implants was still oedematous and somewhat hyperaemic. One implant appeared to have pus around its posterior end. All showed growth and in seven this was marked at both ends giving them a "dumb-bell" appearance.

Light Microscopy

After seven days <u>in situ</u>, six of the ten specimens still show some metachromasia of the epiphyseal cartilage (Zone A) of either or both ends. In six ends which have lost their metachromasia, small irregular areas of growth which are still metachromatic can be seen. Two sections show very eosinophilic accumulations of cells adjacent to the cartilage of the head. These

contain large numbers of heterophil leucocytes. Their staining properties are very similar to the amophous debris seen over the metaphysis in the one day old donor series. The chondrocytes are becoming pyknotic.

The periosteum is less well defined. Its fibrous layer now consists of younger less dense collagenous tissue. A large number of cellular trabeculae have formed beneath it. Each trabecula is lined with one or two layers of plump basophilic osteoblasts (in nine specimens). Areas of young cellular cartilage with thin-walled lacunae, whose matrix stains metachromatically, are present mainly in the metaphyseal regions (Fig. 18). The connective tissue surrounding the new cartilage and bone is very mesenchymal in character near the implant. It becomes more condensed and appears more basophilic as the periosteum is approached. It is very vascular.

The osteocytes of the original trabeculae are viable except for regions close to the marrow cavity where osteocytic osteolysis is occuring. Occasional osteoclasts are seen along the shaft.

The marrow is acellular and cell outlines are no longer visible.

Just outside the fibrous periosteum, a moderate infiltration of medium and large lymphocytes is present. In scattered areas about thin-walled vessels, rounded

accumulations of large pale cells can be found which resemble the germinal centres of mammalian lymphoid tissue.

A_3 - one-day old chick tibia - implanted fourteen days

Gross Observations

The small blood vessels in the capsule now were fewer in number but larger in calibre. The large vein from the runner feathers was no longer engorged. The capsule was composed of opaque white connective tissue continuous with the implanted bone. Four of the ten implants in this group showed no growth but only two were partly decalcified. Of the six which showed signs of growth, three grew at the proximal end only. The three that had grown at both ends were "dumb-bell" shaped as if most of the growth had occurred around the metaphyses and over the articular surfaces.

Light Microscopy

Two weeks after implantation, the edges of the now more eosinophilic cartilage of the head of the bone are surrounded by patches of granular debris which are in turn surrounded by a layer of vacuolated multinucleated cells with a pale staining cytoplasm (Fig. 7).

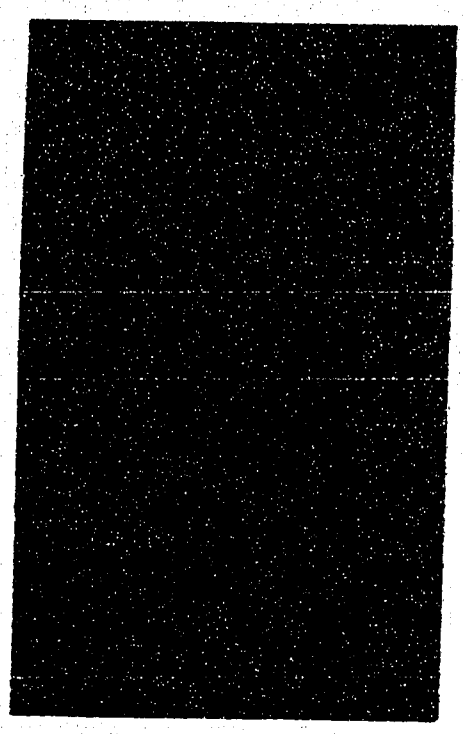
The eosinophilia of this cartilage may be homogeneous or in the form of a network similar in distribution to the interlacunar network visualized when the articular cartilage was stained with PAS and alcian blue (Bélanger and Migicovsky, 1961). The original chondrocytes are all

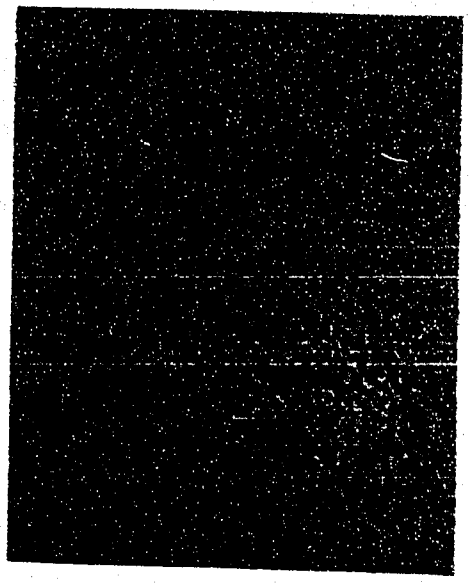
Fig. 20 - A_2 - one-day old donor - implanted seven days. Accumulations of cells with an indefinite rounded outline can be seen around some of the small blood vessels in the capsule. Many of these cells are large with large pale nuclei and a prominent eosinophilic nucleolus (arrow).
Dilute Wright stain. 220x.

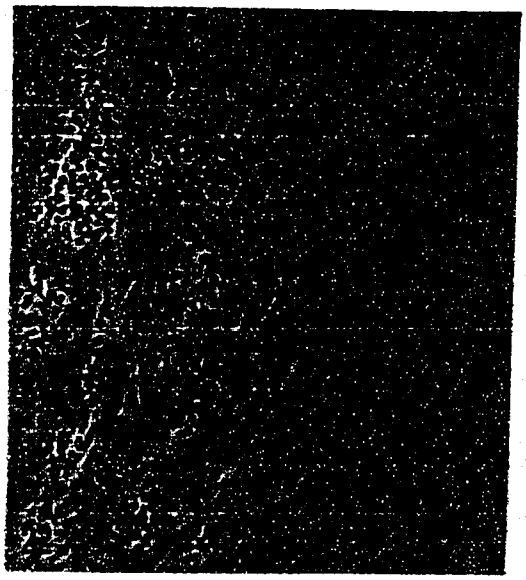
Fig. 21 - B_3 - one-week old donor - implanted fourteen days. The outlines of the accumulations of cells are now more definite. The areas appear darker because of an increased number of mitotic figures (solid arrow).
Dilute Wright stain. 220x.

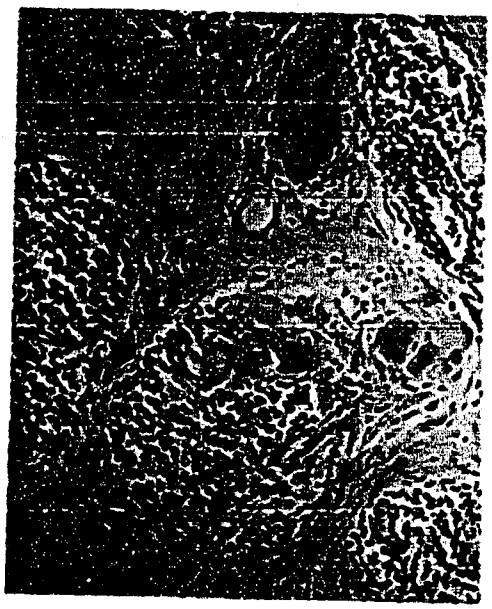
Fig. 22 - A_4 - one-day old donor - implanted twenty-one days. The areas now contain darkly staining plasma cells (solid arrow) as well as small lymphocytes.
Dilute Wright stain. 220x.

Fig. 23 - B_5 - one-week old donor - implanted twenty-eight day The cells composing the accumulations are now mainly small lymphocytes. There is also the beginning of a connective tissue capsule around each rounded area (solid arrow).
Dilute Wright stain. 220x.









pyknotic. Areas of new cellular metachromatic cartilage are forming an irregular cap over the ends of six implants just under the connective tissue capsule. This new cartilage is separated from the implanted cartilage by a layer of cellular loose connective tissue (Fig. 27).

The implant is now totally surrounded by a connective tissue capsule of maturing fibrous tissue rather than a periosteum. Inside the capsule in the loose cellular tissue, the cells toward the periphery now appear more like fibroblasts with their cytoplasm prolonged at either end into filamentous processes rather than the stellate mesenchymal cells which are still present close to the implant. In eight of the specimens, immature cellular bone trabeculae can be found in this layer. These trabeculae are no longer covered with osteoblasts and are even, in limited areas, being resorbed by osteoclasts (Fig. 13). In implants where these new trabeculae are frequent and well developed, they appear to be oriented mainly at right angles to the long axis of the bone. Large numbers of small blood vessels can be found between the trabeculae.

The new cartilage in the region of the metaphysis appears to be becoming hypertrophied. Many of the chondrocytes it contains have disappeared. Some of the lacunae are broken and there are large basophilic granules in the walls of the lacunae as well as in the empty lacunae

themselves (Fig. 10). In a few areas the cartilage is being resorbed by multinucleated chondroclasts.

The loose connective tissue under the capsule and around the new bone and cartilage is infiltrated with a few plasma cells and some small lymphocytes. Throughout the capsule rounded accumulations of cells, containing some small lymphocytes and other cells with a large pale nucleus and a prominent eosinophilic nucleolus, are found. Frequent mitotic figures are found within these areas (Fig. 21). These accumulations are especially frequent around the epiphyses just outside the region where the new cartilage caps are forming. They bear a marked resemblance to the germinal centres of mammalian primary lymphatic nodules without the surrounding rim of small lymphocytes (Ham, 5th ed., 1965).

Amorphous debris is all that remains of the marrow.

Most of the osteocytes of the original bone are now pyknotic or undergoing lysis within their lacunae. Along the outer borders of the shaft, osteoclasts are frequently seen (Fig. 13).

Alpharadiography

The cartilage of the head now shows a network of increased density corresponding to the network of deep eosinophilic material seen in several of the histological

specimens. The trabeculae remain essentially unchanged (Fig. 25).

The specimen which showed a homogeneous deep eosinophilic staining of the head in the routine preparation, on the alpharadiograph shows a homogeneous density (Fig. 26).

Histochemistry to Demonstrate Mucopolysaccharides in the Cartilage

Thionine-eosin

The network of eosinophilic material seen in the Wright stain stains metachromatically with the thionine. The hypertrophied calcified cartilage still retains its metachromatic stain. Most of the old bone trabeculae now stain metachromatically.

The specimen with the homogeneously eosinophilic head also stains with uniform metachromasia with the thionine.

PA-Schiff

The network in the epiphyseal cartilage as well as the layer of amorphous debris at its surface gives a positive reaction to this test. Fragments of PAS positive material can be seen in the vacuoles of some of the multinucleated cells which surround the amorphous debris.

The uniformly eosinophilic specimen is also homogeneously PAS-positive.

Fig. 24 - Alpharadiograph

A_2 - one-day old donor - implanted seven days. The cartilage of the head which is pale pink with the Wright stain is homogeneously less dense than the bone trabeculae seen at the bottom of the picture.

Decalcified section. 10x.

Fig. 25 - Alpharadiograph

A_3 - one-day old donor - implanted fourteen days. The cartilage of the head shows a network of eosinophilic material with the Wright stain. The same network is apparent on the alpharadiograph under high power. At this magnification only a general moderate increase in density of the cartilage can be seen.

Decalcified section. 10x.

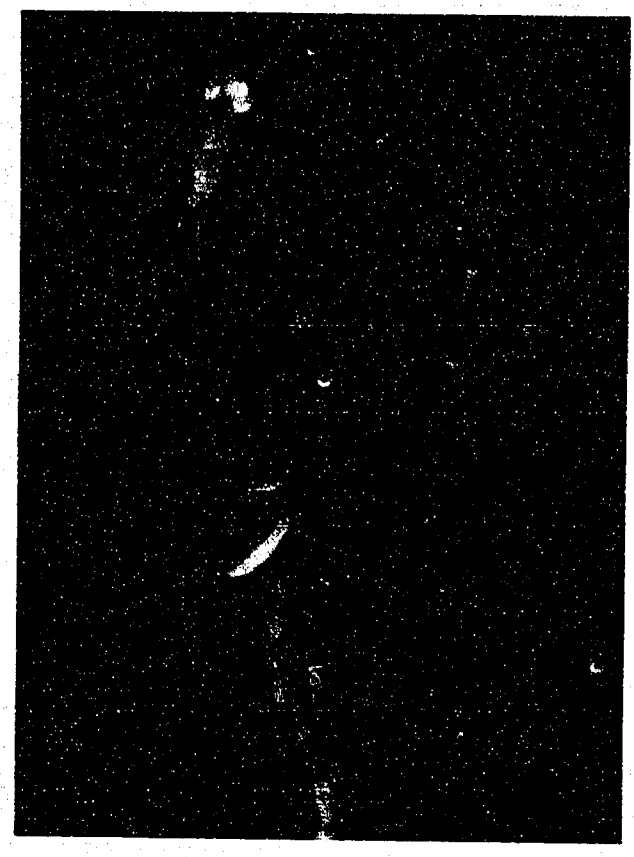


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Fig. 26 - Alpharadiograph

A_3 - one-day old donor - implanted fourteen days. This specimen showed a dark uniform eosinophilia with the routine Wright stain. On the alpha-radiograph a much increased density can be noted. Declacified section. 10x.

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Fig. 27 - A_3 - one-day old donor - implanted fourteen days. An irregular new cartilage cap has formed over the end of the bone and a patch of cartilage can be distinguished near the metaphysis. The marrow cavity is now empty.
Dilute Wright stain. 4½x.

Fig. 28 - A_4 - one-day old donor - implanted twenty-one days. A more extensive cartilage cap has formed over the bone end. Large numbers of new bone trabeculae can be seen around the shaft (see Fig. 14).
Dilute Wright stain. 4½x.

Fig. 29 - A_5 - one-day old donor - implanted twenty-eight days. The original bone is smaller than when implanted. The new cartilage at the metaphysis is resorbing (solid arrow) (see also Fig. 11).
Dilute Wright stain. 4½x.

Fig. 30 - A_5 - one-day old donor - implanted twenty-eight days. In contrast to Fig. 29 the general architecture of the bone has been preserved. There has also been a marked increase in size from that of the original bone which was transplanted (see Fig. 1).
Dilute Wright stain. 4½x.

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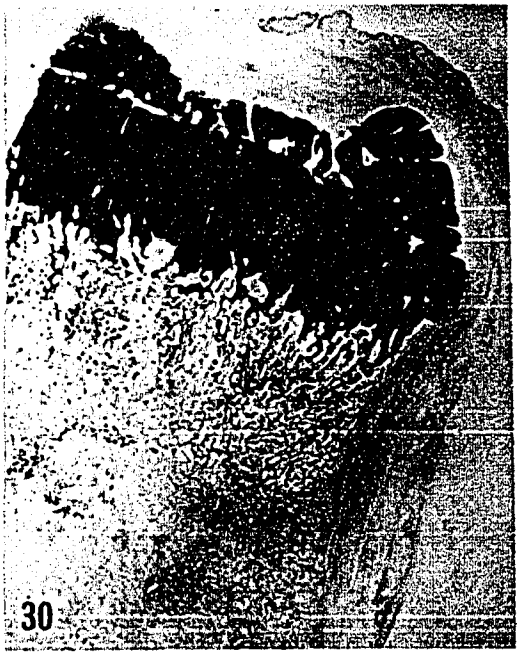

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The matrix of the new cartilage which is hypertrophying as well as areas in the centre of the newly formed trabeculae are also positive. The edges of the new cartilage caps are positive also.

B_3 - one week old chick tibia - implanted fourteen days

Gross Observations

The implants were each contained in an opaque connective tissue capsule which was continuous with them. The capsule was not oedematous and now had fewer but larger blood vessles traversing it. The vein from the runner feathers, though larger than the one on the opposite side, was not engorged. All the implants appeared to have increased in size and were more cylindrical than the previous group except for three specimens which had grown mainly at the ends retaining their "dumb-bell" shape.

Light Microscopy

After two weeks <u>in situ</u>, the cartilage of the head is pink and irregular, surrounded by eosinophilic debris covered by a layer of vacuolated multinucleated cells. The chondrocytes are mostly indistinct outlines with a few pyknotic nuclei. Areas of new metachromatic cartilage distinct from the original epiphysis which are present in half of the specimens, are losing their metachromasia at the edges and are being infiltrated (Fig. 37) by small lymphocytes.

The connective tissue capsule contains accumulations of large lymphocytes with frequent mitotic figures. These are more numerous over the ends of the bone in the region of the new cartilage cap. The loose connective tissue inside the capsule is infiltrated with a few plasma cells and moderate numbers of small lymphocytes.

In nine of the ten bones new bone and cartilage have formed. Eight of these show loss of the osteoblasts on the new cellular trabeculae (Fig. 33). Moderate numbers of small lymphocytes and plasma cells can be seen between the trabeculae. A few osteoclasts are found on the trabeculae. The new cartilage is hypertrophied; many of the chondrocytes have disappeared. A few basophilic granules can be seen within the empty lacunae and their walls.

One specimen with new bone formation has retained its plump osteoblasts and has only a minimal infiltration of its loose connective tissue, (Fig. 31). The tenth shows no new bone or cartilage formation and has large accumulations of lymphoid cells in its capsule.

The old trabeculae contain many pyknotic osteocytes and others which are becoming indistinct. Large numbers of osteoclasts are clustered around the shaft.

Fig. 31 - B_3 - one week old donor - implanted fourteen days. The arrangement of the new bone and cartilage which have formed around the shaft (lower left hand corner) can be well seen in this picture. The cartilage is just under the capsule and between it and the trabeculae are areas of hypertrophied cartilage.

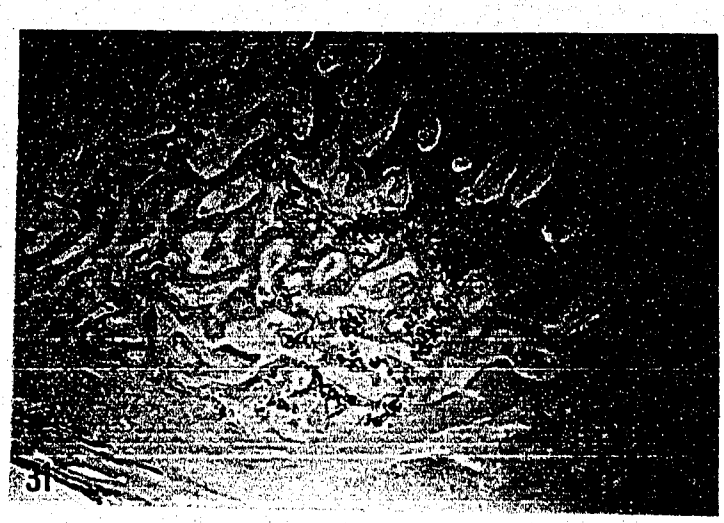
Dilute Wright stain. 32x.

Fig. 32 - B_2 - one week old donor - implanted seven days. In comparison with the trabeculae seen around the one-day old donors (Fig. 12) after this length of time in situ, these trabeculae are relatively mature. The osteoblasts are flattened rather than being plump.

Dilute Wright stain. 110x.

Fig. 33 - B_3 - one week old donor - implanted fourteen days. The new trabeculae have lost their osteoblasts. A moderate infiltration of small round cells can be seen between the trabeculae.

Dilute Wright stain. 110x.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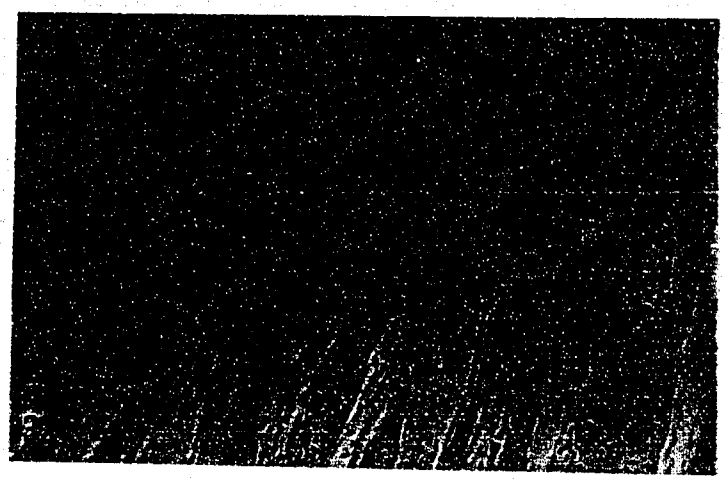


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A_4 - one-day old chick tibia - implanted twenty-one days

Gross Observations

Five of the specimens appeared smaller than the original implants and several of these were very rubbery. One was very brittle and broke while it was being removed. Only one implant was noticeably larger than the normal day-old tibia. The capsule was well supplied by blood vessels in all cases but did not appear hyperaemic in any of the specimens.

Light Microscopy

After three weeks in situ the implanted bone is surrounded by a well vascularized capsule of mature dense connective tissue. The epiphyses now are smaller in size and irregular in outline (Fig. 28). Their staining properties vary from almost colourless to deeply eosinophilic. The chondrocytes have either completely disappeared or show only as faint outlines. The now ragged edges of the cartilage are surrounded by a confluent area of eosinophilic debris covered by a layer of vacuolated multinucleated cells. In seven specimens, one of the epiphyses was not found.

The connective tissue under the capsule is now infiltrated with moderate numbers of plasma cells and small lymphocytes. Frequent round or oval accumulations

of lymphoid cells are present in the capsule and in the loose connective tissue just under it, especially about the ends of the implant. These areas stain more deeply basophilic than those in the previous group because of the increased numbers of mitotic figures present. Plasma cells can also be seen (Fig. 22) within them.

The original trabeculae stain more deeply eosinophilic though the outlines of many osteocytes can still be faintly distinguished. The shaft is surrounded by an almost unbroken layer of osteoclasts.

The new trabeculae now contain some oseocytes which are dying. Many areas of osteocytic osteolysis with a basophilic matrix and metachromasia around the lacunae can be distinguished (Fig. 14). The vascular connective tissue between these trabeculae is infiltrated with lymphocytes and plasma cells (Fig. 14). Moderate numbers of osteoclasts are seen on this bone. The lacunae of the new cartilage around the shaft and metaphysis are recognizable mainly by remnants of their walls in some of which the granules indicating calcification can be seen (Fig. 10). Chondroclasts are present in moderate numbers.

The new cartilage around the epiphyses is now losing its metachromasia at the edges and these pale areas are

beginning to be infiltrated by small lymphocytes.

B_4 - one week old chick tibia - implanted twenty-one days

Gross Observations

At twenty-one days the capsule was not oedematous and appeared to have a good blood supply. Six of the implants had grown and were still calcified. Four were smaller than the original week-old bone which had been implanted. Three of these were rubbery and decalcified; the fourth was brittle and broke during removal.

Light Microscopy

Three weeks after implantation the epiphyseal cartilage of the original ends of the bone is pink or deeply eosinophilic. Its outline is now very irregular and it is decreased in size. The ragged edge of the cartilage is surrounded by eosinophilic debris around which vacuolated multinucleated cells are clustered.

Nine of the ten implanted bones show some new bone or cartilage formation. Around the one which shows no new growth and one which shows only small remnants of new cartilage being resorbed near the metaphysis, there is a heavy infiltration of cells, chiefly plasma cells with some small lymphocytes. In the capsules surrounding these two specimens there are large basophilic accumulations of lymphoid cells containing many mitotic figures

(Fig. 21). The original trabeculae stain eosinophilic and appear amorphous. Large numbers of osteoclasts surround the shaft (Fig. 34).

Five of the specimens have a moderate to heavy infiltration of plasma cells and lymphocytes around them. New trabeculae are identifiable but the osteocytes are dead and there are no osteoblasts present (Fig. 35). In many areas the new trabeculae are being resorbed by osteoclasts. The old trabeculae contain only dead osteocytes; the shaft is surrounded by osteoclasts (Fig. 34). Moderate to large basophilic accumulations of lymphoid cells are present in the capsule and just under it, especially at the ends of the implant. The new cartilage at the ends, present in about one-half of these specimens, is losing its metachromasia at the edges and is being infiltrated by small lymphocytes.

In the remaining four implants the picture is different in that the cartilage over the ends is still growing and the new trabeculae contain healthy osteocytes. This new bone is still lined by plump osteoblasts in most areas. The lymphoid infiltration is mainly around the original implant and not between the new trabeculae.

Fig. 34 - B_4 - one week old donor - implanted twenty-one days. The old trabeculae at the bottom have lost their osteocytes and the outer surface is covered with osteoclasts. The surrounding connective tissue is heavily infiltrated with round cells.
Dilute Wright stain. 110x.

Fig. 35 - B_4 - one week old donor - implanted twenty-one days. The new trabeculae have lost their osteoblasts. The loose connective tissue between them is infiltrated with moderate numbers of small round cells, in contrast to the preceding Fig. 34 where the infiltrate was much heavier.
Dilute Wright stain. 110x.

Fig. 36 - B_5 - one week old donor - implanted twenty-eight days. At four weeks the large numbers of small round cells have given way to numerous darkly staining plasma cells (arrow).
Dilute Wright stain. 220x.

Fig. 37 - A_5 - one-day old donor - implanted twenty-eight days. The new cartilage cap which was formed (see Fig. 27) by fourteen days is now losing its metachromasia and its edges are being infiltrated.
Dilute Wright stain. 110x.


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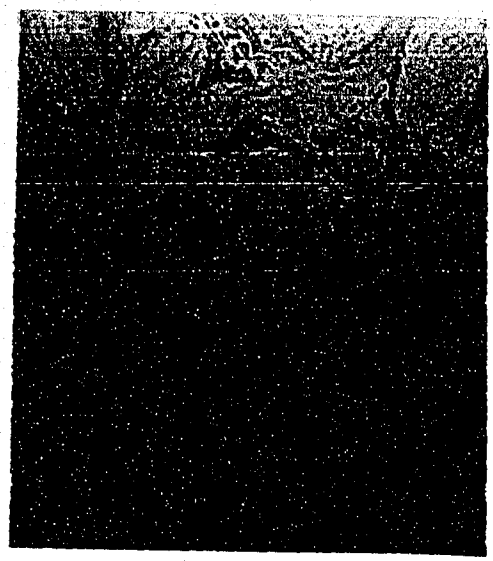

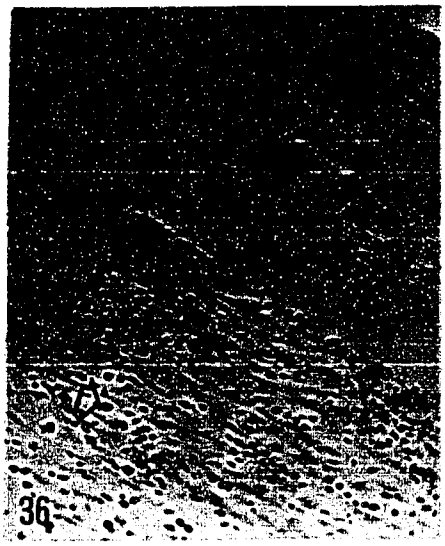
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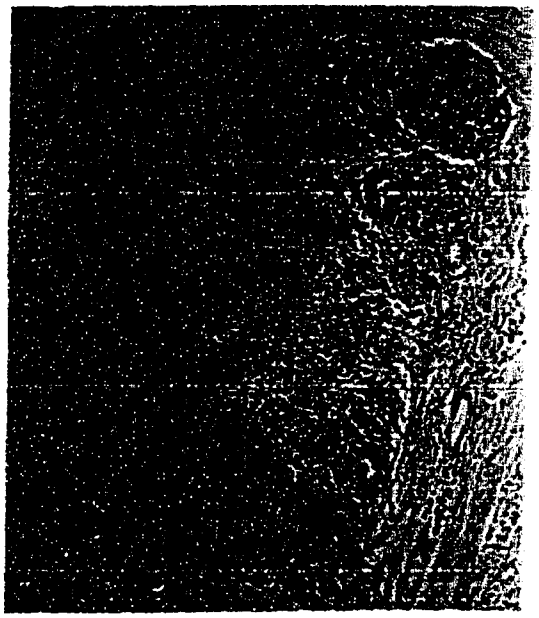


A_5 - one-day old chick tibia - implanted twenty-eight days

Gross Observations

Seven of the grafts were large and showed obvious new growth. One of these had grown only at the proximal end and the distal end was decalcified. The remaining three were small and rubbery. The surrounding capsule of all the implants showed fewer blood vessels than the preceding group.

Light Microscopy

Four weeks after implantation this group showed more variation than the preceding ones.

Six of the implants are small. The dense connective tissue capsule surrounding them contains large accumulations of lymphoid cells (Fig. 29). These are now composed chiefly of small lymphocytes though some mitotic figures are still present. The loose connective tissue under the capsule is heavily infiltrated with large numbers of plasma cells and small lymphocytes.

The epiphyses have been largely resorbed. Some eosinophilic material surrounded by vacuolated multinucleated cells is all that remains.

The original trabeculae are more eosinophilic and the outlines of the lacunae difficult to distinguish. Some of the trabeculae are partly resorbed and the shaft

is still surrounded by large numbers of osteoclasts. Granular basophilic material can be seen in patches between the trabeculae.

The connective tissue between the new trabeculae contains many thin-walled blood vessels and is heavily infiltrated with lymphocytes and plasma cells (Fig. 15). In some areas rounded accumulations of these cells are forming between the trabeculae. Many of the osteocytes are becoming pyknotic. Areas of osteocytic osteolysis can be seen as well as many osteoclasts on the trabeculae. Small metachromatic remnants of the wells of the cartilage lacunae still remain (Fig. 11).

The new cartilage caps at the ends are losing more of their metachromasia. The edges are becoming irregular as they are invaded by small lymphocytes (Fig. 37).

Three of the implants have large amounts of new bone and cartilage formed around them. The new trabeculae show healthy osteocytes and are covered with osteoblasts (Fig. 30).

The new cartilage around the metaphysis has hypertrophied and many of the lacunae are empty. The cartilage over the ends is deeply metachromatic with no infiltration of the edges.

The connective tissue contains some small accumulations

of lymphoid cells. The loose connective tissue around the new trabeculae has a light to moderate infiltrate of plasma cells and small lymphocytes.

The remaining implant has retained much of its original architecture. The cartilage at the ends is cellular and deeply metachromatic. This progresses through a zone of flattened cells, then a layer of hypertrophied cartilage cells to merge with new bone trabeculae covered with plump osteoblasts (Fig. 30).

There is minimal lymphocytic infiltration of the capsule and underlying tissues.

The marrow spaces of this implant are filled with many thin-walled blood vessels and islands of haematopoietic cells.

B_5 - one week old chick tibia - implanted twenty-eight days

Gross Observations

The capsule surrounding each of the implants had noticeably fewer blood vessels than the preceding groups. Grossly six of the implants had increased in size; two only at one end, but all, large or small, were very rubbery and decalcified.

Light Microscopy

Twenty-eight days after implantation marked differences are noted among the implants.

Five of the implants show no new bone or cartilage, or at most, minimal remains of new cartilage lacunae at the metaphysis. The accumulations of lymphoid cells in and just under the thick connective tissue capsule, are large and strikingly basophilic. They consist mainly of small lymphocytes and mitotic figures are not prominent (Fig. 23). The capsule itself shows fewer vessels than previously. There is a heavy infiltrate under the capsule composed of large numbers of plasma cells and a few small lymphocytes (Fig. 36). The lacunae of the old trabeculae are empty. The original shaft is still covered by large numbers of osteoclasts though it appears smaller than when implanted.

Four of the remaining implants have new bone trabeculae which are being resorbed. The osteocytes are dead or absent. Remnants of cartilage lacunae can be seen at the metaphysis. Where new cartilage is present over the ends of the bone it is losing its metachromasia and its edges are irregularly infiltrated with lymphocytes. The capsule contains accumulations of lymphoid cells among which mitotic figure are frequent. The looser tissue under it has a moderate to heavy infiltrate of many plasma cells (Fig. 36) and some small lymphocytes. The lacunae of the trabeculae in the original implant

are empty. The original shaft is surrounded by large numbers of osteoclasts and is diminished from its original size.

The tenth implant differs in having new trabeculae still lined with osteoblasts. The tissues surrounding the original implant are infiltrated with lymphoid tissue similar to that in the preceding specimens, but there is little infiltration actually between the new trabeculae.

E - Quantitation of the Rejection Reaction and Statistical Analysis of the Results

Three weeks after implantation, the tibiae of the day-old chick showed considerable variation both in the amount of growth present and in the response of the host, i.e. lymphoid infiltration of capsule and periosteal proliferations.

Based on a review of the total of ninety-nine specimens in this group (A_4 from Series A was included) a rough quantitation of the amount of rejection which was present three weeks after a day-old chick tibia had been implanted subcutaneously was arrived at. The scoring system used is shown next.

1 - Estimate of the Degree of Rejection

Category	Description
I	- No rejection - either retains original architecture with minimal or no infiltration - or - large numbers of bone trabeculae covered with plump osteoblasts are present - may have some lymphocytic infiltration around the original implant.
II	- Beginning rejection - recognizable new bone and cartilage are present - areas of flattened osteoblasts are still present on some of the new trabeculae - the new cartilage is hypertrophied - some lacunae are broken and empty- light to moderate infiltration with lymphocytes and some plasma cells - accumulations of lymphoid cells have frequent mitotic figures or large pale cells similar to those of mammalian germinal centres.
III	- Established rejection - recognizable new bone and cartilage still present - no osteoblasts present on the new trabeculae - the new osteocytes becoming pyknotic - only remnants

of the walls of the cartilage lacunae still visible - moderate to heavy infiltration with lymphocytes and plasma cells - large accumulations of lymphoid cells - either mainly small lymphocytes or with frequent mitotic figures.

IV - Completed rejection - no new bone - only minimal remains, if any, of new cartilage lacunae - infiltrate of many plasma cells and some lymphocytes - accumulations, mainly of small lymphocytes, smaller and less frequent - implant appears walled off - fewer blood vessels in the capsule.

There was little difficulty placing each specimen into the appropriate category though some overlap did occur between categories III and IV.

2 - Student's T-test for Paired Variates

Twenty-eight pairs of implants were scored. Each pair had differing hosts but the same donor. A Student's t-test for paired variates was applied to the differences in category between each pair (Table IV).

Table IV - Calculation of the Significance of Differences in Implants from the Same Donor in Varying Hosts. Student's t-test for Paired Variates.

Donor	Host A	Host B	Number of Categories Difference D = X-Y	$(D - \bar{D})$	$(D - \bar{D})^2$
1	III	IV	1	0.29	0.084
2	III	III	0	-0.71	0.504
3	III	II	1	0.29	0.084
4	III	III	0	-0.71	0.504
5	III	III	0	-0.71	0.504
6	I	I	0	-0.71	0.504
7	III	IV	1	0.29	0.084
8	IV	III	1	0.29	0.084
9	IV	III	1	0.29	0.084
10	III	IV	1	0.29	0.084
11	III	I	2	1.29	1.664
12	IV	I	3	2.29	5.244
13	I	IV	3	2.29	5.244
14	III	III	0	-0.71	0.504
15	IV	IV	0	-0.71	0.504
16	III	III	0	-0.71	0.504
17	II	IV	2	1.29	1.664
18	III	III	0	-0.71	0.504
19	III	III	0	-0.71	0.504
20	II	III	1	0.29	0.084
21	IV	IV	0	-0.71	0.504
22	IV	IV	0	-0.71	0.504
23	III	III	0	-0.71	0.504
24	IV	IV	0	-0.71	0.504
25	IV	III	1	0.29	0.084
26	II	III	1	0.29	0.084
27	III	III	0	-0.71	0.504
28	III	IV	1	0.29	0.084
			$\Sigma D = 20$		$21.714 = \Sigma (D-\bar{D})^2$

$D = X - Y$

$\bar{D} = \frac{\Sigma(X - Y)}{n} = \frac{\Sigma D}{n}$

S_d = estimate of the standard deviation of the differences

$= \sqrt{\frac{\Sigma(D - \bar{D})^2}{N - 1}}$

n = number of pairs = 28

v = degrees of freedom

$= n - 1 = 27$

$S_{\bar{d}} = \frac{S_d}{\sqrt{n}}$

$m_d = \frac{\Sigma D}{N} = \frac{\Sigma(X - Y)}{N} = \frac{\Sigma X}{N} - \frac{\Sigma Y}{N} = 0$

$t = \frac{\bar{D} - m_d}{S_{\bar{d}}}$

$= \frac{0.71 - 0}{0.17} - 13.03$

for $v = 27$, $t_{.05} = 2.052$

$t_{.01} = 2.771$

The results thus indicate that there is a significant difference in identical grafts placed in different hosts.

All eighty-nine implants in this series plus the ten from group A_4 which were performed in a comparable manner were used to give a total of ninety-nine specimens for scoring. The figures obtained by Craig and McDermid (1963) for their total series of three hundred and fifty-four skin grafts between chickens of varying genetic background were used as an estimate of expectation. They divided their

results into two main groups: those grafts rejected before eighteen days and those surviving longer. Since the implants in this experiment were observed on day twenty-one they were divided into two groups;

I and II - viable at twenty-one days

III and IV - definitely rejected at twenty-one days.

Craig and McDermid showed 11.6% survival of the skin grafts in their total series for more than eighteen days. A χ^2 test to measure the discrepancy between the observed frequencies in this series and the corresponding "expected" frequencies was carried out (Table V).

Table V - χ^2 test for the Comparison of Observed and Predicted Frequency of Rejection of Bone Implants in an Unselected Population of Chickens.

$$\chi^2 = \frac{\Sigma(o - e)^2}{e}$$

Groups	o	e	o - e	$(o - e)^2$	$\frac{(o - e)^2}{e}$
I & II	13	11.48	1.52	2.3104	0.201
III & IV	86	87.52	-1.52	2.3104	0.025
	99	99			$\chi^2 = 0.226 = 0.23$

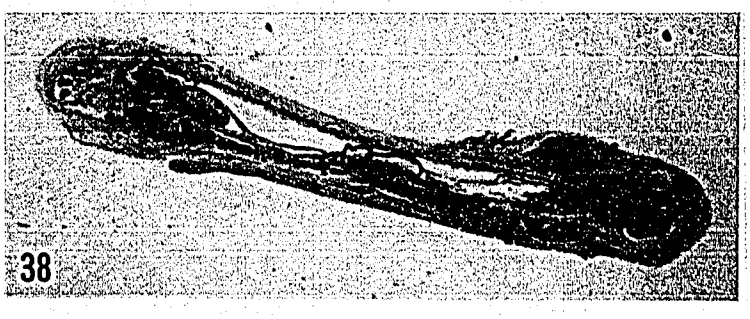
for $v = 1$, $\chi^2_{.05} = 3.84$

Since the closer the agreement between the observed and expected frequencies the smaller the value of χ^2 will be, this result can be interpreted as a good indication that observation agrees with expectation.

Figs. 38 and 39 - one-day old tibiae from one donor implanted in different hosts.
Both bones showed complete rejection histologically at three weeks after transplantation.
Dilute Wright stain. 3x.

Figs. 40 and 41 - one-day old tibiae from one donor implanted in different hosts.
One bone (Fig. 40) showed growth and the other was rejected at three weeks (Fig. 41).
Dilute Wright stain. 3x.

Figs. 42 and 43 - one-day old tibiae from one donor implanted into different hosts.
Both these bones showed a large and approximately equal amount of growth at three weeks. The signs of rejection were minimal at that time.
Dilute Wright stain. 3x.



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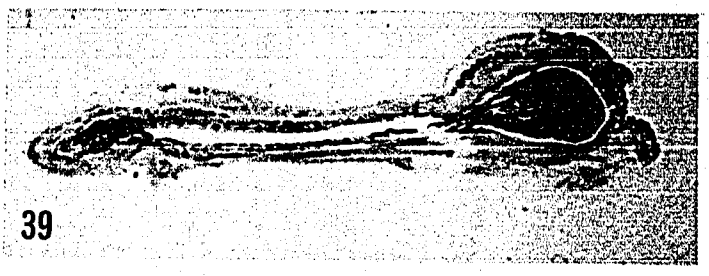

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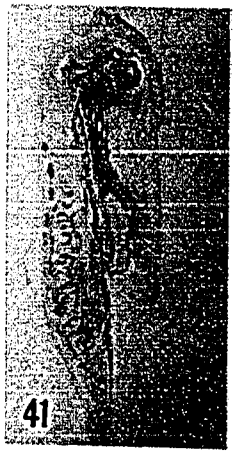

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IV - DISCUSSION

A - Source of New Bone and Cartilage Around the Transplanted Bone

Part of the original purpose of this study was to determine the nature of the tissue around bones transplanted to ectopic sites and, if possible, to elucidate its source.

By following the progress with time of the transplanted bones it has been shown in our study that the new partly calcified tissue around them described by Migicovsky and Nielson (1952) is composed of young cartilage and new bone trabeculae.

The source of the new bone and cartilage at the level of the zone of hypertrophied cartilage (Zone C) appears to be the osteoblastic layer of the original periosteum. At three days after implantation this layer shows increased activity by "dedifferentiation" of its cells to a form more closely resembling primitive mesenchyme in the metaphyseal region. This change is not obvious in the week old donor group (B_1) since the periosteum in this region presents a dedifferentiated appearance in the unimplanted control group (B_0).

By one week after transplantation this layer of very young tissue formed by the osteoblastic periosteum has increased markedly in depth and extended for some distance

along the shaft. In the one-day old donor group (A_2) very young cartilage and pale cellular new bone trabeculae can be seen condensing from this surrounding tissue in the manner in which intramembranous bone formation can be visualized in the embryo (Ham, 1965) and in the healing of bone defects (Siffert, 1955). In the one week old donor group (B_2) the cartilage and bone were more mature suggesting that some degree of differentiation was already present in this layer when the bone was transplanted (Siffert, Barash, 1961; Balinsky, 1965).

Two weeks after transplantation, the layer of cells which now resemble primitive mesenchyme completely surrounds the entire length of the shaft and new bone trabeculae are present throughout this layer. The new bone thus forms a cuff around the transplanted bone and is continuous with the cartilage formed at the level of the hypertrophied cartilage (Zone C).

The origin of the new cartilage cap over the bone ends is more obscure. This cartilage resembles the original Zone A cartilage in several ways: the cells towards the periphery resemble the transitional forms seen between the flat cells of the articular surfaces and the main bulk of the epiphysis and the chondrocytes in the centre are separated by large amounts of metachromatic matrix

unlike the cartilage formed near the metaphysis.

At one week after transplantation there are irregular, dividing areas of cartilage at the articular surfaces; the new cartilage cap is not noted until two weeks after transplantation when there is no other sign of the previously dividing areas. Thus it appears probable that the source of this new cartilage which does not hypertrophy is the irregular growth from the edges of the Zone A cartilage.

B - Organization of the New Bone and Cartilage

In our study the periosteum adjacent to the hypertrophied chondrocytes (Zone C) was the first to proliferate but instead of bone (Lacroix, 1951) it produced new cartilage which in its turn hypertrophied in a partial recapitulation of the events seen in the cartilage of Zones B and C preceding endochondral bone formation. In some areas the large basophilic granules indicative of slow calcification (Bélanger and Migicovsky, 1961b) could be seen in the walls and within the lacunae demonstrating that this cartilage could also become calcified.

The new cartilage cap formed over the ends of the bones did not hypertrophy lending support to the concept of some "organizing substance" (Lacroix, 1951) which is produced by the hypertrophied chondrocytes. The further

observation from our work that when the hypertrophied cartilage was implanted alone new bone and cartilage formed under the periosteum surrounding it also reinforces the concept.

In general the new bone trabeculae appeared to arise de novo out of the surrounding dedifferentiated tissue which originated from the donor periosteum (Siffert, 1955). In some areas, however, where the new tissue was exceptionally thick new cartilage could be seen just under the periosteum. This appeared to hypertrophy and become covered with osteoid as the centre of the transplant was approached. These areas strongly resembled areas of fracture callus which have out-stripped their blood supply, formed cartilage and are then converting the cartilage to bone (Bonfiglio, 1958). Since this phenomenon appeared around our transplants only where a large amount of growth was observed, it may be supposed that the blood supply, while insufficient for bone formation, could support new cartilage.

C - Vascularization of the Graft

Hammack and Enneking (1960) showed that the periosteal cells of cortical bone could survive transplantation by means of an efficient extravascular mode of nutrition until the area became vascularized. In our present study

vessels could be seen grossly between the host and the proximal end of the implant by three days but in a very limited area, so that microscopically they were not seen unless the section passed through that specific site. One week after transplantation large numbers of thin-walled blood vessels were observed throughout the mesenchymal tissue between the new trabeculae. Most authorities conclude that these vessels are of host origin (Hammack, Enneking, 1960; Zeiss et al., 1960) except in the case of small bone fragments transplanted to the chorio-allantoic membrane of the chicken where, since circulating cells were observed within the bone within five hours, donor vessels were assumed to have survived and united with host capillaries (Hancox, 1947).

Both the initial formation and the subsequent amount of periosteal new bone and cartilage appeared to be related to the early establishment of an adequate vascular connection with the host but because of the difficulty of finding and identifying the tiny blood vessels no reliable quantitative data could be obtained. In this regard injection techniques, such as those developed by Trueta and Morgan (1960), carried out at varying time intervals during the first week after transplantation, especially in the first three days (Hammack, Enneking, 1960), could provide

more information regarding both the number of vessels and their pattern of growth.

Grossly the large vein from the runner feathers appears to be the source of the developing vasculature. Perfusion of this vessel with the injection medium would establish the extent of the vascular connection between host and graft and its pattern of development. It would also aid in distinguishing between blood vessels whose walls consist only of one layer of flattened endothelium and the thin-walled lymphatic channels which follow both arteries and veins in the chicken (Dransfield, 1945). In the present study many of the endothelium-lined spaces around the periosteal proliferations and in the capsule were empty of cells and since many had accumulations of lymphoid cells about them, it was unclear whether there were blood vessels, lymphatic channels or both were present.

Unlike the transplanted foetal bones in mammals (Sabet et al., 1961; Chalmers, Ray, 1962) in which actual revascularization of the diaphysis could be demonstrated within the first week the chick bones in our study, showed no signs of revascularization at any period. In fact they showed no signs of invasion by blood vessels either which is also dissimilar to studies on mammalian bone which have shown that even cortical homografts are invaded by host vessels (Zeiss et al., 1960). This finding was

confirmed by the radioautographs in which the hosts had been injected with Ca^{45}. In these the periosteal new bone was highly radioactive but the original bone showed no signs of activity so that there can have been no exchange of Ca^{45} between the trabeculae and the blood. Since on microincineration it was shown that the shaft had retained its mineralization, it might be expected that some Ca^{45} exchange, even though limited in extent, would take place if the shaft were supplied with blood vessels.

D - Reaction of Chick Cartilage to Transplantation

The epiphyseal cartilage (Zone A) of the whole chick bones began to lose its metachromasia, showed death of the chondrocytes by one week and by two weeks was being resorbed by large numbers of vacuolated, multinucleated cells. In contrast to the cartilage with the whole bone, when the Zone A cartilage fragments were implanted alone, the matrix retained its metachromasia and the chondrocytes remained viable at three weeks even though some sepcimens had a moderate lymphoid infiltration in the surrounding tissues. In other words, the cartilage survived and the appearance of any rejection reaction was greatly delayed. No growth of the transplanted cartilage was observed. Our observations on the behaviour of the cartilage

fragments when transplanted are in keeping with the previous findings that homografts of mammalian cartilage, unlike homologous grafts of other tissues, could survive for long periods in the new host with no special precautions (Peer, 1958; Peacock, 1960).

The death of the cartilage when transplanted as part of the whole bone, appears to be attributable to some interference with its source of nutrition since it appears within three days of transplantation. The layer of flattened cells over the articular surfaces which proliferates in the first three days and has an underlying zone of necrosis at that time, could well be the factor responsible for the failure of the cartilage to survive by causing interference with the diffusion of the necessary nutrients. This layer of flattened cells has not been described covering the articular surfaces in mammalian diarthrodial joints but was described by Fell (1925) in the day-old chick tibia.

The changes in the staining properties of the Zone A cartilage with time after transplantation, i.e. the decrease of metachromasia and the increase of eosinophilia, when investigated by histochemical techniques appear to represent a loss of the sulphated mucopolysaccharides (loss of metachromasia with thionine) and a condensation of the protein-bound mucopolysaccharides (PAS-positive), (Bélanger and Migicovsky, 1961a). The interlacunar network

of PAS-positive fibers (Bélanger and Migicovsky, 1960a) was not noted in our unimplanted specimens but was apparent one week after transplantation. The chicks in their study were at least one week old at the time of sacrifice and so this may well be partly an age difference.

E - Transplantation Immunity

The loss of the osteoblasts from the newly formed trabeculae and the lymphocytic infiltration between them are the first signs of bone homograft rejection (Chalmers, 1959; Enneking, 1962). Our present observations at two weeks after transplantation show a very similar picture and support our contention that the new bone trabeculae and the new cartilage are of donor origin.

The histological signs of a first set rejection phenomenon appear approximately ten days after homologous bone transplantation (Chalmers, 1959). The time sequence observed in the histological signs of rejection in our material agrees well with these previous observations.

At three weeks after transplantation the evidences of rejection were increasingly obvious. The grafts at this time could be divided into two broad categories corresponding roughly to those of Craig and McDermid (1963): those rejected before eighteen days (twenty-one

days in this study) and those either just beginning to show rejection or showing no signs of rejection at that time. These differences were postulated in their study to be due to the presence of a major histocompatibility locus in some way associated with the B-locus erythrocyte antigens.

Since the inheritance of a major histocompatibility locus with the B-locus antigens was noted by Schierman and Nordskog (1961) more investigation has revealed an increasingly complex picture. There are at least seven separately inherited blood groups in chickens (Gilmour, 1962) and though the B-locus antigens still appear to be the only ones associated with a histocompatibility locus at least twenty-one specific and separate alleles are involved in the inheritance of this blood group alone (Craig, McDermid, 1963). This considerably lessens its value as a pregraft test of compatibility.

The overall percentage of grafts showing late rejection in this present series (Part III - Section B-3) corresponds rather closely to the total percentage in the studies of skin grafting in chickens by Craig and McDermid (1963). This leads us to the conclusion that in this group of non-inbred chickens, though they were all of the same breed (White Rock), a very similar histocompatibility locus must be involved in the rejection of whole bone

grafts. This is, in all probability, associated with the B-locus erythrocyte antigens as no other histocompatibility loci of equal importance have been demonstrated (Schierman, Nordskog, 1961).

Some studies (Burwell, 1964) have implicated bone marrow as the main antigenic factor in homograft rejection. Other experiments with foetal bone grafting (Saber et al., 1961; Chalmers, Ray, 1962) indicate that the bone is readily revascularized and the marrow cavity invaded thus bringing the marrow cells into close contact with host cells. In the case of homografts the haematopoietic marrow is actually replaced by loose connective tissue. In the chick bones studied in the present experiments, the vascularization was confined to the periosteum. There was no invasion of the bone and the marrow cavity remained isolated from contact with the host. Thus it would appear that the bone and cartilage cells of the periosteal proliferations are responsible for the rejection reaction lending further support to our thesis that the new bone and cartilage is of donor origin.

No investigations have been reported which demonstrate a sex-linked histocompatibility antigen in the chicken such as has been found in some strains of mice (Eichwald et al., 1955).

F - Induction and Cell Survival

While the origin of the subperiosteal new bone and cartilage is evidently from surviving cells of the donor there are unanswered questions as to the source of some of the cell types around the graft. All the blood vessels supplying the graft have been assumed to be of host origin (Hammack, Enneking, 1960) but the survival of at least some of the donor vessels already present in the periosteum at the time of transplantation cannot be completely discounted.

The large numbers of vacuolated, multinucleated cells around the cartilaginous ends of the bone which are evident at one week are probably of host origin as there is no source of precursors of this type of macrophage in that region of the graft, consisting as it does only of various forms of cartilage cells. The layer of flattened cells over the articular surface might have unexpected potentialities, however, since it is continuous with the fibrous layer of the periosteum but a major degree of dedifferentiation would have to be postulated in order for this specialized tissue to form macrophages.

More obscure is the origin of the osteoclasts which appear around the original shaft of the bone when most of the osteocytes have finally died at two weeks after transplantation. They appear at a time, when because of

cell death, the bone is definitely abnormal and formation by modulation of osteoclasts from the proliferating periosteal cells might reasonably be expected (Donna, Cronkite, 1961). On the other hand, if they are host cells they are an example of induction since their precursors in that case are the primitive connective tissue cells which accompany the invading host blood vessels. The stimulus then is bone which is foreign as well as abnormal.

The fate of the fibrous periosteum is also in some doubt. At one week after transplantation it appeared less dense and some mitoses could be seen, but by two weeks the host and graft are completely continuous with no line of demarcation. This means that the relative contribution of host and donor cells to the final dense connective tissue capsule cannot be assessed at present. It is interesting to note that some of the largest accumulations of lymphoid cells containing large numbers of plasma cells were found within the connective tissue capsule.

In their attempt to answer the basic question of cellular survival versus induction in bone grafts, Sabet and Ray (1963) used tritiated thymidine as a cell marker. Other studies (Kember, 1960) have shown that this label disappears after one week in regions of active endochondral bone formation and so the suspicion arises that in actively

dividing tissues, such as foetal bones, this label may be diluted too rapidly to trace accurately the modulation and/or induction of various cell types.

The sex chromatin (Barr, Bertram, 1949) has been found in some species of mammalian bone, specifically rabbits (Arora, Laskin, 1964; Vernino, Laskin, 1960), though not in others, e.g. mice and rats (Sabet, Ray, 1963). Studies on the incidence of sex chromatin in the chicken (Gallus Domesticus) by Kosin and Ishizakh (1959) show that a good chromatin marker is present in the female though it is not analogous to the mammalian sex chromatin (Ohno et al., 1960). A method of accurately sexing young chicks by cytological means has been described (Krishan, 1962). This method combined with improved techniques of staining sex chromatin (Beckert, Garner, 1966) should prove to be a useful tool in the future investigation of induction and transplantation in the chicken.

V - SUMMARY

1. The nature of the partly calcified tissue found around whole bones transplanted to ectopic sites by Migicovsky and Nielson (1952) was studied.

2. Two ectopic sites, subcutaneous and intra-abdominal, were investigated in order to find the most reliable growth around the transplanted bone.

3. The subcutaneous site was chosen because of a high mortality rate among the intra-abdominal group.

4. The new tissue around the transplanted bones was found to be new bone and cartilage.

5. Fragments from various parts of the whole bones were investigated to elucidate their contribution to new bone and cartilage formation.

6. The fragments of Zone C cartilage (hypertrophied) were found to have the largest amount of new bone and cartilage around them.

7. Studies with Ca^{45} and by microincineration showed that the original bone retained its calcification but did not deposit any Ca^{45} at all though the newly formed trabeculae were highly radioactive indicating

rapid calcium deposition.

8. The progress with time of the new cartilage and bone was followed from three days to four weeks.

9. The source of the new bone and cartilage could be seen to be the osteoblastic layer of the periosteum.

10. The development of the vasculature has been discussed and injection techniques suggested as a means for further study.

11. The first histological signs of rejection were apparent at two weeks after transplantation and were well developed by three weeks.

12. At three weeks variation in the strength of the rejection reaction were noted.

13. A large series of transplants was undertaken to study the statistical behaviour of the rejection phenomenon.

14. Tibiae from the same donor transplanted to different hosts occasionally showed marked differences in the amount of growth and the vigor of the rejection reaction.

15. In the total series the percentage of grafts showing tolerance at three weeks was remarkably similar to the total percentage of skin grafts in chickens which had not been rejected at eighteen days (Craig and McDermid, 1963).

16. We have postulated that the B-locus erythrocyte antigens are probably the histocompatibility locus implicated in rejection of the transplanted bones before three weeks in situ in our study.

17. Use of the sex chromatin as a cellular label has been suggested as a tool for the elucidation of the problem of induction versus cell survival.

BIBLIOGRAPHY

1. Albee, F. H.: Evolution of Bone Graft Surgery. Am. J. Surg., 63: 421, 1944.

2. Alder, H. L., and Roessler, E. B.: Introduction to Probability and Statistics. W. H. Freeman and Company, San Francisco and London, 1960.

3. Algire, G. H.: Diffusion-Chamber Techniques for Studies of Cellular Immunity. Ann. N. Y. Acad. Sci., 69: 663, 1957-58.

4. Amano, M., Messier, B., and Leblond, C. P.: Specificity of Labelled Thymidine as a Deoxyribonucleic Acid Precursor in Radioautography. J. Histochem. & Cytochem., 7: 153, 1959.

5. Amprino, R.: Cellular Interactions in Cell Differentiation. J. Emb. & Exp. Morph., 1: 283, 1953.

6. Amprino, R.: Autoradiographic Research on the S^{35}-Sulphate Metabolism in Cartilage and Bone Differentiation and Growth. Acta Anatomica, 24: 121, 1955.

7. Anderson, K. J.: The Behavior of Autogenous and Homogenous Bone Transplants in the Anterior Chamber of the Rat's Eye. A Histological Study of the Effect of the Size of the Implant. J. Bone & Joint Surg., 43A: 980, 1961.

8. Anderson, K. J., Dingwall, J. A., Schmidt, J., Lacoste, J. P., and Clawson, D. K.: Induced Connective Tissue Metaplasia. I. Heterogenous Bone Extract Implants in the Rat Anterior Eye Chamber. A Preliminary Report. Plastic & Reconstr. Surg., 25: 399, 1960.

9. Andrew, W.: Textbook of Comparative Histology, pp. 346, 378. Oxford University Press, New York, 1959.

10. Andrew, W.: Comparative Hematology, pp. 118, 120, 126. Grune and Stratton, New York, 1965.

11. Arnold, J. S., and Jee, W. S. S.: Bone Growth and Osteoclastic Activity as Indicated by Radioautographic Distribution of Plutonium. Am. J. Anat., 101: 367, 1957.

12. Arora, B. K., and Laskin, D. M.: Sex Chromatin as a Cellular Label of Osteogenesis by Bone Grafts. J. Bone & Joint Surg., 46A: 1269, 1964.

13. Balinsky, B. I.: An Introduction to Embryology, 2nd ed., W. B. Saunders Company, Philadelphia, 1965.

14. Barr, M. L., and Bertram, E. G.: A Morphological Distinction between Neurones of the Male and Female, and the Behavior of the Nucleolar Satellite during Accelerated Nucleoprotein Synthesis. Nature, 163: 676, 1949.

15. Bassett, C. A. L.: Current Concepts of Bone Formation. A.A.O.S. Instructional Course Lecture. J. Bone & Joint Surg., 44A: 1308, 1962.

16. Bassett, C. A. L., and Becker, R. O.: Generation of Electric Potential by Bone in Response to Mechanical Stress. Science, 137: 1063, 1962.

17. Bassett, C. A. L., and Herrmann, I.: Influence of Oxygen Concentration and Mechanical Factors on Differentiation of Connective Tissue in Vitro. Nature, 190: 460, 1961.

18. Barth, L. G.: Embryology, revised. Dryden Press, New York, 1953.

19. Becker, R. O.: The Bioelectric Factors in Amphibian Limb Regeneration. J. Bone & Joint Surg., 43A: 643, 1961.

20. Beckert, W. H., and Garner, J. G.: Staining Sex Chromatin: Biebrich Scarlet and Fast Green FCF as a Mixture versus their use in Sequence. Stain Tech., 41: 141, 1966.

21. Bélanger, L. F.: Autoradiographic Visualization of the Entry and Transit of S^{35} in Cartilage, Bone and Dentine of young Rats and the Effect of Hyaluronidase in Vitro. Can. J. Biochem. & Physiol., 32: 161, 1954.

22. Bélanger, L. F.: Observations on the Manifestations of Osteolathyrism in the Chick. J. Bone & Joint Surg., 41B: 581, 1959.

23. Bélanger, L. F.: Staining Processed Radioautographs. Stain Tech., 36: 313, 1961.

24. Bélanger, L. F., and Bélanger, C.: Alpharadiography: A Simple Method for Determination of Mass Concentration in Cells and Tissues. J. Biophys. & Biochem. Cytology, 6: 197, 1959.

25. Bélanger, L. F., Copp, D. H., and Morton, M. A.: Demineralization with EDTA by Constant Replacement. Anat. Rec., 153: 41, 1965.

26. Bélanger, L. F., Jarry, L., and Uhthoff, H. K.: Osteocytic Osteolysis in Paget's Disease. Rev. Can. de Biol., 27: 37, 1968.

27. Bélanger, L. F., and Migicovsky, B. B.: Comparison Between Different Mucopolysaccharide Stains as Applied to Chick Epiphyseal Cartilage. J. Histochem. & Cytochem., 9: 73, 1961a.

28. Bélanger, L. F. and Migicovsky, B. B.: Extratrabecular Crystallization in Rickets; Effects of Vitamin D, Calcium and Norethandrolone. Proc. Exp. Biol. & Med., 106: 19, 1961b.

29. Bélanger, L. F., Van Erkle, G. A., and Jakerow, A.: Behavior of the Dermal Mast Cells In Magnesium-Deficient Rats. Science, 126: 29, 1957.

30. Benoit, J. A. A.: Induction de cartilage in vitro par l'extrait d'otocystes d'embryons de poulet. J. Emb. & Exp. Morph., 8: 33, 1960a.

31. Benoit, J. A. A.: L'otocyste exerce-t-il une action inductrice sur le mésenchyme somatique chez l'embryon de poulet. J. Emb. Exp. Morph., 8: 39, 1960b.

32. Billingham, R. E., Brent, L., and Medawar, P. B.: Tolerance of Red Cell Antigens and Transplantation Immunity in Chickens. Experientia, 11: 444, 1955.

33. Billingham, R. E., Brent, L., and Medawar, P. B.: The Antigenic Stimulus in Transplantation Immunity. Nature, 178: 514, 1956.

34. Birge, E. A., and Imhoff, C. E.: Versenate as a Decalcifying Agent for Bone. Am. J. Clin. Path., 22: 192, 1952.

35. Bloom, M. A., Domm, L. V., Nalbandov, A. V., and Bloom, W.: Medullary Bone of Laying Chickens. Am. J. Anat., 102: 411, 1958.

36. Boake, W. H., and Muir, H.: The Non-Antigenicity of Chondroitin Sulphate. Lancet, 1955-II: 1222.

37. Bonfiglio, M.: Repair of Bone-Transplant Fractures. J. Bone & Joint Surg., 40A: 446, 1958.

38. Bonfiglio, M., Jeter, W. S., and Smith, C. L.: The Immune Concept: Its Relation to Bone Transplantation. Ann. N. Y. State Acad. Sci., 59: 417, 1955.

39. Bridges, J. B., and Pritchard, J. J.: Bone and Cartilage Induction in the Rabbit. J. Anat., 92: 28, 1958.

40. Burger, M., Sherman, B. S., and Sobel, A. E.: Observations of the Influence of Chondroitin Sulphate on the Rate of Bone Repair. J. Bone & Joint Surg., 44B: 675, 1962.

41. Buring, K., and Urist, M. R.: Transfilter Bone Induction. Clin. Orthop., 54: 235, 1967a.

42. Buring, K., and Urist, M. R.: Effects of Ionizing Radiation on the Bone Induction Principle in the Matrix of Bone Implants. Clin. Orthop., 55: 225, 1967b.

43. Burnet, F. M.: The Clonal Selection Theory of Acquired Immunity. Vanderbilt University Press, Nashville, 1959.

44. Burnett, F. M.: Immunological Recognition of Self. Science, 133: 307, 1961.

45. Burwell, R. G.: Studies in the Transplantation of Bone. IV. The Immune Responses of Lymph Nodes Draining Second-set Homograft of Fresh Cancellous Bone. J. Bone & Joint Surg., 44B: 688, 1962.

46. Burwell, R. G.: The Role of Lymphoid Tissue in Morphostasis. Lancet, 285: 69, 1963a.

47. Burwell, R. G.: Studies in the Transplantation of Bone. V. The Capacity of Fresh and Treated Homografts of Bone to Evoke Transplantation Immunity. J. Bone & Joint Surg., 45B: 386, 1963b.

48. Burwell, R. G.: Studies in the Transplantation of Bone. VII. The Fresh Composite Homograft-Autograft of Cancellous Bone. J. Bone & Joint Surg., 46B: 110, 1964.

49. Burwell, R. G.: Studies in the Transplantation of Bone. VIII. Treated Composite Homograft-Autografts of Cancellous Bone: An Analysis of Inductive Mechanisms in Bone Transplantation. J. Bone & Joint Surg., 48B: 532, 1966.

50. Burwell, R. G., and Gowland, G.: Studies in the Transplantation of Bone. I. Assessment of Antigenicity-Serological Studies. J. Bone & Joint Surg., 45B: 814, 1963.

51. Burwell, R. G., and Gowland, G.: Studies in the Transplantation of Bone. II. The Changes Occurring in the Lymphoid Tissue after Homograft and Autografts of Fresh Cancellous Bone. J. Bone & Joint Surg., 45B: 820, 1963.

52. Burwell, R. G., and Gowland, G.: Studies in the Transplantation of Bone. III. The Immune Responses of Lymph Nodes Draining Components of Fresh Homologous Cancellous Bone and Homologous Bone Treated by Different Methods. J. Bone & Joint Surg., 44B: 131, 1962.

53. Burwell, R. G., Gowland, G., and Dexter, F.: Studies in the Transplantation of Bone. VI. Further Observations Concerning the Antigenicity of Homologous Corticol and Cancellous Bone. J. Bone & Joint Surg., 45B: 807, 1963.

54. Bush, L. F., and Garber, C. Z.: The Bone Bank. J. Am. Med. Assn., 137: 588, 1948.

55. Carleton, H. M., and Drury, R. A. B.: Histological Technique, 3rd ed. Oxford University Press, London, New York, Toronto, 1957.

56. Chalmers, J.: Transplantation Immunity in Bone Homografting. J. Bone & Joint Surg., 41B: 160, 1959.

57. Chalmers, J.: Bone Transplantation. Symp. Tissue Org. Transplant. Suppl. J. Clin. Path., 20: 540, 1967.

58. Chalmers, J., and Ray, R. D.: The Growth of Transplanted Foetal Bones in Different Immunological Environments. J. Bone & Joint Surg., 44B: 149, 1962.

59. Chase, S. W., and Herndon, C. H.: The Fate of Autogenous and Homogenous Bone Grafts. (A Historical Review). J. Bone & Joint Surg., 37A: 809, 1955.

60. Cheng, H. F., Dicks, M., Shellhamer, R. H., Brown, D. S., Roberts, A. N., and Haurowitz, F.: Localization of Antigens by Autoradiography. Proc. Exp. Biol. & Med., 106: 93, 1961.

61. Cooley, L. M., and Goss, R. J.: The Effects of Transplantation and Xirradiation on the Repair of Fractured Bones. Am. J. Anat., 102: 167, 1958.

62. Coons, A. H.: Fluorescent Antibodies in the Study of Antigens. Ann. N. Y. Acad. Sci., 69: 658, 1957-58.

63. Cooper, G. W.: Induction of Somite Chondrogenesis by Cartilage and Notochord: A Correlation between Inductive Activity and Specific Stages of Cytodifferentiation. Devel. Biol., 12: 185, 1965.

64. Craig, J. V., and McDermid, E. M.: Prolong Skin Homograft Survival and Erythrocyte (B-locus) Antigens in Young Chicks. Transplant., 1: 191, 1963.

65. Curtiss, P. H. Jr., Herndon, C. N., and Chase, S. W.: Immunologic Factors in Homogenous Bone Transplantation. I. Serologic Studies. II. Histologic Studies. J. Bone & Joint Surg., 38A: 103 & 324, 1956.

66. De Palma, A., Tsaltas, T. T., and Mauler, G. G.: Viability of Osteochondrial Grafts as Determined by Uptake of S^{35}. J. Bone & Joint Surg., 45A: 1503, 1963.

67. De Vries, P. H., Bagdley, C. E., and Lindman, J.: Radiation Sterilization of Homogeneous-Bone Transplants Utilizing Radioactive Cobalt. Preliminary Report. J. Bone & Joint Surg., 40A: 187, 1958.

68. Dransfield, J. W.: The Lymphatic System of the Domestic Fowl. Veterinary Journal, 101: 171, 1961.

69. Dubuc, F. L., and Urist, M. R.: The Reversibility of the Bone Induction Principle in Surface-Demineralized Bone Implants. Clin. Ortho., 55: 217, 1967.

70. Dudley, H. R., and Spiro, D.: The Fine Structure of Bone Cells. Increase in Cellular Complexity with Differentiation of Osteoblasts. J. Biophys. & Biochem. Cytol., 11: 627, 1961.

71. Eichwald, E. J., Silmser, C. R., and Weissman, I.: Sex-Linked Rejection of Normal and Neoplastic Tissue. I. Distribution and Specificity. J. Nat. Cancer Instit., 20: 563, 1958.

72. Engfeldt, B., Engstrom, A., and Bostrom, H.: The Localization of Radiosulfate in Bone Tissue. Exp. Cell Res., 6: 251, 1954.

73. Enneking, W. F.: Histological Investigation of Bone Transplants in Immunologically Prepared Animals. J. Bone & Joint Surg., 39A: 597, 1957.

74. Enneking, W. F.: Immunologic Aspects of Bone Transplantation. South. Med. J., 55: 894, 1962.

75. Enneking, W. F., and Gratch, A.: The Effect of Total Body Irradiation on Bone Transplants in Parabiosed Animals, J. Bone & Joint Surg., 41A: 463, 1959.

76. Entin, M. A., Alger, J. R., and Baird, R. M.: Experimental and Clinical Transplantation of Autogenous Whole Joints. J. Bone & Joint Surg., 44A: 1518, 1962.

77. Fell, H. B.: The Histogenesis of Cartilage and Bone in the Long Bones of the Embryonic Fowl. J. of Morph. & Physiol., 40: 417, 1925.

78. Fell, H. B.: Experimental Transformation of Cells. Nature, 185: 882, 1960.

79. Forkner, C. E.: Blood and Bone Marrow Cells of the Domestic Fowl. J. Exp. Med., 50: 121, 1929.

80. Freiburg, R. A., and Ray, R. D.: Studies of Devitalized Bone Implants. Arch. Surg., 89: 417, 1964.

81. Gibson, T.: The Transplantation of Cartilage. Symp. Tiss. Org. Transplant. Suppl. J. Clin. Path., 20: 513, 1967.

82. Gilman, S. H., and Enneking, W. F.: Prehistologic Changes in the Rejection Mechanism of Bone Transplants. J. Surg. Res., 5: 31, 1965.

83. Gilmour, D. G.: Current Status of Blood Groups in Chickens. Ann. N. Y. Acad. Sci., 97: 166, 1962.

84. Glick, D.: Techniques of Histo- and Cytochemistry. Interscience Publishers, Inc., London, New York, 1949.

85. Goldhaber, P.: Preliminary Observations on Bone Isografts within Diffusion Chambers. Proc. Soc. Exp. Biol. Med., 98: 53, 1958.

86. Goldhaber, P.: Osteogenic Induction across Millipore Filters in vivo. Science, 133: 2065, 1961.

87. Goldhaber, P.: Calcification within Diffusion Chambers Containing Bone Isografts. Clin. Ortho., 25: 204, 1962.

88. Grobstein, C.: Autoradiography of the Interzone between Tissues in Inductive Interaction. J. Expt. Zool., 142: 203, 1959.

89. Hall, B. K.: The Formation of Adventitious Cartilage By Membrane Bones under the Influence of Mechanical Stimulation Applied in Vitro. Life Sci., 6: 663, 1967.

90. Ham, A. W.: Histology (Fifth Edition). J. B. Lippincott Company, Philadelphia, 1969.

91. Hammack, B. L., and Enneking, W. F.: Comparative Vascularization of Autogenous and Homogenous - Bone Transplants. J. Bone & Joint Surg., 42A: 811, 1960.

92. Hancox, N. M.: The Survival of Transplanted Embryo Bone Grafted to Chorioallantoic Membrane and Subsequent Osteogenesis. J. Physiol., 106: 276, 1947.

93. Heller, M., McLean, J. C., and Bloom, W.: Cellular Transformations in Mammalian Bones Induced by Parathyroid Extract. Am. J. Anat., 87: 315, 1950.

94. Hendricks, S. B.: The Nature of Bone Salt. Ann. N. Y. Acad. Sci., 60: 660 (abstract), 1954-55.

95. Herndon, C. H., and Chase, S. W.: The Fate of Massive Autogenous and Homogenous Bone Grafts Including Articular Surfaces. Surg. Gyn. Obs., 99: 275, 1954.

96. Heslop, B. F., Zeiss, I. M., and Hisket, M. M.: Studies on Transference of Bone. I. A Comparison of Autologous and Homologous Bone Implants With Reference to Osteocyte Survival, Osteogenesis and Host Reaction. Brit. J. Exp. Path., 41: 269, 1960.

97. Hirschman, A., and Dziewiatkowski, D. D.: Sturgeon Polysaccharide Loss During Endochondral Ossification: Immunochemical Evidence. Science, 154: 393, 1966.

98. Hodge, H. C.: Some Achievements and Problems in Studying the Solubility of the Mineral of Bone and Tissues. Ann. N. Y. Acad. Sci., 60: 661, 1954-55.

99. Holborow, E. J.: An A.B.C. of Modern Immunology. I. Links between the Old and the New. II. Antibody Anatomized. III. Immunoglobulin Physiology. IV. Cell and Immune Faculties - Competence and Memory. V. The Cytological Capability of Small Lymphocytes. VI. Cell-Mediated Immunity. VII. The Genesis of Immunological Competence. VIII. Defects and Evasions. Lancet, 1: 839, 898, 942, 995, 1049, 1098, 1148, 1208, 1967.

100. Holtzer, H., Abbott, J., Lash, J., and Holtzer, S.: The Loss of Phenotypic Traits by Differentiated Cells in Vitro. I. Dedifferentiation of Cartilage Cells. Proc. Nat. Acad. Sci., 46: 1533, 1960.

101. Holtzer, H., and Detwiler, S. R.: An Experimental Analysis of the Development of The Spinal Column. XII. Induction of Skeletogenous Cells. J. Expt. Zool., 123: 335, 1953.

102. Hommes, F. A., Van Leeuwen, G., and [illegible] T.: Induction of Cell Differentiation. III. The Isolation of a Chondrogenic Factor from Embryonic Chick Spinal Cords and Notochords. Biochem. Biophys. Acta, 56: 320, 1962.

103. Huggins, C. B., McCarrol, N. R., and Blocksom [illegible] Jr.: Experiments on the Theory of Osteogenesis. [illegible] of Local Calcium Deposits on Ossification. The Osteogenic Stimulus of Epichelium. Arch. Surg., 18: 915, 1930.

104. Humphrey, J. H.: Antigenic Properties of Hyaluronic Acid. Biochem. J., 37: 460, 1943.

105. Hurley, L. A., Stinchfield, F. E., Bassett, A. L., and Lyon, W. K.: The Role of Soft Tissues in Osteogenesis - An Experimental Study of Canine Spinal Fusions. J. Bone & Joint Surg., 41A: 1245, 1959.

106. Hutchison, J.: The Fate of Experimental Bone Autografts and Homografts. Brit. J. Surg., 39: 552, 1952.

107. Karges, D. E., Anderson, K. J., Dingwall, J. A., and Jowsey, J.: Experimental Evaluation of Processed Heterogenous Bone Transplants. Clin. Ortho., 35: 230, 1965.

108. Karphishka, I., and Carneiro, J.: Incorporation of three Methionine Labels, Radioautographic Comparison of the Sites of C^{14}, S^{35}, and H^{3}. Anat. Rec., 137: 73, 1960.

109. Kember, N. F.: Cell Division in Endochondral Ossification. A Study of Cell Proliferation in Rat Bones by the Method of Tritiated Thymidine Autoradiography. J. Bone & Joint Surg., 42B: 824, 1960.

110. Khvorostukhin, I. I.: Antigenic Properties of Arthritic Cartilage. Bull. Exp. Biol. Med., 45: 90, 1958.

111. Kiehn, C. L., Cebul, F., Berg, M., Satoneay, J., and Glover, D. M.: A Study of the Vascularization of Experimental Bone Grafts by means of Radioactive Phosphorus and the Transparent Chamber. Ann. Surg., 136: 404, 1952.

112. Kingma, M. J., and Hampe, J. F.: The Behavior of Blood Vessels After Experimental Transplantation of Bone. J. Bone & Joint Surg., 40A: 141, 1964.

113. Kosin, I. L., and Ishizaki, K.: Incidence of Sex Chromatin in Gallus Domesticus. Science, 111: 43, 1950.

114. Krishan, A.: A Cytological Method for Sexing Young Chicks. Experientia, 18: 100, 1962.

115. Lacroix, P.: The Organization of Bones, p. 136. McGraw-Hill (Blakiston), New York, 1951.

116. Lacroix, P.: Bone: Symposium. Lancet, II: 824, 1956.

117. Lambert, P. B.: Local Recognition of Histocompatibility Differences in Skin. Science, 155: 99, 1967.

118. Lash, J., Holtzer, S., and Holtzer, H.: An Experimental Analysis of the Development of the Spinal Column. VI. Aspects of Cartilage Induction. Exper. Cell Res., 13: 292, 1957.

119. Lash, J. W., Hommes, F. A., and Zilliken, F.: Induction of Cell Differentiation. I. The IN VITRO Induction of Vertebral Cartilage with a low Molecular-Weight Tissue Component. Biochem. Biophys. Acta, 56: 313, 1962.

120. Laskin, D. M., and Sarnat, B. G.: The Metabolism of Fresh, Transplanted and Preserved Cartilage. Surg. Gyn. Obs., 96: 493, 1953.

121. Leblond, C. P., Bélanger, L. F., and Greulich, R. C.: Formation of Bones and Teeth as Visualized by Radioautography. Ann. N. Y. Acad. Sci., 60: 681, 1954-55.

122. Leblond, C. P., Wilkinson, G. W., Bélanger, L. F., and Robichon, J.: Radioautographic Visualization of Bone Formation in the Rat. Am. J. Anat., 86: 265, 1950.

123. Liébecq-Hutter, S.: Action de la "Diamnéine" sur la Croissance et l'Ossification d'Ébauche de Tibias et de Fémurs d'embryons de poulet cultivés in Vitro. J. Emb. & Exp. Morph., 4: 279, 1956.

124. Lucas, A. M., and Jamroz, C.L:Atlas of Avian Hematology. Agricultural Monograph 25, U.S. Dept. of Agric., 1961.

125. McDonald, J. C.: Serum Antibodies in Transplantation. N. Y. State J. Med., 66: Part I, 1631, 1966.

126. McLean, F. C., and Urist, M. R.: Bone - An Introduction to the Physiology of Skeletal Tissue (2nd edition). University of Chicago Press, Chicago, 1961.

127. McManus, J. F. A., and Mowry, R. W.: Staining Methods - Histological and Histochemical. Hoeber, New York, 1960.

128. Malein, M.: Osteogenesis Induced by Vesical Mucosal Transplant in the Guinea-Pig. J. Bone & Joint Surg., 44B: 165, 1962.

129. Mankin, H. J.: Osteogenesis in the Subchondral Bone of Rabbits. J. Bone & Joint Surg., 46A: 1253, 1964.

130. Mareel, M.: Recherches sur la relation réciproque entre chondrocytes et périoste dans le tibia embryonnaire de poulet. Arch. Biol., 78: 145, 1967.

131. Medewar, P. B.: Immunological Tolerance. Science, 133: 303, 1961.

132. Melcher, A. H., and Irving, J. T.: The Healing Mechanism in Artificially Created Circumscribed Defects in the Femora of Albino Rats. J. Bone & Joint Surg., 43B: 528, 1962.

133. Messier, B., and Leblond, C. P.: Cell Proliferation and Migration as revealed by Radioautography after Injection of Thymidine-H^3 into Male Rats and Mice. Am. J. Anat., 106: 247, 1960.

134. Migicovsky, B. B., and Nielson, A. M.: Bone Metabolism as a Means of Studying Vitamin D action. Science, 115: 354, 1952.

135. Mjassojedoff, S. W.: Die Zellformen des Bindegewebes und des Blutes und die Blutbildung beim erwachsenen Menschen. Folia Haem., 31-32: 263, 1926.

136. Moller, G., and Moller, E.: Plaque-formation by Non-Immune and X-irradiated Lymphoid Cells on Monolayers of Mouse Embryo Cells. Nature, 208: 260, 1965.

137. Moscona, A.: Development of Heterotypic Combinations of Dissociated Chick Cells. Proc. Soc. Exp. Biol. & Med., 92: 410, 1956.

138. Moss, M. L.: Extraction of an Osteogenic Inductor Factor from Bone. Science, 127: 755, 1958.

139. Munson, P. L.: Studies on the Role of the Parathyroids in Calcium and Phosphorus Metabolism. Ann. N. Y. Acad. Sci., 60: 776, 1954-55.

140. Murray, J. W. G., Levine, L. S., and [illegible]: Comparative Study of Healing of Organic and Inorganic Bone Grafts. N. Y. State J. Med., 60: Part II, 3017, 1960.

141. Ohno, S., Kaplan, W. D., and Kinosita, R.: On the Sex Chromatin of Gallus Domesticus. Exp. Cell Res., 21: 160, 1960.

142. O'Steen, W. K., and Walker, B. E.: Radioautographic Studies of Regeneration in the Common Newt. II. Physiological Regeneration. Anat. Rec., 137: 501, 1960.

143. Peacock, E. E. Jr., Weeks, P. M., and Petty, J. M.: Some Studies on the Antigenicity of Cartilage. Ann. N. Y. Acad. Sci., 87: 175, 1960.

144. Peer, L. A.: Sex Chromatin Study to Determine the Survival of Cartilage Homografts. Transpl. Bull., 5: 404, 1958.

145. Polley, C. R., Crosse, C. R., and Craig, J. V.: A Skin-Grafting Technique for Use in Genetic Studies with Chickens. Plast. & Reconstr. Surg., 25: 425, 1960.

146. Post, R. H., Heiple, K. G., Chase, S. W., and Herndon, C. H.: Bone Grafts in Diffusion Chambers. Clin. Ortho., 44: 265, 1966.

147. Pritchard, J. J.: A Cytological and Histochemical Study of Bone and Cartilage Formation in the Rat. J. Anat., 86: 259, 1952.

148. Quinn, R. W., and Cerroni, R.: Antigenicity of Chondroitin Sulphate. Proc. Soc. Exp. Biol. Med., 96: 268, 1957.

149. Quinn, R. W., and Singh, K. P.: Antigenicity of Hyalyronic Acid. Proc. Soc. Exp. Biol. Med., 95: 296, 1957.

150. Ray, R. D., and Holloway, J. A.: Bone Implants. J. Bone & Joint Surg., 39A: 1119, 1957.

151. Ray, R. D., and Sabet, T. Y.: Bone Grafts: Cellular Survival vs. Induction. J. Bone & Joint Surg., 45A: 337, 1963.

152. Robinson, R. A.: An Electron-Microscopic Study of the Crystalline Inorganic Component of Bone and Its Relationship to the Organic Matrix. J. Bone & Joint Surg., 34A: 389, 1952.

153. Robinson, R. A., and Watson, M. L.: Crystal-Collagen Relationships in Bone as Observed in the Electron Microscope. III. Crystal and Collagen Morphology as a Function of Age. Ann. N. Y. Acad. Sci., 60: 596, 1954-55.

154. Rose, G. G., and Shindler, T. O.: The Cytodifferentiation of Osteoblasts in Tissue Culture. A Description of Cellular Emigrations from Embryo Chick-Leg Bones. J. Bone & Joint Surg., 42A: 485, 1960.

155. Rosin, A., Frieberg, H., and Zajicek, G.: The Fate of Rat Bone Marrow Spleen and Periosteum Cells Cultivated In Vivo in the Diffusion Chamber with Special Reference to Bone Formation. Exp. Cell Res., 29: 176, 1963.

156. Sabet, T. Y., Hedvegi, E. B., and Ray, R. D.: Bone Immunology. II. Comparison of Embryonic Mouse Isografts and Homografts. J. Bone & Joint Surg., 43A: 1037, 1961.

157. Sabet, T. Y., and Ray, R. D.: The Immune Mechanism of Transplantation and Possible Role of DNA and RNA. Bone Biodynamics, International Symposium, Henry Ford Hospital, p. 71, 1964.

158. Saunders, J. W. Jr., and Gasseling, M. T.: Effects of Reorienting the Wing-Bud Apex in the Chick Embryo. J. Expt. Zool., 142: 553, 1959.

159. Schierman, L. W., and Nordskog, A. W.: Relationship of Blood Type to Histocompatibility in Chickens. Science, 134: 1008, 1961.

160. Scott, B. L., and Pease, D. C.: Electron Microscopy of the Epiphyseal Apparatus. Anat. Rec., 126: 465, 1956.

161. Sedar, J. D.: The Influence of Direct Current Fields upon the Developmental Patterns of the Chick Embryo. J. Exp. Zool., 133: 47, 1956.

162. Selle, R. W., and Urist, M. R.: Calcium Deposits and New Bone Formation in Muscle in Rabbits. J. Surg. Res., 1: 132, 1961.

163. Siffert, R. S.: Experimental Bone Transplants. J. Bone & Joint Surg., 37A: 742, 1955.

164. Siffert, R. S., and Barash, E. S.: Delayed Bone Transplantation. An Experimental Study of early Host-Transplant Relationships. J. Bone & Joint Surg., 43A: 407, 1961.

165. Silvers, W. K., Wilson, D. B., and Palm, J.: Mixed Leucocyte Reactions and Histocompatibility in Rats. Science, 155: 703, 1967.

166. Simonsen, M.: The Impact on the Developing Embryo and Newborn Animal of Adult Homologous Cells. Acta Path. Microbiol. Scand., 40: 480, 1957.

167. Sisken B. F., and Glueckshohn-Waelsch, S.: A Developmental Study of the Mutation "Phocomelia" in the Mouse. J. Expt. Zool., 142: 623, 1959.

168. Sledge, C. B.: Some Morphologic and Experimental Aspects of Limb Development. Clin. Ortho., 44: 241, 1966.

169. Smith, J. W.: Age Changes in the Organic Fraction of Bone. J. Bone & Joint Surg., 45B: 761, 1963.

170. Sobel, A. E.: Local Factors in the Mechanism of Calcification. Ann. N. Y. Acad. Sci., 60: 713, 1954-55.

171. Sonck, M. V.: The Chemical Nature of the Organic Matrix of Bone, Dentin and Enamel. Ann. N. Y. Acad. Sci., 60: 585, 1954-55.

172. Stark, O., Kren, V., and Frenzl, B.: Dissociation of Isohaemagglutinin Formation and Tolerance of Skin Grafts in Chickens. Nature, 190: 281, 1961.

173. Stowell, R. E.: Feulgen Reaction for Thymonucleic Acid. Stain Technol., 19-21: 45, 1944-46.

174. Terasaki, P. J., Cannon, J. A., and Longmire, W. P. Jr.: The Specificity of Tolerance to Homografts in the Chicken. J. Immunol., 81: 246, 1958.

175. Thomas, W. C. Jr.: Comparative Studies on Bone Matrix and Osteoid by Histochemical Techniques. J. Bone & Joint Surg., 43: 419, 1961.

176. Tonna, E. A.: Osteoclasts and the Aging Skeleton: A Cytological, Cytochemical and Autoradiographic Study. Anat. Rec., 137: 251, 1960.

177. Tonna, E. A.: The Cellular Complement of the Skeletal System Studied Autoradiographically with Tritiated Thymidine (H^3TDR) during Growth and Aging. J. Biophys. & Biochem. Cytol., 9: 813, 1961.

178. Tonna, E. A., and Cronkite, E. P.: Use of Tritiated Thymidine for the Study of the Origin of the Osteoclast. Nature, 190: 459, 1961.

179. Trautz, O. R.: X-ray Diffraction of Biological and Synthetic Apatites. Ann. N. Y. Acad. Sci., 60: 696, 1954-55.

180. Trinkaus, J. P.: The Differentiation of Tissue Cells. Am. Naturalist, 90: 273, 1956.

181. Trinkaus, J. P., and Gross, M. C.: The Use of Tritiated Thymidine for Marking Migratory Cells. Exp. Cell Res., 24: 52, 1961.

182. Trueta, J.: The Role of the Vassels in Osteogenesis (Lecture). J. Bone & Joint Surg., 45B: 402, 1963.

183. Trueta, J., and Amato, V. P.: The Vascular Contribution to Osteogenesis. III. Changes in the Growth Cartilage caused by Experimentally Induced Ischaemia. J. Bone & Joint Surg., 42B: 571, 1960.

184. Trueta, J., and Buler, A. J.: The Vascular Contribution to Osteogenesis. V. The Vasculature Supplying the Epiphyseal Cartilage in Rachitic Rats. J. Bone & Joint Surg., 45B: 572, 1963.

185. Trueta, J., and Little, K.: The Vascular Contribution to Osteogenesis. II. Studies with the Electron Microscope. J. Bone & Joint Surg., 42B: 367, 1960.

186. Trueta, J., and Morgan, J. D.: The Vascular Contribution to Osteogenesis. I. Studies by the Injection Method. J. Bone & Joint Surg., 42B: 97, 1960.

187. Urist, M. R.: Bone: Formation by Autoinduction. Science, 150: 893, 1965.

188. Urist, M. R., and McLean, F. C.: Osteogenetic Potency and New Bone Formation by Induction in Transplants to the Anterior Chamber of the Eye. J. Bone & Joint Surg., 34A: 443, 1952.

189. Urist, M. R., and McLean, F. C.: The Local Physiology of Bone Repair with Particular Reference to the Process of New Bone Formation by Induction. Am. J. Surg., 85: 444, 1953.

190. Urist, M. R., Silverman, B. F., Buring, K., Dubuc, F. L., and Rosenberg, J. M.: The Bone Induction Principle. Clin. Ortho., 53: 243, 1967.

191. Urist, M. R., Wallace, T. M., and Adams, T.: The Function of Fibrocartilaginous Callus. J. Bone & Joint Surg., 47B: 304, 1965.

192. Van de Putte, K. A., and Urist, M. R.: Osteogenesis in the Interior of Intramuscular Implants of Decalcified Bone Matrix. Clin. Orthop., 43: 257, 1966.

193. Van Rood, J. J., Van Leeuwen, A., and Bruning, J. W.: The Relevance of Leucocyte Antigens for Allogenic Renal Transplantation. Symp. Tiss. Org. Transpl., Suppl. J. Clin. Path., 20: 504, 1967.

194. Vernino, D. M., and Laskin, D. M.: Sex Chromatin in Mammalian Bone. Science, 132: 675, 1960.

195. Vincent, J., Bélanger, L. F., and Migicovsky, B. B.: Minéralization dans l'os rachitique. Exper. Cell Res., 26: 168, 1962.

196. Voisin, G. A., and Maurer, P.: Studies on the Role of Antibodies in the Failure of Homografts. Ann. N. Y. Acad. Sci., 64: 1053, 1956-57.

197. Walker, D. G.: Citric Acid Cycle in Osteoblasts and Osteoclasts. A Histochemical Study of Normal and Parathormone-Treated Rats. Bull. Johns Hopkins Hosp., 108: 80, 1961.

198. Watson, M. L., and Robinson, R. A.: Collagen - Crystal Relationships in Bone. II. Electron Microscope Study of Basic Calcium Phosphate Crystals. Am. J. Anat., 93: 25, 1953.

199. Weinburg, H., Makin, M., Nelkin, D., and Genevitch, A.: A and B Antigens in Human Bone Tissue. J. Bone & Joint Surg., 41B: 151, 1959.

200. Weiss, P.: Some Introductory Remarks on the Cellular Basis of Differentiation. J. Emb. & Exp. Morph., 1: 181, 1953.

201. Weiss, P., and James, R.: Skin Metaplasia in Vitro Induced by Brief Exposure to Vitamin A. Exp. Cell Res., Suppl. 3: 381, 1955.

202. Weiss, P., and Taylor, A. C.: Reconstitution of Complete Organs from Single Cell Suspensions of Chick Embryos in Advanced Stages of Differentiation. Proc. Nat. Acad. Sci., (U.S.), 46: 1177, 1960.

203. Wilkins, L.: Hormonal Influences on Skeletal Growth. Ann. N. Y. Acad. Sci., 60: 763, 1954-55.

204. Williams, R. G.: Observations on the Repair of Injuries to Bone Growing from Autografts in Rabbits. Anat. Rec., 137: 107, 1960.

205. Young, M. H.: The Repair of Experimental Defects in Rabbit Skulls. J. Bone & Joint Surg., 46B: 329, 1964.

206. Young, R. W.: Cell Proliferation and Specialization During Endochondral Osteogenesis in Young Rats. J. Cell Biol., 14: 357, 1962a.

207. Young, R. W.: Regional Differences in Cell Generation Time in Growing Rat Tibiae. Exper. Cell Res., 26: 562, 1962b.

208. Young, R. W.: Nucleic Acids, Protein Synthesis and Bone. Clin. Ortho., 26: 147, 1962c.

209. Zeiss, I. M., Nisbet, N. W., and Heslop, B. F.: II. Vascularization of Autologous and Homologous Implants of Cortical Bone in Rats. Brit. J. Exper. Path., 41: 345, 1960.