STUDIES IN THE TRANSPLANTATION OF BONE

VI. Further Observations Concerning the Antigenicity of Homologous Cortical and Cancellous Bone

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Recent studies have shown that the red marrow is the principal antigenic component of a fresh homograft of iliac bone and that iliac bone tissue washed free from marrow is not demonstrably antigenic (Burwell and Gowland 1962, Burwell 1963).

These findings clearly emphasise the necessity, when investigating the antigenicity of fresh cortical and cancellous bone, of cleansing the homograft of red marrow before its insertion into the host. In several previous studies in which immunity was evoked by homografts of bone from which the marrow had not been so removed, it seems likely that the immunological effects observed resulted more from the marrow than from the bone tissue itself (Bonfiglio, Jeter and Smith 1955; Curtiss and Herndon 1955, 1956; Enneking 1957; Enneking, Gratch and Ethridge 1957; Urist, Macdonald and Jowsey 1958; Enneking and Gratch 1959; Chalmers 1959; Hancox, Owen and Singleton 1961). Likewise in the recent experiments of Ray and his colleagues (Sabet, Hidvegi and Ray 1961; Chalmers and Ray 1962) in which embryonic femora were transplanted as homografts into adult mice, it is not possible to apportion the transplantation antigenicity of their grafts to bone, to cartilage or to marrow.

In the present studies the primary and secondary immune responses of regional lymph nodes have been used as histological indicators of the antigenicity of foreign cortical and cancellous bone grafts inserted into their drainage areas. Previous work has shown that the secondary response which occurs in lymph nodes draining second-set homografts of marrow-containing iliac bone occurs more rapidly and is of greater magnitude than that of the primary response (Burwell 1962*a, b*). Hence it was considered that the insertion of bone homografts into the drainage areas of lymph nodes previously sensitised to donor tissue might reveal antigenicity which was not detected after the insertion of first-set homografts into the drainage areas of lymph nodes of non-sensitised animals (Burwell and Gowland 1962).

The findings of the present work are related to previous knowledge of bone immunology. It is concluded that: 1) fresh homologous cortical bone contains transplantation antigens; 2) fresh homologous cancellous bone has yet to be shown indisputably to contain such antigens; and 3) some methods of physical and chemical treatment of marrow-containing iliac bone permit tissue antigens in the marrow to remain immunologically active.

MATERIALS AND METHODS

Eighty-four adult male rabbits weighing from 2.4 to 3.7 kilograms were used in the investigation. Six rabbits of Albino strain served as donors of homologous bone and the seventy-eight recipient animals were of Flemish Giant strain. In the analysis of the material the findings from experiments with forty-two additional rabbits included in previous reports have also been utilised (Burwell and Gowland 1961, Burwell 1962*a*).

OPERATIVE PROCEDURES

First-set bone grafts—All animals were anaesthetised by the injection of sodium pentobarbitone into the marginal vein of the right ear. The bone grafts were prepared and inserted as follows.

Fresh homologous cortical bone blocks—From a donor rabbit the right femoral shaft was excised, split longitudinally and the periosteum removed by thorough scraping. The specimens were then

TABLE I
WEIGHTS OF THE FIRST REGIONAL LYMPH NODES
Lymph nodes draining first-set bone grafts

Bone material	Five days after grafting			Eight days after grafting		
	Serial number	Weight of node on side of operation (milligrams)	Weight of node on control side (milligrams)	Serial number	Weight of node on side of operation (milligrams)	Weight of node on control side (milligrams)
Fresh homologous cortical bone blocks	452	120	80	455	180	140
	453	90	50	456	120	80
	454	130	130	457	160	140
Fresh homologous cortical bone chips	458	115	75	461	120	100
	459	75	50	462	260	140
	460	160	60	463	120	95
Fresh autologous cortical bone blocks	566	120	80	560	165	200
	567	110	130	561	145	145
	568*	19	22	562	120	80
Fresh autologous cortical bone chips	569	135	145	563	100	115
	570	155	105	564	180	140
	571	145	120	565	135	130
Fresh autologous marrow-free iliac bone	572	120	80	575	90	60
	573	155	125	576	80	65
	574	65	60	577	110	125

* Excluded from the statistical analysis.

washed free from marrow with 0.9 per cent saline ejected from a dental chip syringe. Blocks of cortical bone weighing 82 to 100 milligrams were prepared, one block being inserted subcutaneously into the left ear of six recipient animals 1 to 2 centimetres from the external auditory meatus. The wound edges were carefully apposed by one suture of braided nylon.

Fresh homologous cortical bone chips—Clean portions from the femoral shaft prepared as described above were cut into chips and washed with 0.9 per cent saline; excess moisture was removed by placing on surgical gauze. A small cluster of chips weighing 100 milligrams was then inserted subcutaneously into the left ear of each of six recipient animals and the wound closed.

Fresh autologous cortical bone blocks—Each specimen was procured from the right femoral shaft of the same animal which received the graft. The excised femur was divided longitudinally and cleansed of soft tissues and marrow by thorough scraping and washing. Blocks of cortical bone weighing 90 to 100 milligrams were then prepared. One autograft was inserted subcutaneously into the left ear of each of six rabbits.

Fresh autologous cortical bone chips—Cortical chips from the femoral shaft of each animal receiving a graft were prepared by the method described above. A cluster of chips weighing 100 milligrams was then inserted subcutaneously into the left ear of each of six rabbits as an autograft.

Fresh autologous marrow-free iliac bone—The right ilium was removed from each of six rabbits and grafts were prepared as described previously (Burwell and Gowland 1961). Each graft was washed free from marrow and inserted subcutaneously as an autograft into the left ear.

Second-set bone grafts—A first-set homograft of fresh marrow-containing iliac bone, prepared as described in an earlier paper (Burwell and Gowland 1961), was inserted subcutaneously into the left ear of each of the recipient rabbits at a site about 2 centimetres from the external auditory meatus. Each of these homografts served to sensitise the host to tissue from the donor. Three weeks later a second-set bone graft obtained from the donor was inserted subcutaneously into the same ear of the corresponding recipient at a site 1 centimetre from the external auditory meatus. The second-set grafts comprised: autografts of fresh marrow-containing iliac bone; homografts of fresh marrow-free iliac bone; boiled iliac homografts; frozen (—20 degrees Centigrade) iliac homografts; freeze-dried iliac homografts; iliac homografts treated with merthiolate solution; fresh homologous cortical bone blocks; fresh homologous cortical bone chips. The methods of preparation of these grafts are described, either above or in a previous paper (Burwell and Gowland 1961). No attempt was made to remove

TABLE II
WEIGHTS OF THE FIRST REGIONAL LYMPH NODES
Lymph nodes draining second-set bone grafts

Bone materials First-set iliac homograft followed three weeks later by one of a variety of second-set grafts as indicated	Two days after second-set graft			Four days after second-set graft		
	Serial number	Weight of node on side of operation (milligrams)	Weight of node on control side (milligrams)	Serial number	Weight of node on side of operation (milligrams)	Weight of node on control side (milligrams)
Fresh autologous marrow-containing iliac bone	408	180	130	411	210	130
	409	155	120	412	115	50
	410	130	105	413	155	90
Fresh homologous marrow-free iliac bone	437	110	115	440	140	40
	438	120	60	441	175	90
	439	195	90	442	120	140
Boiled iliac bone	443	140	110	446	80	30
	444	140	230	447*	570	130
	445	230	110	448	200	100
Frozen iliac bone	429	95	80	432	350	140
	430	275	130	433	150	70
	431	425	400	434	125	60
Freeze-dried iliac bone	414	155	160	417	200	160
	415	140	150	418	170	120
	416	155	125	419	95	70
Merthiolated iliac bone	478	160	90	481	150	90
	479	230	160	482	150	90
	—	—	—	483	240	140
Fresh homologous cortical bone blocks	465	270	170	468	210	210
	466	210	140	470	170	130
Fresh homologous cortical bone chips	472	310	210	475	370	160
	473	210	130	476	200	125
	474	180	90	477	175	110

*Excluded from the statistical analysis.

the marrow from the treated iliac homografts. One rabbit, which should have received a second-set graft of merthiolated bone, died eleven days after the insertion of the first-set homograft. Two rabbits, each of whom should have received a second-set cortical bone block, died before the second graft could be inserted.

HISTOLOGICAL TECHNIQUES

The recipient animals were killed by the intravenous injection of sodium pentobarbitone. The rabbits which received first-set grafts were killed five or eight days after operation; those animals which received second-set grafts were killed two or four days after the insertion of the second graft. The first regional (parotid) lymph nodes draining the grafts were removed together with the contralateral control nodes. The nodes were weighed and the weight increment of the node on the side draining the graft was calculated. The bone grafts were also removed but their microscopical appearances will not be described in the present paper. The fixation, processing and staining of the material were similar to that described in an earlier work (Burwell and Gowland 1961).

STERILITY TESTS

A sample of each bone tissue used in the investigation was taken for aerobic and anaerobic culture. All specimens were found to be bacteriologically sterile with the exception of: 1) the specimen from the first-set homografts of cortical bone which was found to be contaminated with a coagulase-positive staphylococcus; and 2) the specimens from five of the six second-set iliac autografts which yielded growths of either *Neisseria catarrhalis*, *proteus vulgaris* or an aerobic spore-forming bacillus.

OBSERVATIONS

WEIGHT CHANGES

Tables I and II show the weights of the first regional lymph nodes from the grafted and control sides.

Lymph nodes draining first-set bone grafts—Figure 1 shows the means, and standard errors, of the combined weight increments of lymph nodes removed five and eight days after the insertion of various first-set bone grafts.

FIG. 1

Weight increments in milligrams (expressed as means and standard errors) of the first regional lymph nodes removed five and eight days after the insertion of first-set tissue grafts into their drainage areas. The grafts include the fresh autografts and the fresh homografts of bone, as indicated.

It was shown previously (Burwell and Gowland 1961) that first-set homografts of fresh marrow-containing iliac bone evoked a highly significant weight increase of the first regional lymph nodes as compared with first-set autografts of fresh marrow-containing iliac bone ($P < 0.001$). Moreover the red marrow was shown to be the principal antigenic component of such fresh homologous iliac bone. The present experiments show that the mean weight increment of the lymph nodes draining the homografts of marrow-free iliac bone is not significantly different from the mean weight increment of lymph nodes draining the autografts of marrow-free iliac bone ($0.1 < P < 0.2$). Neither is there a significant difference between the mean weight increment of the lymph nodes draining the homografts of cortical blocks and those draining the autografts of cortical blocks ($0.1 < P < 0.2$). However, in the experiments using cortical chips the difference between the mean weight increment of the lymph nodes draining the homografts and that of those draining the autografts approaches significance ($0.05 < P < 0.1$); with a larger number of animals this difference might have been significant.

Lymph nodes draining second-set bone grafts—Figure 2 shows the means, and standard errors, of the combined weight increments of lymph nodes removed two and four days after the insertion of the various second-set bone grafts.

The second-set homografts of fresh marrow-containing iliac bone evoke a highly significant weight increase of the first regional lymph nodes compared with that of lymph nodes draining second-set autografts of fresh marrow-containing iliac bone ($P < 0.001$). These figures have been evaluated from findings previously published (Burwell 1962a).

The following statistical analysis has been undertaken to determine if the various grafts are less antigenic than homologous iliac bone. The combined mean weight increment of lymph nodes removed two and four days after the insertion of second-set homografts of fresh marrow-containing iliac bone have been used for statistical comparison (*t*-test) with the combined mean weight increments of the lymph nodes draining the various second-set grafts. The results of this analysis are as follows. *Fresh bone*—Autografts of iliac bone ($P < 0.001$); homografts of marrow-free iliac bone ($P < 0.001$); homologous fresh cortical blocks ($P < 0.001$);

FIG. 2

Weight increments in milligrams (expressed as means and standard errors) of the first regional lymph nodes removed two and four days after the insertion of the various second-set bone grafts into their drainage areas, as indicated.

homologous fresh cortical chips ($0.4 < P < 0.5$). *Treated bone*—Boiled bone ($0.001 < P < 0.01$); frozen bone ($0.01 < P < 0.02$); freeze-dried bone ($P < 0.001$); merthiolated bone ($P < 0.001$).

In an attempt to establish whether any residual antigenicity remained within the various grafts, the following statistical analysis was performed. The combined mean weight increments of lymph nodes previously sensitised to a fresh homograft of marrow-containing iliac bone and removed two and four days after the insertion of a second-set iliac autograft into each of their drainage areas have been compared statistically (*t*-test) with the combined mean weight increment of lymph nodes draining a variety of second-set grafts. The results of this analysis are as follows. *Fresh bone*—Homografts of marrow-free iliac bone ($P > 0.9$); homologous fresh cortical blocks ($P > 0.9$); homologous fresh cortical chips ($0.3 < P < 0.4$). *Treated bone*—Boiled bone ($0.7 < P < 0.8$); frozen bone ($0.2 < P < 0.3$); freeze-dried bone ($0.02 < P < 0.05$); merthiolated bone ($0.1 < P < 0.2$).

These results suggest: 1) that fresh homologous cortical chips are possibly antigenic; 2) the fresh homologous cortical blocks and fresh marrow-free homologous iliac bone are not demonstrably antigenic; 3) that the use of a more sensitive test-system than an unsensitised lymph node (Burwell and Gowland 1961) to assess bone antigenicity, namely that of a lymph node previously sensitised to donor tissue, does not reveal any residual antigenicity in grafts of homologous marrow-containing iliac bone treated by boiling, freezing (-20 degrees Centigrade), freeze-drying or by immersion in merthiolate solution for six weeks.

HISTOLOGICAL OBSERVATIONS: THE REGIONAL LYMPH NODES

First-set bone grafts. *Fresh homologous cortical bone blocks*—There was slight hyperplasia of the diffuse cortical lymphoid tissue of each node draining these grafts. The content of large and medium lymphoid cells in the cortices appeared normal in four of the six lymph nodes. But in two lymph nodes (452, 453) there was a considerable increase in the number of pyroninophilic lymphoid cells in an activated sector of each node; the distribution of these cells was peripheral in the one node, and was throughout the activated sector in the other node. The germinal centres of all the nodes resembled those of their respective control nodes. The medullary trabeculae, in general, were slightly thickened and contained mainly small lymphocytes and mature plasma cells. The medullary sinuses of all the nodes contained small lymphocytes, macrophages and a few cells having pyroninophilic cytoplasm.

Fresh homologous cortical bone chips—There was slight hyperplasia of the diffuse cortical lymphoid tissue of each of these nodes. The content of large and medium lymphoid cells in the cortices appeared normal in all the nodes. The germinal centres appeared similar to those of their respective control nodes. The medullary trabeculae were slightly thickened and contained small lymphocytes and mature plasma cells. In the medullary sinuses were seen small lymphocytes, a few macrophages with ingested red blood corpuscles and occasional cells with pyroninophilic cytoplasm.

Fresh autologous cortical bone blocks—Each of these nodes resembled its respective control lymph node.

Fresh autologous cortical bone chips—The lymph nodes were similar in microscopical appearances to their respective control nodes.

Fresh autologous marrow-free iliac bone—The lymph nodes were similar in microscopical appearances to their respective control nodes.

Second-set bone grafts. *Fresh autologous marrow-containing iliac bone*—The lymph nodes were slightly larger than their respective contralateral nodes. In the cortices of the nodes the germinal centres were in general a little larger and slightly more numerous, and the diffuse cortical tissue was thicker, than in the control nodes. There was, however, no increase in the number of large and medium lymphoid cells in the cortices of five of the six lymph nodes. One node (413), however, showed a slight increase of pyroninophilic lymphoid cells at the periphery of a sector of its diffuse cortical lymphoid tissue. The medullary trabeculae of each of the nodes were slightly thicker than those of their respective control nodes and contained small lymphocytes and mature plasma cells. The medullary sinuses of the nodes draining the grafts contained small lymphocytes, macrophages with ingested red blood corpuscles and a few cells having pyroninophilic cytoplasm.

Fresh homologous marrow-free iliac bone—The microscopical appearance of each of these lymph nodes was similar to most of the nodes draining first-set iliac homografts followed three weeks later by second-set iliac autografts and described above; but there was no increase in the number of large and medium lymphoid cells in the cortices of any of these lymph nodes. Macrophages with ingested red blood corpuscles were less numerous in the nodes draining the homografts of marrow-free iliac bone than in the nodes draining the second-set iliac autografts.

Boiled iliac bone—Five of the six lymph nodes were similar in appearance to most of the nodes draining the second-set iliac autografts described above. One node (448) was much larger than its respective contralateral node and showed peripheral collections of a moderate number of pyroninophilic medium-sized lymphoid cells in a sector of its diffuse cortical tissue.

Frozen iliac bone—Three of the six nodes showed accumulations of large and medium lymphoid cells in a sector of their cortices. The distribution of these cells tended to be peripheral, rather than throughout the activated sector of the cortex. The medullary trabeculae of these nodes

were thickened and contained mainly small lymphocytes and mature plasma cells. In the sinuses of the medullae small lymphocytes, medium lymphoid cells and occasional macrophages with ingested red blood corpuscles were seen. Three nodes (432, 433, 434) had microscopical appearances similar to most of the nodes draining the second-set iliac autografts described above.

Freeze-dried iliac bone—Five of the six nodes had microscopical appearances similar to most of the nodes draining the second-set iliac autografts described above. One node (418), however, showed considerable numbers of large and medium lymphoid cells distributed in a peripheral position in a sector of its diffuse cortical lymphoid tissue. The medullary trabeculae of this node were thickened and contained accumulations of large and medium lymphoid cells; its medullary sinuses contained small lymphocytes, medium lymphoid cells and a few macrophages.

Merthiolated iliac bone—Three of five lymph nodes showed peripheral accumulations of large and medium lymphoid cells in a sector of each of their diffuse cortical lymphoid tissue. The medullary trabeculae and sinuses of these nodes contained large and medium lymphoid cells. Two of the nodes (482, 483) were similar in appearance to most of the nodes draining the second-set iliac autografts described above.

Fresh homologous cortical bone blocks—The four lymph nodes in this group were each similar in microscopical appearance to most of the nodes draining the second-set iliac autografts described above.

Fresh homologous cortical bone chips—Each of the six lymph nodes showed a moderate increase in the number of large and medium lymphoid cells in the peripheral part of a sector of its cortex. The medullary trabeculae of the nodes were slightly thickened and contained small lymphocytes, mature plasma cells and a few medium lymphoid cells. In the sinuses of the medullae small lymphocytes, medium lymphoid cells and occasional macrophages were seen.

DISCUSSION

The findings described above will be related to previous knowledge concerning the antigenicity of fresh and treated homologous bone tissues.

FRESH HOMOLOGOUS BONE TISSUES

Marrow-free cortical bone—The presence of transplantation antigens in homografts of fresh cortical bone is suggested from the studies of Nisbet and his colleagues (Heslop, Zeiss and Nisbet 1960; Zeiss, Nisbet and Heslop 1960). These workers transplanted homografts of tibial cortex, which had been freed from periosteal and medullary tissues by thorough scraping, subcutaneously into rats. They found, firstly, that the cellular infiltration was more marked about homografts than about autografts; and secondly, that the vascularisation of homografts was significantly slower than that of autografts. Subsequent work has shown that homografts of fresh femoral cortical blocks and fresh femoral cortical chips, freed from soft tissues by thorough scraping and washing before being inserted subcutaneously into rats, evoked transplantation immunity (Burwell 1963). The latter observation is consistent with those of Nisbet and his colleagues, and demonstrates that the cortical bone of rats contains transplantation antigens.

In view of the conclusion that tissue antigens are present in rat cortical bone, it might be expected that homografts of rabbit cortical bone inserted subcutaneously into the ear would evoke immune responses in regional lymph nodes. But in the present work it is shown that in the cortices of ten of twelve lymph nodes draining first-set homografts of fresh cortical bone (blocks and chips) there was no increase in the number of large and medium lymphoid cells. The immune responses which were seen in two lymph nodes (452, 453) removed five days

after the insertion of homologous fresh cortical bone blocks into their drainage areas might be due either to the contaminating micro-organism (a coagulase-positive staphylococcus) or to a histo-incompatibility that was not present in the other ten rabbits. The insertion for periods of two to four days of fresh homologous cortical bone blocks into the drainage areas of lymph nodes previously sensitised to donor tissue failed to cause any cytological change suggestive of an immune response. But when fresh homologous cortical bone chips instead of blocks were similarly transplanted a secondary response was found in all of the six lymph nodes studied. Thus it is concluded that fresh homologous cortical bone of the rabbit contains transplantation antigens. The failure of the blocks of fresh homologous cortical bone to evoke a secondary response in the draining lymph nodes is probably due to the smaller surface area of the bone tissue from which transplantation antigens could diffuse into the tissues of the host.

Tissue antigens in cortical bone could probably be demonstrated more readily after heterologous transplantation than in homologous systems. In this connection Curtiss and Herndon (1955, 1956) showed that dog cortical bone, from which the marrow had been mechanically removed before the graft was inserted intramuscularly into rabbits, evoked the production of haemagglutinins. They concluded that the agglutinating antibodies may have developed as a result of an antigenic stimulus in the bone tissue itself. But Weinberg, Makin, Nelken and Gurevitch (1959) were unable to find the blood group antigens A and B in thoroughly washed human cortical bone.

Marrow-free cancellous bone—The antigenic properties of fresh homologous cancellous bone are known less securely than those of cortical bone. In a previous study (Burwell and Gowland 1962) it was found that first-set homografts of fresh marrow-free iliac bone did not usually produce cytological evidence of a primary response in lymph nodes draining the grafts. In the present work it is shown that similar homografts inserted into the drainage areas of lymph nodes previously sensitised to donor tissue also failed to evoke a detectable secondary response in the regional lymph nodes.

In experiments with rats (Burwell 1963) it was reported that homografts of fresh marrow-free iliac bone inserted subcutaneously did not evoke transplantation immunity. However, the histological examination of such homografts removed three weeks after their subcutaneous transplantation showed an impairment of new bone formation similar to that seen in homografts of bone tissues which did evoke transplantation immunity. At first sight this histological finding suggests that the immunity elicited by homografts of fresh marrow-free iliac bone, while too feeble to be detected by a "skin test," is sufficiently strong to impede the proliferation of newly forming homologous bone. But because in these experiments the action of natural cytotoxins and metabolic differences between the foreign graft and the host could not be excluded, the presence of transplantation antigens in fresh homologous marrow-free iliac bone has still to be detected indisputably.

Curtiss and Herndon (1955, 1956) using a heterologous system have, however, detected haemagglutinins in rabbits which had received intramuscular transplants of minced-and-washed iliac bone from dogs. Moreover Weinberg *et al.* (1959) found the blood group antigens A and B in thoroughly washed human cancellous bone.

TREATED HOMOLOGOUS BONE TISSUES CONTAINING MARROW

Boiled iliac homografts—The evidence suggests that in homografts of boiled marrow-containing iliac bone the iso-antigens do not remain immunologically active, for 1) such iliac homografts inserted subcutaneously into rats did not evoke any detectable change in the sensitivity of the host to donor tissue (Burwell 1963); 2) first-set homografts of boiled iliac bone inserted subcutaneously into the rabbit's ear did not elicit cytological evidence of a primary response in any one of the six lymph nodes studied (Burwell and Gowland 1962); and 3) in the present

paper it is shown that second-set homografts of boiled iliac bone inserted subcutaneously into the drainage areas of lymph nodes previously sensitised to donor tissue did not evoke evidence of a secondary response in five of six lymph nodes. The one lymph node (448) which did show an immune response should probably be discounted, because 1) one of four lymph nodes removed twenty-one days after the insertion of first-set fresh marrow-containing iliac homografts into their drainage areas had peripheral collections of large and medium lymphoid cells in a sector of its cortex (Burwell 1962a); and 2) one of six lymph nodes draining the iliac autografts inserted into the drainage areas of lymph nodes previously sensitised by an iliac homograft showed cytological evidence of an immune response.

Frozen iliac homografts—There is evidence suggesting that certain tissue antigens remain immunologically active in bone grafts which have been treated by freezing in air at -15 to -37 degrees Centigrade before being inserted into a homologous host.

Bonfiglio *et al.* (1955) transplanted fresh and frozen homografts obtained from ulnar shafts to defects prepared in the ulnae of rabbits. They found that the inflammatory reaction about the fresh homografts was most intense two and four weeks after their insertion; the cellular reaction around the frozen homografts, though less intense initially, persisted for periods up to twelve and eighteen weeks. In their conclusion these workers implied that such frozen bone grafts contain transplantation antigens, but as they omitted any description of the marrow content of their grafts it is not possible to apportion this antigenicity to bone or to marrow. Bonfiglio and his colleagues also found that the inflammatory infiltration about such frozen bone homografts was markedly diminished by previous immunisation of the host with injections of a bone extract prepared from donor tissue. In a previous discussion of this work it was concluded that this diminished infiltration should be regarded as an immunological enhancement effect (Burwell 1963). Urist *et al.* (1958) inserted homografts of fresh and frozen bone (compacta with spongiosa) into the upper tibial metaphysis of young rabbits. They found that the infiltration of lymphocytes and plasma cells around their frozen grafts was equally as great as that about two similar grafts of fresh bone removed twenty-eight and forty-two days after their insertion into similar sites. Hancox *et al.* (1961) implanted frozen homografts of cancellous bone into the left femoral condyle of each of nine sheep. In the grafts, recovered after thirty-six days, they detected “. . . occasional large gatherings of plasma cells and lymphocytes, suggesting a lingering antigenic response.” Again, because both Urist *et al.* and Hancox *et al.* omitted any description of the marrow content of their grafts, it is not possible to apportion this antigenicity to bone or to marrow.

In recent studies it was found that homografts of frozen (-20 degrees Centigrade) marrow-containing iliac bone implanted subcutaneously into rats failed to evoke transplantation immunity (Burwell 1963). Moreover first-set homografts of frozen marrow-containing iliac bone inserted subcutaneously into the rabbit's ear did not evoke detectable evidence of a primary response in the regional lymph nodes (Burwell and Gowland 1962). But in the experiments reported in the present paper it is shown that similar frozen iliac homografts transplanted into the drainage areas of lymph nodes previously sensitised to tissue from the same donor elicited cytological evidence of a secondary response in three of six lymph nodes. It seems unlikely that these immune responses were caused by micro-organismal contaminants, because the specimen of frozen bone was found to be bacteriologically sterile. Moreover the possibility that these immune responses were due to a lingering release of transplantation antigens from the first-set homografts is improbable because 1) only one of four nodes removed twenty-one days after their insertion of first-set fresh marrow-containing iliac homografts into their drainage areas showed peripheral collections of large and medium lymphoid cells in a sector of its cortex (Burwell 1962a); and 2) only one of six lymph nodes draining the iliac autografts inserted into the drainage areas of lymph nodes previously sensitised by an iliac homograft showed cytological evidence of an immune response. Hence the conclusion seems justified that the second-set homografts of frozen iliac bone have evoked a secondary (or

anamnestic) response in three of the lymph nodes. Furthermore, since the principal antigenic component of a fresh iliac homograft is the red marrow, the conclusion implies that some transplantation antigens have persisted in the red marrow of such grafts after storage in air at -20 degrees Centigrade for one week. It is possible, however, that longer periods of storage would have diminished this antigenicity. Why three of the lymph nodes (432, 433, 434) did not have a similar immune response is not clear, but it might be that there was a weaker histo-incompatibility between the donor tissue and these three animals, or that the tissue antigens of these three grafts had been more rapidly neutralised by freezing.

Freeze-dried iliac homografts—Freeze-drying has been shown to destroy the iso-antigens (T-antigens) of homografts which evoke the production of transplantation immunity; the H-antigens which elicit the production of humoral antibodies are relatively stable to this form of physical treatment (Medawar 1959). Hence it might be expected that H-antigens would remain immunologically active in homografts of freeze-dried marrow-containing iliac bone. More recent work (Brent, Medawar and Ruszkiewicz 1961), however, has suggested that the distinction between H-antigens and T-antigens is false. The immunologically-determinant group of each may be identical, the T-form having some extra-attachment, perhaps a lipid or a lipoprotein, which excites a predominantly cellular response (Medawar 1960).

Chalmers (1959) inserted homografts of freeze-dried marrow-containing iliac bone intramuscularly into rats; three weeks later skin homografts removed from the respective donors were applied to each of the recipients. It was found that the mean survival time of these skin homografts was significantly longer than that of other skin homografts applied to normal rats, an observation which has recently been confirmed (Burwell 1963). In a previous paper it was suggested that such an enhancement effect might be caused by the persistence of tissue antigens within the dead marrow of the grafts (Burwell 1963).

The implantation of first-set homografts of freeze-dried marrow-containing iliac bone subcutaneously into the rabbit's ear did not evoke detectable evidence of a primary response in the regional lymph nodes (Burwell and Gowland 1962). Likewise the insertion of second-set homografts of similar grafts into the drainage areas of lymph nodes previously sensitised to donor tissue, did not elicit evidence of a secondary immune response in five of the six lymph nodes. The one lymph node (418) which did show cytological evidence of a secondary response should again be discounted for the same reasons as discussed above (*see* boiled iliac homografts).

The reason why the second-set homografts of frozen iliac bone commonly elicit a secondary response in sensitised lymph nodes and the second-set homografts of freeze-dried iliac bone do not, is not clear. But it is possible that freezing and drying is a more efficient method of destroying transplantation antigens than is freezing and thawing of the tissue. However, it is also conceivable that the β -propiolactone (BPL) which was used to sterilise the bone grafts before the freezing and drying may have caused a chemical alteration of the iso-antigens and so prevented an immune response in sensitised lymph nodes. In this connection there is recent work showing that BPL may indeed alter the antigenicity of tissues which have been chemically sterilised by this agent, for Gowland and Burwell (1962) have found in rabbits that though the injection of freeze-dried reconstituted autologous serum does not elicit the production of serum antibodies, the injection of BPL-treated freeze-dried reconstituted autologous serum evokes the formation of precipitating antibodies.

Merthiolated iliac homografts—The evidence concerning the antigenicity of homografts of merthiolated marrow-containing iliac homografts is conflicting. Burwell and Gowland (1962) found that first-set homografts of such grafts inserted subcutaneously into the rabbit's ear failed to produce cytological evidence of a primary response in any of the six lymph nodes studied. But in the present paper it is shown that homografts of merthiolated iliac bone inserted subcutaneously into the drainage areas of lymph nodes previously sensitised to donor tissue elicited secondary responses in three of five nodes. This finding suggests that certain tissue

antigens may remain immunologically active in the marrow of iliac bone grafts treated by immersion in 1:1,000 aqueous merthiolate solution for six weeks. Further investigation of the antigenicity of merthiolated bone is desirable.

SUMMARY

1. The antigenicity of homologous cortical and cancellous bone has been investigated in eighty-four rabbits.
2. The primary immune responses which occur in lymph nodes draining homografts of fresh tissues (Burwell and Gowland 1961, 1962) have been used as a histological indicator of the antigenicity of fresh homologous cortical bone freed from soft tissues.
3. The secondary immune responses which occur in lymph nodes draining homografts of fresh marrow-containing iliac bone (Burwell 1962*a*, *b*) have been used also as a histological indicator of the antigenicity of homografts of 1) fresh cortical bone freed from soft tissues, 2) fresh marrow-free iliac bone, and 3) marrow-containing iliac bone treated by boiling, freezing, freeze-drying and merthiolate solution.
4. It is found that whereas fresh homologous cortical bone fails usually to produce cytological evidence of a primary response in the regional lymph nodes, fresh homologous cortical bone chips inserted into the drainage areas of lymph nodes sensitised previously to donor tissue evoke constantly cytological evidence of a secondary response.
5. Fresh homologous marrow-free iliac bone inserted into the drainage areas of lymph nodes sensitised previously to donor tissue does not produce detectable evidence of a secondary response.
6. Homografts of boiled marrow-containing iliac bone do not elicit a secondary response in lymph nodes previously sensitised to donor tissue.
7. Previous work has shown that homografts of frozen (—20 degrees Centigrade) marrow-containing iliac bone do not evoke a primary response in lymph nodes draining such grafts. In the present work it is shown that similar frozen homografts inserted into the drainage areas of lymph nodes previously sensitised to donor tissue evoked a secondary response in three of six lymph nodes.
8. Homografts of freeze-dried marrow-containing iliac bone fail usually to evoke a secondary response in lymph nodes sensitised to donor tissue.
9. Homografts of marrow-containing iliac bone treated by immersion in merthiolate solution before being inserted into the drainage areas of lymph nodes previously sensitised to tissue from the donor elicited a secondary response in three of five lymph nodes.
10. Knowledge concerning the antigenicity of fresh and treated homologous bone is discussed in the light of recent work.

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