STUDIES IN THE TRANSPLANTATION OF BONE

IV. The Immune Responses of Lymph Nodes Draining Second-set Homografts of Fresh Cancellous Bone

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In a previous paper it was reported that a fresh homograft of cancellous bone implanted subcutaneously into the rabbit's ear causes evidence of an immunological response in the regional lymph node draining the graft (Burwell and Gowland 1961). The lymph nodes showed increased numbers of pyroninophilic large and medium lymphoid cells in a sector, activated through afferent lymphatics by tissue antigens from the graft. A relationship was suggested between the greatest production of such cells in the first regional lymph node, the invasion of the graft bed with small lymphocytes and the inhibition of new bone formation by the homograft.

Medawar (1944) found that first-set skin homografts (in high dosage) in rabbits had a median survival time of 10.4 ± 1.1 days. Furthermore, second-set homografts transplanted to a freshly prepared raw area on the opposite side of the thoracic wall sixteen days after a rabbit had received first-set skin homografts (in high dosage) from the same donor, had a median survival time of 6.0 ± 0.6 days. Medawar concluded that the accelerated rejection of second-set skin homografts was consistent with an actively acquired immune reaction. These experiments with rabbits have been confirmed by Medawar and his colleagues using inbred strains of mice (Billingham, Brent and Medawar 1954). They found also that the principal immunological response to a first-set skin homograft occurs in the regional lymph nodes draining the graft.

However, it is not known whether the rejection of a second-set homograft of skin is caused entirely by sensitised lymphoid cells which have persisted after the primary immune response in the regional lymph nodes has subsided, or is contributed to by a secondary immune response in the lymph nodes draining the second graft (Hildemann 1959; Brent and Medawar 1959; Medawar 1959; Hildeman, Peters and Thaxter 1960; Steinmuller 1960).

Though the appearance of pyroninophilic cells in lymph nodes draining first-set skin homografts has been described by many workers (Gallone, Radici and Riquier 1952; Scothorne and McGregor 1955; Radici and Piredda 1957; Burwell, Smiddy and Parsons 1962), the responses of lymph nodes draining second-set homografts of skin have not been reported. Orthotopic homologous skin is not however an ideal tissue with which to investigate secondary immune responses in lymph nodes, because the application of a second-set skin homograft to the drainage area of the lymph node might evoke changes in a node modified, not only by tissue antigens from the first graft, but also by antigens from a micro-organismal flora consequent upon the rejection and scab formation of the first graft. Better experimental conditions for a study of secondary responses in lymph nodes are attained if the homologous tissue is transplanted to a subcutaneous site so that primary healing of skin over the first graft occurs.

Craigmyle (1958) showed that a first-set homograft of cartilage transplanted to the subcutaneous tissues of the rabbit's ear did not evoke any definite change of weight or cytological detail in the draining lymph node. When, however, four days after the implantation of diced homologous cartilage into one ear of each of six rabbits, a second implant of diced cartilage from the original donor was made into the same ear, a moderate increase of weight of the regional node occurred and in most cases large pyroninophilic cells were found within the cortices of the nodes.

Because homologous cartilage is antigenically weak and does not excite a primary immune response in the draining lymph nodes, it is clearly unsuitable for a study of the secondary responses of lymph nodes to tissue antigens. Homologous red bone marrow is, however, highly antigenic (Burwell and Gowland 1962). In the present investigation fresh cancellous bone containing red marrow is used to study the immune responses of lymph nodes draining second-set homografts inserted into the subcutaneous tissues of the rabbit's ear.

It is shown that not only does a secondary immune response occur in lymph nodes draining second-set homografts of fresh cancellous bone, but the reaction is earlier and is of greater magnitude than that of the primary response. The distribution of the large and medium lymphoid cells in the cortices of the nodes is also different in such primary and secondary immune responses. A preliminary report of these findings has been published (Burwell 1961a).

MATERIAL AND METHODS

Sixty adult male rabbits weighing 2 to 3.6 kilograms were used in the investigation. The five donor animals were of Albino strain and all the recipients were Flemish giants.

Operative procedure—The grafts of fresh cancellous bone, each weighing 95 to 100 milligrams, were obtained from the ilium and prepared as described previously (Burwell and Gowland 1961). The first-set grafts were inserted into the subcutaneous tissues of the left ear on the ventral aspect two centimetres from the external auditory meatus; three weeks later a second-set graft, obtained from the same rabbit as the first-set graft, was inserted into the subcutaneous tissue of the ear proximal to the first graft and about one centimetre from the external auditory meatus. The skin wound over each graft was carefully apposed with one suture of braided nylon.

The thirty animals which received two homografts of cancellous bone were killed at one, two, three, four, five, six, eight, fourteen or twenty-one days after the second operation. The fifteen rabbits each receiving two autografts were killed at two, four, six, eight, fourteen or twenty-one days after the second-set grafts were inserted.

A further nine rabbits which received only first-set homografts of fresh iliac cancellous bone were killed at one, three or twenty-one days after operation. One rabbit received a first-set autograft of cancellous bone and was killed twenty-one days later. These animals supplemented an earlier investigation and provided material for a comparison with the second-set responses at one, three and twenty-one days.

Histological techniques—The animals were killed by intravenous sodium pentobarbitone and the first regional lymph nodes draining the grafted and control ears were removed and weighed separately. The second regional lymph node on each side, the spleen and the bone graft were also removed from each rabbit. The lymph nodes and a segment of spleen (4 to 5 millimetres thick) were fixed in Carnoy's fluid. The bone grafts were fixed in 10 per cent neutral formal-saline and then decalcified with ethylenediamine tetra-acetic acid. The material was prepared for histological study as described in an earlier paper (Burwell and Gowland 1961). The sections of lymph nodes and spleens were stained with methyl green-pyronin and the bone grafts with haematoxylin and eosin, periodic-acid-Schiff and methyl green-pyronin.

OBSERVATIONS

WEIGHT CHANGES

The weights of the first regional lymph nodes from the side of operation and the control side, together with the weights of the spleens from the homografted and the autografted animals are shown in Tables I and II.

The mean weight increment (together with the standard error of each mean) of the first regional lymph nodes for each time interval studied after the insertion of first-set and second-set homografts and autografts of fresh cancellous bone into the subcutaneous tissues of the rabbit's ear are shown in Figures 1 and 2. These figures include the analyses obtained from seventy rabbits which had received either first-set homografts or first-set autografts of fresh cancellous bone and which were reported in an earlier paper (Burwell and Gowland 1961).

After the insertion of second-set homografts of cancellous bone the weight increment of the node draining the graft was more rapid and reached a higher level than after the insertion of first-set homografts of cancellous bone; the decline of the weight increment was more rapid

in second-set than in first-set responses. A statistical analysis in which cubic curves were fitted over the ranges of time one to fourteen days in the primary response and nothing to eight days in the secondary response revealed that the primary curve of weight increments rose to a maximum of 119 ± 17 milligrams at 7.9 ± 2.3 days and the secondary curve to a maximum of 189 ± 27 milligrams at 2.6 ± 0.4 days. The difference between the greatest weight increments of the lymph nodes draining the first-set and the lymph nodes draining the second-set homografts of cancellous bone is 70 ± 32 milligrams. The difference in time between these maxima after the insertion of the first-set and the insertion of the second-set homografts was 5.3 ± 2.4 days.

The insertion of second-set autografts produced a curve of weight increments of the first regional lymph nodes similar to that obtained for nodes draining first-set autografts (Fig. 2).

TABLE I
WEIGHTS OF THE FIRST REGIONAL LYMPH NODES AND OF THE SPLEENS:
PRIMARY RESPONSES

Serial number	Days after grafting	Weight of node on side of operation (milligrams)	Weight of node on control side (milligrams)	Weight of spleen (grammes)
Homografted animals				
422	1	105	95	1.48
423	1	105	75	1.03
424	1	155	120	1.48
425	3	200	100	1.12
426	3	190	110	0.92
427	3	125	90	1.14
383	21	260	140	1.90
397	21	180	200	1.23
403	21	120	120	1.76
Autografted animals				
406	21	160	110	1.25

The mean absolute weight and standard error of the control nodes were 146 ± 11 milligrams after second-set homografts and 129 ± 11 milligrams after second-set autografts; the difference between these means is not statistically significant. After first-set grafts of cancellous bone the mean absolute weight of the control nodes was 125 ± 6 milligrams after homografts (sixty rabbits) and 111 ± 8 milligrams after autografts (twenty rabbits); the difference between these two means is not significant. There was no statistical difference between the mean absolute weights of the control nodes removed from animals which received first-set or second-set homografts ($0.05 < P < 0.1$), or from animals which received first-set or second-set autografts ($0.5 < P < 0.6$).

The mean absolute weights and standard errors of the spleens from animals which received second-set homografts was 1.49 ± 0.12 grammes and from animals which received second-set autografts was 1.64 ± 0.08 grammes, a difference which is not statistically significant.

HISTOLOGICAL OBSERVATIONS

The regional lymph nodes

Lymph nodes draining first-set homografts inserted one, three and twenty-one days previously— The lymph nodes removed one day after the insertion of first-set homografts into their drainage areas were similar in histological appearance to their respective control nodes, apart from numerous macrophages containing ingested red blood corpuscles lying within the medullary sinuses. All three nodes removed three days after the insertion of first-set homografts contained accumulations of large and medium lymphoid cells throughout a sector of their cortices (Fig. 3); however, in one (427) of the three nodes the large and medium lymphoid

cells were not generally distributed throughout the activated sector of the cortex, but were found in foci, some of which were confluent. The medullary sinuses of all three nodes contained macrophages with ingested red blood corpuscles, small lymphocytes and a few large and medium-sized pyroninophilic cells; the medullary trabeculae consisted mainly of small

TABLE II
WEIGHTS OF THE FIRST REGIONAL LYMPH NODES AND OF THE SPLEENS:
SECONDARY RESPONSES

Serial number	Days after second graft	Weight of node on side of operation (milligrams)	Weight of node on control side (milligrams)	Weight of spleen (grammes)
Homografted animals				
340	1	260	140	2.04
362	1	260	130	0.70
363	1	210	100	1.08
342	2	280	140	1.84
356	2	340	110	1.31
357	2	340	140	1.22
341	3	230	100	1.90
359	3	360	210	1.65
360	3	360	90	0.73
60	4	340	170	1.16
346	4	230	65	2.33
352	4	250	80	1.75
353	4	240	90	0.55
347	5	165	125	1.34
354	5	450	170	1.70
355	5	300	120	0.85
349	6	245	235	2.90
361	6	210	240	0.89
364	6	260	100	0.70
62	8	160	100	0.83
376	8	250	220	1.77
378	8	180	140	1.65
384	8	160	70	0.77
63	14	270	110	2.06
379	14	210	280	1.75
380	14	Mainly fat	290	2.45
385	14	150	110	1.65
381	21	220	140	0.94
386	21	190	190	1.18
387	21	220	180	2.98
Autografted animals				
404	2	130	100	2.10
405	2	120	120	2.15
70	4	130	120	1.52
391	4	100	60	1.11
393	4	150	130	1.17
394	6	210	180	1.41
396	6	170	70	1.79
72	8	150	150	1.63
398	8	130	135	1.26
399	8	240	190	1.84
76	14	75	75	1.59
400	14	210	190	1.45
401	14	140	120	1.73
389	21	140	170	1.70
392	21	140	120	2.10

lymphocytes, mature plasma cells and occasional large and medium lymphoid cells. Medium lymphoid cells are round or ovoid in outline, about 10–12 μ in greatest diameter, and have large pale nuclei, one or more prominent nucleoli and strongly pyroninophilic cytoplasm.

Three of the four lymph nodes removed twenty-one days after the insertion of first-set homografts had histological appearances similar to their respective control nodes. One node

(383) was, however, considerably heavier than its control node, germinal centres of Flemming type were prominent and collections of large and medium lymphoid cells were found beneath the marginal sinus and on the medullary side of the cortical nodules; the medullary trabeculae of the lymph node were thickened and contained small lymphocytes, mature plasma cells and

FIG. 1

Graph to show the mean weight changes (with standard errors) of the first regional lymph nodes draining first-set and second-set homografts of fresh iliac bone (ninety rabbits).

FIG. 2

Graph to show the mean weight changes (with standard errors) of the first regional lymph nodes draining first-set and second-set autografts of fresh iliac bone (thirty-five rabbits).

a few large and medium lymphoid cells. A small amount of dead new bone was present in the graft which drained to this lymph node.

Control lymph nodes from animals receiving second-set homografts—The histological appearances of these control nodes resembled those of control nodes removed from rabbits which received first-set homografts of fresh cancellous bone into the subcutaneous tissues of the opposite ear (Burwell and Gowland 1961) (Fig. 4).

Lymph nodes draining second-set homografts inserted one to twenty-one days previously—The lymph nodes removed one day after the insertion of second-set homografts into their drainage area were enlarged compared with their respective control nodes (Fig. 3). The germinal centres were prominent, the diffuse cortical tissue was little thicker than in the control nodes, and there was no increase in the numbers of large and medium lymphoid cells. The medullae were, however, considerably larger than the medullae of either the control nodes or of the nodes draining first-set homografts of cancellous bone; the medullary trabeculae were thickened and contained mature plasma cells, small lymphocytes and occasional large and medium lymphoid cells. The medullary sinuses contained macrophages (with ingested red blood corpuscles), small lymphocytes and an occasional medium-sized pyroninophilic cell. The cells in the medullary sinuses were more widely separated than in nodes draining first-set homografts, and it appeared that some of the medullary enlargement was due to oedema.

FIG. 3

Camera lucida tracings of a section from each of six lymph nodes removed one, two and three days after the insertion of either first-set homografts (primary response) or second-set homografts (secondary response) of fresh iliac bone. The stippled areas indicate the sectoral distribution of large and medium lymphoid cells within the cortices of the nodes. (S.P. = superior pole; P.C.S.L. = peripheral collections of small lymphocytes; G.C. = germinal centre; M. = medulla). For further details see text.

Lymph nodes removed two, three, four, five, six and eight days after the insertion of second-set homografts into their drainage areas were larger than their control nodes (Fig. 5) and showed peripheral collections of large and medium lymphoid cells in the diffuse cortical tissue both beneath the marginal sinus and on the medullary sides of the germinal centres (Figs. 3, 8, 9 and 11). A sectoral distribution of these cells in the cortex was seen in nodes responding to second-set homografts, but in general was less marked than the sectoral distribution of these cells in nodes draining first-set homografts of cancellous bone. The diffuse lymphoid tissue of the cortices was thicker in nodes removed two to eight days after insertion of the second graft than in the control nodes. The distribution of the pyroninophilic large and medium lymphoid cells was, in general, peripheral within the cortices, and mitoses

were frequently seen in these cells. In certain sectors of some of the nodes, however, large and medium lymphoid cells were found throughout the whole thickness of their cortices. The germinal centres, mainly of Flemming type, became larger and more numerous on and after the fourth day, whereas they tended to disappear from lymph nodes draining first-set grafts between the fourth and the eighth days (Figs. 8 and 9). The medullae of nodes removed two to eight days after insertion of the second-set grafts were larger, and their medullary trabeculae

FIG. 4

Control lymph node showing germinal centres, moderately thick diffuse cortical tissue and poorly developed medullary trabeculae. ($\times 15$. Rabbit 342.)

were thicker, than in the control lymph nodes; large and medium lymphoid cells, small lymphocytes and mature plasma cells were found in the trabeculae. The medullary sinuses contained large and medium-sized pyroninophilic cells, small lymphocytes and, in nodes removed at two to five days, macrophages containing ingested red blood corpuscles.

The lymph nodes removed fourteen and twenty-one days after insertion of the second graft showed considerably more germinal centres in the cortices than did the control nodes; the germinal centres tended to be larger in the fourteen-day than in the twenty-one-day nodes (Fig. 9). The diffuse cortical tissue was slightly thicker in some of the nodes compared with their controls but the concentration of large and medium lymphoid cells was normal; collections of mature plasma cells were found on the medullary side of some germinal centres in certain of the nodes. The medullae of the nodes removed at fourteen and twenty-one days were larger than those of their respective control nodes and contained germinal centres; the thin medullary trabeculae contained principally mature plasma cells and small lymphocytes. The medullary sinuses contained small lymphocytes and occasional large and medium lymphoid cells.

The main histological differences between primary and secondary immune responses in lymph nodes draining homografts of fresh cancellous bone were as follows. 1) The appearance of large and medium lymphoid cells was earlier after second-set than after first-set homografts (Fig. 3). 2) In general the distribution of large and medium lymphoid cells in the cortex was peripheral in the secondary responses and was throughout the cortex in the primary responses (Figs. 10 and 11). 3) The germinal centres became larger and more numerous in nodes draining second-set grafts after the fourth day, whereas they tended to disappear in nodes draining first-set grafts between the fourth and the eighth day (Fig. 8). 4) The medullary enlargement was earlier in secondary than in primary immune responses (Fig. 3).

Second regional lymph nodes—The second lymph nodes on the side draining the graft were in general larger than their corresponding nodes on the opposite side. The diffuse lymphoid tissue of the cortex was thickened in some nodes, but without any increase in the numbers of large and medium lymphoid cells; the germinal centres appeared normal. The medullary

trabeculae appeared similar to those of the control nodes; the sinuses of the medullae contained small lymphocytes and in some nodes large and medium lymphoid cells and macrophages with ingested red blood corpuscles were seen.

First and second regional lymph nodes from animals receiving second-set autografts—The first regional lymph nodes differed only slightly from their control nodes (Figs. 6 and 7). The diffuse

FIG. 5

Lymph node removed two days after the transplantation of a second-set homograft of fresh iliac bone into its drainage area. The diffuse cortical tissue is thickened at the upper pole and on one side of the node. Germinal centres are present. The medullary trabeculae are hyperplastic. ($\times 15$. Rabbit 342.)

cortical tissue was a little thickened in nodes removed at four, six and eight days, but no increase in the numbers of large and medium lymphoid cells was detected. The germinal centres appeared normal in size, form and number. The medullary trabeculae in certain nodes were slightly thickened and consisted mainly of small lymphocytes and mature plasma cells: the medullary sinuses contained small lymphocytes and, in some of the four-day and six-day nodes, a few macrophages containing ingested red blood corpuscles.

The second regional lymph nodes had a similar histological appearance to their respective control nodes.

Spleens—Careful histological examination of the spleens did not reveal any difference between the spleens of animals receiving either second-set homografts or second-set autografts of cancellous bone. All the spleens appeared microscopically normal.

Bone grafts from animals receiving second-set autografts—The second-set autografts of cancellous bone removed two to twenty-one days after their insertion (Fig. 12) are similar in histological appearance to that described for first-set autografts of cancellous bone removed at the same time intervals (Burwell and Gowland 1961).

FIG. 6

FIG. 7

Figure 6—Control lymph node showing germinal centres, thin diffuse cortical tissue and poorly developed medullary trabeculae. ($\times 15$. Rabbit 404.) Figure 7—Lymph node removed two days after the transplantation of a second-set autograft of fresh iliac bone into its drainage area. The cortex consists of germinal centres and thin diffuse lymphoid tissue. The medullary trabeculae are moderately hyperplastic. ($\times 15$. Rabbit 404.)

The first-set autografts removed twenty-three to forty-two days after their insertion (obtained from animals which received second-set autografts on the twenty-first day) showed in general a progressive fibrovascular replacement of the necrotic marrow; in some of the later specimens small foci of healthy haemopoietic marrow were found. Some osteocytes survived in all the grafts; new bone formation was present in association usually with trabeculae and less frequently with the periosteal surface of the graft. The outer cortical surface of the bone occasionally showed marked erosion. The surrounding connective tissue was fibrous and contained small round cells, either in tiny foci or arranged between the collagenous bundles.

Bone grafts from animals receiving second-set homografts—Some osteocytes survived in most of the second-set homografts removed one to twenty-one days after insertion; generally the number of living osteocytes diminished with the increasing time intervals after implantation. No definite evidence of new bone formation was seen in any of the grafts. The marrow appeared necrotic. Some oedema was present in the connective tissues surrounding the three second-set grafts removed at one day; however, no differences were detected in the local inflammatory

FIG. 8

Camera lucida tracings of a section from each of six lymph nodes removed four, five and six days after the insertion of either first-set homografts (primary response) or second-set homografts (secondary response) of fresh iliac bone. For further details see Figure 3 and text.

FIG. 9

Camera lucida tracings of a section from each of six lymph nodes removed eight, fourteen and twenty-one days after the insertion of either first-set homografts (primary response) or second-set homografts (secondary response) of fresh iliac bone. For further details see Figure 3 and text.

response until the third day, when two of the three second-set grafts were more infiltrated with small round cells and eosinophils than were any of the first-set grafts removed at the same time interval. On the fourth day no differences were seen in the inflammatory responses

FIG. 10

The cortex of a lymph node removed five days after the insertion of a first-set homograft of fresh iliac bone into its drainage area. Note the distribution of the pyroninophilic large and medium lymphoid cells throughout the cortex. ($\times 130$. Ektachrome. Rabbit 185.)

FIG. 11

The cortex of a lymph node removed five days after the insertion of a second-set homograft of fresh iliac bone into its drainage area. Note the peripheral concentration of the pyroninophilic large and medium lymphoid cells. ($\times 130$. Ektachrome. Rabbit 347.)

between the second-set and the first-set grafts. After five days, however, there was a more prominent infiltration of small round cells in all the second-set than in the first-set grafts; these differences were present also at six days, but only in two of the three second-set grafts.

At eight days (Fig. 13) there were marked differences, the second-set grafts being surrounded by considerable infiltrations of small round cells with marked local fibrosis. In the second-set grafts removed fourteen and twenty-one days after insertion, the collections of small round cells were more localised by fibrous tissue than in second-set grafts studied at earlier time intervals, and generally the infiltration was greater about second-set than about first-set grafts.

The first-set homografts removed twenty-two to forty-two days after their insertion (obtained from animals which received second-set homografts on the twenty-first day) showed, in general, a progressive replacement of necrotic marrow by fibrovascular connective tissue. Some osteocytes survived in most of the grafts, especially in the cortex and in the sub-cortical trabeculae. New bone formation was found only in two grafts which were removed twenty-nine and forty-two days after implantation and was small in amount (Fig. 14). The connective tissue surrounding all grafts became increasingly fibrous the longer the interval after insertion; the small round-cell infiltration was not marked and tended to occur in loculi between the collagenous bundles.

DISCUSSION

The different behaviour of the mammalian antibody-forming system to an antigen met for the first time and the same antigen met a second time was established by von Dungern (1903). He showed that a second intravenous injection of a soluble antigen (crab plasma) into rabbits caused not only a greater rate of antibody formation and a higher ultimate titre, but also a longer duration of antibody production than after a single injection of the antigen. These findings have been confirmed and extended by many workers with a wide variety of antigens (Glenny and Südmersen 1921, Glenny 1925, Topley 1930, Barr and Glenny 1945, Burnet and Fenner 1949, *vide* Freund 1953 and McMaster 1961). The differences between primary and secondary immune responses to classical antigens are particularly evident when bacterial toxins or toxoids are used as the antigen (Burnet and Fenner 1949).

Previous work has shown that the formation of humoral iso-antibodies to transplants of homologous tissues follows the orthodox pattern of primary and secondary responses (Brent and Medawar 1959). Mitchison (1955), using an experimental technique, showed that secondary responses occur in lymph nodes draining homologous tumours in mice. The observations described above show that homografts of a normal tissue (marrow-containing cancellous bone) transplanted subcutaneously into the rabbit evoke a reaction in the regional lymph nodes having characteristics of a secondary immune response, namely a more rapid and greater weight increase than that which occurs in lymph nodes draining first-set homografts of a similar tissue.

At the present time most workers are agreed that under normal conditions of skin homografting the accelerated rejection of the second-set graft is principally due to immunity induced by the first-set graft (Brent and Medawar 1959, Medawar 1959, Steinmuller 1960). According to this belief the transplantation of the second-set homograft is simply a "skin test" which records the existence of a prevailing state of sensitivity to tissue from the donor animal. However, there is evidence that, under appropriate experimental conditions, a second-set graft may reawaken a secondary (or anamnestic) response which contributes to the destruction of the second graft (Hildemann 1959, Hildemann *et al.* 1960, Steinmuller 1960).

The finding, in the present work, of pyroninophilic large and medium lymphoid cells in lymph nodes removed two days after the insertion of fresh cancellous bone into their drainage areas suggests that this secondary response could cause, or at least contribute to, the infiltration of sensitised lymphoid cells necessary for rejection of the second graft, for the median survival time of second-set skin homografts (in high dosage) in the rabbit is 6.0 ± 0.6 days (Medawar 1944). However, this argument cannot be regarded as conclusive because the figure of 6.0 ± 0.6 days represents the time to the completion of graft breakdown and not to the

first signs of graft rejection (Medawar 1961). There seems to be little doubt, however, that lymphoid cells sensitised by the first-set graft do contribute to second-set graft rejection (Brent, Brown and Medawar 1958). These workers found that guinea-pigs which had rejected an initial skin homograft and were then injected intradermally with a cell-free extract made from the tissues of the skin donor developed a tuberculin type of inflammatory reaction after a latent period of only five to eight hours.

It is likely that the tissue antigens leaving a second-set graft gain direct access to the lymphatics of the graft bed, for in the absence of evidence that second-set homografts of skin acquire a lymphatic drainage, the rapid onset of the secondary response in the regional lymph nodes implies that the transplantation antigens enter lymphatics exposed during the surgical

FIG. 12

Second-set autograft of fresh iliac bone inserted into the subcutaneous tissue of the ear eight days previously. Note 1) the new bone formation especially in association with the medullary trabeculae of the graft; 2) the invasion of the red marrow by the surrounding connective tissue; and 3) the absence of inflammatory infiltration about the graft. ($\times 15$. Rabbit 398.)

preparation of the bed of the graft. This conclusion is consistent with observations on first-set homografts. Scothorne (1958) showed that anastomosis between the intrinsic lymphatics of a skin homograft and the lymphatics of the graft bed does not occur until the fifth day after grafting, a finding which has recently been confirmed by Odén (1961); but, as was shown above, the first cytological evidence of an immune response in the regional lymph nodes draining first-set homografts of marrow-containing cancellous bone occurs three days after the insertion of the graft. Hence the evidence suggests that the tissue antigens leaving both first-set and second-set homografts enter the host's lymphatics exposed during the preparation of the graft bed, and at a time before continuity is established between any lymphatics of the graft and the lymphatics of the tissues of the host. In this connection, however, in relation

to homografts of fresh marrow-containing cancellous bone, it must be remembered that lymphatic vessels have not been certainly observed in bone marrow (Clark 1958, Schutt and Gardner 1961).

A primary immune response is seen in all of the lymph nodes removed three days after the insertion of first-set homografts of cancellous bone into their respective drainage areas. In two of the three lymph nodes large and medium lymphoid cells are generally distributed throughout the reactive cortices, the other parts of their cortices being unreactive; the third node (427) shows large and medium-sized pyroninophilic cells in scattered foci in the diffuse lymphoid tissue of the cortex. This early appearance (at three days) of large and medium lymphoid cells in the cortices of lymph nodes draining tissue homografts is consistent with

FIG. 13

Second-set homograft of fresh iliac bone inserted into the subcutaneous tissue of the ear eight days previously. Note 1) the absence of new bone formation; 2) the necrotic marrow; and 3) the marked inflammatory infiltration about the graft. ($\times 15$. Rabbit 378.)

the observation of Mitchison (1955), who showed that, in mice, the competence of regional lymph nodes to transfer immunity caused by a tumour homograft begins three to five days after the implantation of the tumour into the drainage area of the nodes.

The regional lymph nodes draining second-set homografts of cancellous bone first show the appearance of large and medium lymphoid cells at two days; this is one day earlier than in the lymph nodes draining first-set homografts of cancellous bone. Large and medium lymphoid cells are present in the secondary response (as in the primary response—Burwell and Gowland 1961) within an activated sector of the node; this sectoral distribution of antibody-forming cells is however less marked in secondary than in primary responses, but why it should be so is uncertain. Within each activated sector in the primary response, large and

medium lymphoid cells are found distributed throughout the diffuse lymphoid tissue of the cortex (Fig. 10). In secondary responses, however, large and medium-sized pyroninophilic cells are found chiefly to lie peripherally, beneath the marginal sinuses and on the medullary sides of the cortical nodules (Fig. 11).

The significance of the different distribution of pyroninophilic cells within the activated sectors of the cortices of lymph nodes draining first-set and second-set homografts is not clear. An explanation for the peripheral localisation of large and medium lymphoid cells in the secondary response and their distribution throughout the activated sector of the cortex in the primary response could be that, after the implantation of the second-set homograft, some of the tissue antigens are neutralised *in situ* by circulating humoral or cell-bound

FIG. 14

First-set homograft of fresh iliac bone inserted into the subcutaneous tissue of the ear twenty-nine days previously. Note the new bone formation in association with the periosteal surface of the graft. ($\times 120$. Rabbit 376.)

antibodies; hence the regional lymph nodes would receive a smaller dose of tissue antigens than after a first-set graft and thus the secondary response would not extend throughout the cortex but would occur only beneath the marginal sinus of the node. This hypothesis implies that the first appearance of large and medium-sized pyroninophilic cells in the primary response should be peripherally in the cortex. There is insufficient material available at present to generalise with any certainty concerning the earliest localisation of pyroninophilic cells within lymph nodes draining first-set homografts, but in one node (427) removed three days after the implantation of a first-set homograft into its drainage area, collections of large and medium lymphoid cells were found in foci (some of which were confluent) in the diffuse cortical tissue. It is possible that the distribution of large and medium lymphoid cells throughout the activated sector of the cortex in the primary response, and the peripheral

distribution of these cells in the activated sector of the cortex in the secondary response, is due to a fundamental difference in the response of the lymphoid tissue of the host to a tissue antigen met for the first time and the same antigen met for a second time. This problem has been investigated and the results are reported elsewhere (Burwell 1962).

Leduc, Coons and Connolly (1955), using a fluorescent technique, studied the primary and secondary responses of the popliteal lymph node in the rabbit to injections into the foot of either diphtheria toxoid or human gamma-globulin. The primary response in the lymph node was characterised by the appearance on the fourth day of a very few antibody-containing cells situated on the medullary sides of the cortical follicles and in the medullary trabeculae. In lymph nodes responding to a second injection of the same antigen, antibody-containing cells appeared on the second day, and by the fourth day were scattered in much greater profusion but in the same situations as in the primary response. The finding that the "plasma cell reaction" in lymph nodes draining a wide variety of classical antigens is most completely developed in the medulla and on the edges of the cortical follicles has been reported by many workers (Oakley 1959).

After the transplantation of first-set homografts of skin (Burwell *et al.* 1962), iliac cancellous bone (Burwell and Gowland 1961), red marrow (Burwell and Gowland 1962) or renal tissue (Burwell 1961 *b*), the distribution of large and medium lymphoid cells throughout the activated sector of the cortex is different from the distribution of antibody-forming cells in lymph nodes draining a single injection of a classical antigen; first and second injections of classical antigens evoke an immune response in which antibody-forming cells are found in situations similar to those described above in lymph nodes draining second-set homografts of cancellous bone. The difference between the primary response of lymph nodes draining homografts of tissues and lymph nodes draining injected classical antigens is being investigated and will be more fully considered elsewhere.

The germinal centres in the activated sectors of the cortices of lymph nodes draining first-set homografts of cancellous bone generally disappear between the fifth and the eighth day after insertion of the graft; new germinal centres are not found until the tenth day after operation. However, in nodes responding to second-set homografts, germinal centres become markedly evident on and after the fourth day; this appears to be due to enlargement of existing, and the formation of new, germinal centres. In the present work no attempt has been made to quantitate the changes occurring in the lymph nodes, but a few workers using a quantitative camera lucida technique have examined the numbers of secondary nodules in popliteal lymph nodes in rabbits draining injections of a diphtheria anatoxin (Österlind 1938), staphylococci (Rudebeck 1932) and *Pseudomonas pyocyaneus* (Sjövall and Sjövall 1930). It was found that, in lymph nodes draining a single injection, the number of secondary nodules may either remain unchanged (Österlind) or increase (Sjövall and Sjövall, Rudebeck). In secondary responses the number of secondary nodules in the lymph nodes was found to increase after the third day to a maximum at the eighth day after a second injection of the antigen (Österlind).

The medullary enlargement of the lymph nodes draining second-set homografts of cancellous bone occurs earlier than that of nodes draining first-set homografts and probably represents a considerable proportion of the weight increase of the lymph nodes in the first three days after insertion of the second graft. The ratio of cortical to medullary tissue in the popliteal lymph nodes of rabbits responding to injections of diphtheria anatoxin, staphylococci or *Pseudomonas pyocyaneus* has been found to be relatively constant in both primary (Sjövall and Sjövall 1930, Rudebeck 1932) and secondary (Österlind 1938) responses.

An increased infiltration of inflammatory cells in the host's tissues surrounding homografts of bone inserted into animals previously sensitised to fresh tissue from the respective donor has been reported by a number of workers (Bonfiglio, Jeter and Smith 1955, Enneking 1957, Chalmers 1959). Bonfiglio and his colleagues transplanted homografts of ulnar shaft to a defect prepared in the ulna of rabbits. At intervals of one to twelve weeks the right forelimb

was amputated and a second-set graft was inserted into a defect prepared in the left ulna. They found that the inflammatory responses were greater about the second-set than about the first-set homografts at all the stages studied from one week up to and including twelve weeks. These workers also studied the inflammatory responses around homografts of frozen bone transplanted to an ulnar defect in rabbits, each of which had received, or was receiving, injections of an extract (with adjuvant) of bone pooled from eight donor rabbits. They demonstrated that although the titre of complement-fixing antibodies increased to a maximum at about five weeks after the initiation of immunisation, the inflammatory responses about the bone homografts diminished. It seems likely that this reduced inflammatory response about homografts of frozen bone inserted into rabbits previously sensitised by an extract of donor tissue is an immunological enhancement effect (Casey 1941; Snell, Cloudman, Failor and Douglass 1946; Snell 1956; Billingham, Brent and Medawar 1956; Kallis 1958). Though most of the knowledge of enhancement has been gained from studies of tumours, enhancement has been detected as an increased survival time of skin homografts applied to animals which had previously received either injections or implants of freeze-dried tissue (Billingham *et al.* 1956, Chalmers 1959). Enhancement may be due to the presence or production of circulating antibodies which in some as yet undetermined way minimises the ability of transplantation antigens to evoke a cellular immunity (Snell 1956, Billingham *et al.* 1956). This hypothesis is supported further by more recent experimental work (Snell, Winn, Stimpfling and Parker 1960).

Enneking (1957) exchanged a short length of femoral shaft between pairs of female Sprague-Dawley rats. Fourteen to twenty-one days later a similar exchange was performed between the opposite femora of the same pairs of animals. No difference was detected in the inflammatory responses in the tissues around the first-set and the second-set homografts until fifteen days after the respective operations, when in general the responses were greater in the sensitised than in the unsensitised hosts; this difference was still present at 120 days. Enneking (1957) studied also the inflammatory response in the tissues surrounding homografts of femoral shaft exchanged between rats which had been sensitised to each other's tissues, twenty-one to twenty-eight days previously, by parabiotic union. The inflammatory responses in these animals were also greater after the fifteenth day than the inflammatory responses which occurred around the first-set homografts of femoral shaft. Total body irradiation to one member of each pair of parabionts was found to annul the inflammatory response around the femoral homografts in over 95 per cent of the irradiated rats (Enneking, Gratch and Ethridge 1957; Enneking and Gratch 1959). The finding that the total body irradiation of rats sensitised to donor tissue by parabiosis impairs the inflammatory response around homografts of bone is in contrast to the work of Dempster, Lennox and Boag (1950), who found that total body irradiation in rabbits did not modify the survival time of second-set homografts of skin. Enneking and his colleagues revealed, furthermore, that a considerable proportion (almost 60 per cent) of the non-irradiated parabionts also had impaired inflammatory responses around their bone homografts. The interpretation of this additional finding is difficult, but Enneking and Gratch suggested that a humoral factor liberated from the irradiated parabionts may impair immune secondary responses in non-irradiated parabionts.

Chalmers (1959) examined the inflammatory response in the host's tissues surrounding homografts of iliac cancellous bone transplanted to muscle in rats which had been sensitised to the respective donor by a skin homograft three weeks previously. He found that the inflammatory reaction about bone homografts after sensitisation was more intense in the early stages than in the unsensitised host.

The vascularisation of first-set grafts of bone has been studied by several workers, the general conclusion being that homografts are less well vascularised than autografts of bone (Kiehn, Cebul, Berg, Gutentag and Glover 1952; Stringa 1957; Hammack and Enneking 1960; Zeiss, Nisbet and Heslop 1960). There is, however, no published work of attempts to

demonstrate a vascularisation of second-set homografts of either cortical or cancellous bone. Second-set homografts of skin are vascularised feebly, if at all (Lehrfeld, Taylor and Converse 1954). Zeiss and her colleagues (1960), using rats, studied the vascularisation of first-set homografts of fresh cortical bone which had been transplanted principally to subcutaneous sites. The vascularisation of the transplants was assessed by counting the vessels in thick sections of the grafts and statistically analysing the data. These workers revealed a distinct difference in the source of the blood supply of homografts removed on the fourteenth day and those removed on the thirty-fifth day after transplantation. In specimens removed at fourteen days the vascularisation of the grafts was mainly from the periosteal surface of the cortex, but at thirty-five days the main supply was derived from a medullary plexus of vessels. They suggested that the change in the source of the blood supply of their cortical grafts between the second and the fifth weeks after implantation might be due to an immune secondary response consequent upon the continued liberation of transplantation antigens from osteocytes which have continued to survive within the graft. However, a study of regional lymph nodes removed two to four weeks after the insertion of homografts of marrow-free cancellous bone into the subcutaneous tissues of the rabbit did not reveal any changes to suggest that transplantation antigens had been liberated from osteocytes surviving within the grafts (Burwell and Gowland 1962). A study of the vascularisation of homografts of bone implanted into animals which previously have been sensitised to tissue from the respective donor is clearly required.

Some osteocytes appear to survive in most of the second-set homografts of cancellous bone reported in the present paper. In general, the number of living osteocytes diminishes with increasing time after implantation, but there does not appear to be any difference between the proportion of osteocytes surviving in second-set and first-set grafts removed at similar time intervals after implantation. It seems likely that the continued survival of osteocytes, even in the presence of an immune secondary response, is due to the inability of host inflammatory cells to gain access to the bone cells through the canaliculi.

In the present series of experiments no new bone formation was found in any of the second-set homografts implanted subcutaneously into rabbits and removed three to twenty-one days after their insertion. This absence of new bone formation by the second-set homografts is in contrast to our findings with first-set homografts of fresh cancellous bone, in which most grafts, removed six to twenty-one days after implantation, showed evidence of a small amount of newly formed bone (Burwell and Gowland 1961). The lack of new bone formation by homografts of fresh cancellous bone transplanted subcutaneously for periods up to three weeks in rabbits recently sensitised to tissue from the respective donor is consistent with the reports of other workers who have sought for evidence of new bone formation by homografts implanted into animals recently sensitised to donor tissue (Chalmers 1959, Goldhaber 1961). Chalmers found that homografts of fresh iliac bone transplanted intramuscularly into normal rats have two phases, early and late, of new bone formation. In the early phase, which occurred in the first four weeks after transplantation, woven bone appeared at about the fourth day, continued to grow until eight days, and subsequently died. This early phase of new bone formation was abolished if the animal had been sensitised to donor tissue by a skin homograft three weeks previously. Goldhaber, using inbred strains of mice, found in normal animals that implants of neonatal homologous skull bone showed evidence of an early and extensive new bone formation; however, in mice previously sensitised to donor tissue by fragments of homologous spleen implanted subcutaneously, similar bone implants showed little if any attempt at new bone formation. These experimental observations in rabbits, rats and mice are consistent and suggest that it is an acquired immunity on the part of the host to the foreign tissue which prevents the early phase of new bone formation by bone transplanted at a later date from the respective donor; the corollary implies that the early phase of new bone formation is of graft rather than host origin (Chalmers 1959).

In the present experiments new bone formation of the late phase was seen in two first-set homografts which were removed twenty-nine and forty-two days after their subcutaneous insertion into the rabbit's ear. These grafts were excised eight and twenty-one days after a second graft had been inserted from the same donor. Hence in these two first-set transplants new bone was forming and surviving eight and twenty-one days respectively after the initiation of an immune secondary response, suggesting that this late phase new bone is of host rather than of graft type. Other workers who used animals previously sensitised to donor tissue have found little if any new bone formation of the late phase in association with their bone homografts (Chalmers 1959, Goldhaber 1961). In this connection it must be remembered that whereas in the writer's material new bone formation of the late phase is found in two first-set homografts which had not been exposed to a secondary immune response until twenty-one days after their insertion, in the experiments of other workers, which are to be discussed, new bone formation of the late phase was sought for in homografts of bone inserted into hosts at a time when they had already received a graft of donor tissue. Chalmers observed that whereas new bone formation of the late phase occurred in five of fourteen homografts of fresh iliac bone transplanted into normal rats for periods of four weeks or more, the insertion of similar grafts into rats sensitised to donor tissue by a skin homograft three weeks previously abolished the late phase of lamellar bone formation. Goldhaber found also in mice sensitised to donor tissue by three separate subcutaneous transplantations of homologous spleen fragments at two-weekly intervals, that little, if any, attempt at new bone formation occurred in association with grafts of homologous neonatal skull removed two, three or four months after transplantation. Hence it may be concluded that a previously existing state of sensitivity to donor tissue will impair the late phase of new bone formed in association with a bone homograft inserted into that recipient.

The origin of the late phase of new bone formed about homografts of fresh cancellous bone requires further consideration. Chalmers (1959) considered that the new bone which he observed in association with homografts of fresh and freeze-dried iliac bone inserted intramuscularly into normal rats for periods longer than four weeks was due to an inductive effect of the graft upon the local tissues of the host. Previous workers had reached a similar conclusion to explain the new bone which they found about implants of devitalised bone (Bridges 1959). However, as Urist (1960) pointed out, in such experiments it is not clear whether the implanted donor tissue or the injured tissue of the host bed was the inductor, particularly because Bridges and Pritchard (1958) have shown that muscular tissue itself has inductive properties for bone formation. Though there is now conclusive evidence that hypertrophic cartilage has the capacity to evoke new bone formation by induction (Urist and McLean 1952, Urist 1953, Lacroix 1951, 1961, Bridges and Pritchard 1958), unequivocal evidence for induction by bone tissue has been lacking (Ray 1956, Duthie 1958, Goldhaber 1958, Bridges 1959, Urist 1960). Recently, however, Goldhaber (1961) has produced direct experimental evidence to suggest that newly formed bone liberates a diffusible osteogenic inductor. He inserted homologous neonatal skull bone contained within millipore diffusion chambers subcutaneously into mice for periods of two, three and four months. He found, both in normal mice and in mice previously sensitised to donor tissue, that new bone formation occurred not only within the diffusion chambers (where as homologous bone it was protected from the immune response of the host), but also on the host side of the chamber. Goldhaber concluded that the newly formed bone appearing on the outer side of the filter was of autologous type formed in response to an osteogenic inductor diffusing from the new homologous bone laid down on the inner aspect of the filter. These experiments which suggest that newly formed bone contains a diffusible osteogenic inductor cannot as yet be regarded as entirely conclusive because the control experiment of seeking new bone formation on the outer side of empty diffusion chambers implanted into mice of the same strain and recovered at the same time intervals after implantation was not performed. The necessity for this control

experiment is made imperative by the finding of Selye, Lemire and Bajusz (1960) that mechanical factors alone can evoke new bone formation in the subcutaneous tissues of rats.

The evidence obtained from immunological studies discussed above enables considerable light to be shed upon inductive mechanisms of bone formation. There seems to be little doubt that an actively acquired immunity on the part of the host to donor tissue prevents the early phase of new bone formed by homografts of fresh bone inserted into such animals. In view of the conclusion that the late phase of new bone formed in association with bone homografts inserted into normal animals is of host origin, the absence of late bone formation by such grafts in sensitised hosts could be due to two mechanisms: 1) to the neutralisation of donor-specific inductive substances within bone tissue, which would have evoked new bone formation in the tissues of a proportion of normal hosts; or 2) to an abnormality of the host's tissue surrounding the homograft resulting from the increased local infiltration with inflammatory cells. At the present state of knowledge this problem cannot be resolved entirely, but certain observations of Chalmers (1959) are relevant to this discussion. He found that homografts of freeze-dried iliac bone inserted intramuscularly into normal rats without eliciting an early phase of bone formation evoked in three of fourteen grafts a late phase of newly formed bone; the inflammatory reaction was less intense and later in onset than with the fresh homografts. If, however, the freeze-dried grafts were inserted into rats sensitised to donor tissue by a skin homograft four weeks previously, the late phase of new bone formation was abolished, while the inflammatory reaction remained similar to that around freeze-dried homografts inserted into normal rats. These findings, which require to be confirmed, suggest that a mechanism other than cellular infiltration of the host's tissues is preventing the late phase of new bone formed by such grafts.

The question of the liberation of osteogenic inductors from transplants and implants of bone will be more fully considered in subsequent papers.

SUMMARY

1. The response of the first regional lymph node to a homograft of fresh iliac cancellous bone inserted subcutaneously into the rabbit's ear three weeks after the introduction of a similar graft from the same donor into the same ear has been investigated in thirty rabbits. Fifteen rabbits which received second-set autografts of cancellous bone have also been studied.
2. The insertion of second-set homografts of fresh marrow-containing cancellous bone evokes an immune secondary response in the lymph nodes draining the grafts.
3. The increase in weight of the first regional lymph nodes on the side receiving second-set homografts is more rapid and of greater magnitude than that of nodes draining first-set homografts of cancellous bone. Second-set autografts evoke weight changes in the draining nodes similar to those in nodes draining first-set autografts of cancellous bone.
4. The histological changes which occur in the lymph nodes draining the second-set homografts (secondary response) are described and compared with those occurring in lymph nodes draining first-set homografts of cancellous bone (primary response).
5. In the primary response the distribution of large and medium lymphoid cells is throughout an activated sector of the cortex of the lymph node (Burwell and Gowland 1961), but in the secondary response these cells are found peripherally within the activated sector of the node. In both the primary and the secondary responses large and medium lymphoid cells are found in the medullary trabeculae of the lymph nodes.
6. The differences between the primary response of lymph nodes draining a tissue homograft (cancellous bone) and the primary response of lymph nodes draining classical antigens, and reported by other workers, are described.
7. Knowledge concerning the inflammatory response in the tissues of the host surrounding homografts of fresh cortical and cancellous bone implanted into animals previously sensitised to tissue from the respective donor is reviewed.

8. The late phase of new bone formation by homografts of fresh cancellous bone is discussed in the light of immunological studies.

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