**A STUDY OF THE DEVELOPMENT OF CYTOTOXIC ANTIBODIES
PRODUCED IN RECIPIENTS OF XENOGRAFTS
(HETEROGRAFTS) OF ILIAC BONE**

RESULTS

The results of this experiment show clearly that sheep iliac bone with its red marrow is highly antigenic in rats. Its transplantation leads to the production of antibody against sheep transplantation antigens. The fact that antigenicity can be removed from some implants by washing out the marrow clearly indicates that in the present study the red marrow is an important immunogenic component of the graft. That this is not always the case is considered to indicate that attempts to wash the bone marrow out are not uniformly successful.

Rats receiving treated composite xenograft-autografts and examined at death—Rats implanted with variously treated composite xenograft-autografts were bled before grafting, two weeks after grafting and again at death. The results are shown in Table III. These rats were also used for an assessment of osteogenesis in the grafts (Salama *et al.* 1973).

TABLE III
THE NUMBER OF RATS SHOWING THE PRESENCE OF SERUM CYTOTOXIC ANTIBODY
AFTER GRAFTING WITH TREATED COMPOSITE XENOGRAFT-AUTOGRAFTS (CXAs)*

Type of graft	Weeks after grafting			
	2 weeks		6 weeks	
	Numbers	Percentage	Numbers	Percentage
Freeze-dried	3/10	30	4/9	44
Decalcified frozen . . .	12/19	63	5/9	56
Decalcified freeze-dried .	8/22	36	2/9	22
Kiel (partly deproteinised)	3/21	14	2/9	22
Oswestry (deproteinised) .	1/5	20	—	—
Fresh (marrow free)† .	5/8	62	—	—

* Composite xenograft-autograft into one paravertebral muscle and similarly treated iliac bone xenograft into the contralateral muscle.

† Results from Table I.

It was observed that, with the exception of rats receiving decalcified frozen composite xenograft-autografts, none of the groups of rats receiving treated grafts showed the same incidence of anti-sheep antibodies after two weeks as was found in rats receiving fresh washed bone grafts. The incidence of antibodies in rats receiving freeze-dried grafts was considerably less than that found in recipients of either the fresh or decalcified frozen grafts. The decalcified frozen grafts showed the same degree of reaction as was found in recipients of fresh marrow-free bone. Decalcification of the freeze-dried bone did not have any striking effect on its immunogenicity when compared with intact freeze-dried bone, although a slightly increased incidence of antibody was found.

As would be expected, the lowest incidence of antibody formation was found in rats receiving grafts containing deproteinised bone. However, a small number of the recipients did produce antibody. This indicates that some cellular remnants persist within the graft after its treatment. The partially deproteinised Kiel bone gave rise to rather fewer reactions than did the fully deproteinised Oswestry bone. There was only a small number of animals in the latter group however.

In those rats killed six weeks after implantation, the highest incidence of antibody was still seen in the group receiving decalcified frozen bone composite xenograft-autografts. The incidence of antibodies in the group receiving freeze-dried grafts had risen, whereas in animals

TABLE IV
THE PRESENCE OR ABSENCE OF SERUM CYTOTOXIC ANTIBODY IN RATS RECEIVING TREATED ILIAC COMPOSITE
XENOGRAFT-AUTOGRAFTS

Type of graft	Animal number	Days after grafting								
		0	6	8	14	21	28	42	49	56
Fresh* (washed)	1	—	—	—	—	—	—	+		
	2	—	+	+	+	+	+	+		
	3	—	—	—	+	+	+	—		
Frozen composite xenograft-autografts	4	—	—	—	+	+	—	+	+	+
	5	—	+	+	+	+	—	+	+	+
	6	—	—	—	—	—	—	+	—	—
	7	—	—	+	—	—	—	+	—	—
Freeze-dried composite xenograft-autografts	8	—	—	—	—	—	—	—	—	—
	9	—	—	—	—	—	—	+	—	—
	10	—	—	—	—	—	—	+	+	—
	11	—	—	+	+	+	+	+	+	+
Decalcified frozen composite xenograft-autografts	12	—	—	—	—	+	+	+	+	+
	13	—	—	—	+	+	—	—	+	+
	14	—	—	+	+	—	+	+	+	+
	15	—	—	+	+	—	+	+	+	+
Decalcified freeze-dried composite xenograft-autografts	16	—	—	—	+	+	+	Died		
	17	—	—	—	+	—	—	+	+	+
	18	—	—	—	—	—	—	+	—	—
	19	—	—	—	+	+	+	+	+	+
Kiel composite xenograft-autografts	20	—	—	—	—	—	—	—	—	—
	21	—	—	+	+	—	Died			
	22	—	—	—	—	—	—	—	—	—
	23	—	—	—	—	—	—	—	—	—
Oswestry composite xenograft-autografts	24	—	—	—	—	—	—	+	+	—
	25	—	—	—	—	—	—	—	—	+
	26	—	—	—	—	—	—	+	+	—
	27	—	—	—	—	—	—	+	+	—

* Data from Table II.

TABLE V
THE PRESENCE OF SERUM CYTOTOXIC ANTIBODY AFTER GRAFTING WITH FRESH WASHED ILIAC XENOGRAFTS
AND TREATED ILIAC COMPOSITE XENOGRAFT-AUTOGRAFTS

Type of graft	Number of animals	Absence of antibody response	Early antibody response (up to day 28)		Late antibody response only	Early transient and reappearance late
			Continuous	Short-lived		
Fresh washed . . .	3	0	1	1	1	0
Frozen	4	0	1	0	1	2
Freeze-dried . . .	4	1	1	0	2	0
Decalcified frozen .	4	0	2	0	0	2
Decalcified freeze-dried	4	0	3	0	1	—
Kiel	4	3	0	1	0	0
Oswestry	4	0	0	0	4	0

receiving decalcified freeze-dried grafts the proportion of rats with positive serum had fallen. Antibody was found in two out of nine rats receiving grafts of Kiel bone.

Longitudinal studies of rats receiving treated composite xenograft-autografts—Because of the differences in the incidence of antibody production observed in the previous experiment between animals examined early and late after grafting, it was considered desirable to gain some knowledge of the time-course of antibody production after implantation of treated bone xenografts. Small groups of rats were therefore studied at intervals during eight weeks after grafting. The results are shown in Table IV.

Only in the groups receiving freeze-dried or Kiel grafts were individuals found who never developed anti-sheep antibodies during the experiment. In all groups there was a variable pattern of antibody production. In fourteen of the animals shown in the Table antibody appeared within the first four weeks after implantation but was only short lived in three rats. In some individuals antibody appeared in the early period of the experiment and then disappeared, only to become evident again towards the end of the period of observation. Other rats did not develop antibody until late in the experiment. These trends are shown in Table V.

TABLE VI
THE IMMUNE RESPONSE TO XENOGRAFTS COMPARED WITH THE DEGREE OF OSTEOGENESIS
IN COMPOSITE XENOGRAFT-AUTOGRAFTS

Type of graft	Percentage rats with antibody 14 days after grafting	Osteogenesis score at*		Mean pooled† of new bone/grafted bone in composite xenograft-autografts
		2 weeks	6 weeks	
Fresh	75	—	—	—
Decalcified frozen	65.2	0.97	1.12	1.24
Decalcified freeze-dried	42.3	0.77	1.05	1.16
Freeze-dried	28.5	0.60	1.69	1.63
Oswestry	11.1	2.17	2.64	2.34
Kiel	16.6	0.91	1.33	1.38

* From data of Salama and colleagues (1973) Table I and †Table II.

From these observations the types of treated composite xenograft-autografts can be divided into two groups: 1) those that gave an early antibody response in most recipients—namely fresh washed, frozen, decalcified frozen, decalcified freeze-dried; and 2) those that gave a late response or no response in most recipients—namely freeze-dried, Oswestry, Kiel.

These findings are in general agreement with those reported in the previous experiment and would explain the higher incidence of antibodies in those recipients of freeze-dried grafts which were killed after the longer time interval.

The relationship between osteogenesis and antibody production—In order to investigate the possibility that the presence of antibody in the serum may affect the degree of osteogenesis in the composite xenograft-autografts, the results from the second and third series of experiments are combined and presented in Table VI, together with the two weeks, six weeks and overall mean scores for osteogenesis in corresponding groups taken from Tables I and II of the paper by Salama and colleagues (1973).

The greatest degree of early osteogenesis occurred in the Oswestry grafts, in the recipients of which a low frequency of antibody was found at two weeks.

The lowest scores for new bone formation after six weeks were obtained in rats receiving

decalcified frozen and decalcified freeze-dried composite grafts: these are the two groups in which highest incidence of early antibody formation was found. The Kiel grafts on the other hand showed a relatively low degree of osteogenesis at six weeks compared with fresh and freeze-dried grafts but their recipients had a lower incidence of antibody production. It is interesting that the recipients of Oswestry grafts still showed the highest score of osteogenesis after six weeks, at which time 75 per cent of the animals were showing anti-sheep antibodies in their serum (Table IV).

DISCUSSION

The types of treated iliac bone xenografts evaluated in the present study evoked a variable antibody response, directed against cellular antigens. Variability was evident not only in response to the different types of bone-graft material, but also in different animals to the same type of grafting material (Table IV).

The pattern of antibody response to the frozen decalcified bone xenografts was similar to that in the rats which received fresh washed iliac bone xenografts. It seems reasonable to conclude, therefore, that freezing has little or no effect on the immunogenicity of washed iliac bone xenografts. However, from the results presented above, it may be suggested that drying in the frozen state does impair the immunogenicity of bone xenografts; this may be due to a rising salt concentration in the cells during the evaporation of water (Elves 1972). The incidence of antibody production in recipients of freeze-dried decalcified grafts was somewhat higher than in recipients of intact freeze-dried grafts. Why this should be so is not clear, but it could be explained if the decalcification, by removing the mineral, made cellular antigens more accessible to the immunological mechanism.

Grafts of Oswestry bone caused a late antibody response in each of the four recipients. The claim that Oswestry bone is completely non-antigenic (Roaf 1969) cannot therefore be supported by the present results using sheep bone. They indicate that some cellular antigens are present in such "fully-deproteinised" bone xenografts. It is probable therefore that Oswestry bone (extracted with ethylenediamine and hydrogen peroxide) contains residual protein, but we are not aware of any chemical analysis which has examined this possibility (see Kershaw 1963). It is known, however, that "anorganic" bone (ethylenediamine-extracted) contains some residual nitrogen (Losee and Hurley 1956, Wheeler and Hyatt 1960). It may be that there is also residual nitrogen in the Oswestry iliac bone, which may reside in marrow cells incompletely removed in the preparation of this material. The Kiel bone also elicited an antibody response in some rats receiving such bone xenografts.

Kienholz and Kempkes (1956) attempted to demonstrate an immune response against cow bone transplanted into rabbits. They found that some of the recipient animals would develop an anaphylactic reaction when subsequently injected with bovine serum. This test system may be suitable for demonstration of a state of sensitivity to bovine serum components, but it cannot indicate the existence of a state of sensitivity against the cells or other components of the graft. Their results may therefore only suggest that bone contains serum protein. These authors also claimed that Kiel bone was not antigenic, as recipients do not display anaphylactic reactions when challenged with bovine serum. This is, however, not a valid conclusion because the technique has no regard for tissue transplantation antigens.

There have been a number of attempts to investigate the antigenic structure of the bone matrix rather than that of the cellular components. In some instances antibody was found in recipients of bone extracts (Burwell and Gowland 1961a; Millonig, Amrein and Borman 1962; Chandra, Lal, Manocha and Dave 1968; Dickson 1971), whereas in other experiments a negative result was obtained (Siegrist and Enneking 1959; Aoike and Iiji 1960; Kawamura, Hosono, Takahashi, Tomita, Nakazoe, Hirayama and Kido 1960). In most of these studies, however, the same bone preparation was used in the test for antibodies as was used for immunisation, and the possibility has not been excluded that the antibodies are against

transplantation antigens on the cells or cell debris contained in the immunising material. It can therefore be said that, so far, there is no clear evidence which could allow one to claim with confidence that antigens exist that are peculiar to the matrix of bone xenografts. By a simple test, such as the lymphocytotoxicity test used in the present work, this problem could easily be resolved. The demonstration that even treated xenograft bone is capable of eliciting an immune response against cell antigens makes it imperative that antibodies against cellular material are not overlooked in this type of study.

In order to assess the effect of an immune response upon new bone formation in bone grafts three parameters should be investigated: 1) new bone formation in the graft; 2) the presence or absence of antibody in the recipient's blood; and 3) the cellular reactions in the lymph node and in the graft.

In the case of bone allografts some osteocytes in the graft can remain viable for at least three weeks (Heslop, Zeiss and Nisbet 1960; Burwell and Gowland 1961*b*), and new bone formation is evident within the first week of grafting (Chalmers 1959, Burwell and Gowland 1961*b*). This new bone formation is short-lived, however, and osteocytes in the new bone die off, probably as a result of an immune reaction on the part of the host. Several weeks later a second phase of scanty new bone formation can be seen in some cancellous bone grafts. This is probably of host origin (Chalmers 1959). Using grafts of cortical bone, Heslop and her colleagues (1960) found little or no new bone formation in allografts.

The inflammatory cellular response of the host against the graft may influence the degree of osteogenesis in the composite graft. Heslop and colleagues (1960) found a considerably increased cellular reaction around allografts in comparison with autografts. The growth of granulation tissue and vascularisation of allografts of cortical bone was inferior in comparison with autografts (Zeiss, Nisbet and Heslop 1960). These authors considered that the granulation tissue associated with the graft may be of importance in facilitating revascularisation. If there is a blood-borne osteogenic precursor cell, then vascularisation of a bone graft may be of more importance than merely serving a nutritive role. When allografts of cortical bone are placed into immunologically tolerant hosts the cellular reaction around the graft was similar to that around an autograft, and was reduced in comparison to the allograft in the normal animal. The vascularisation also was similar to that seen in autografts and, significantly, osteogenesis occurred in the tolerant recipient (Nisbet, Heslop and Zeiss 1960). The cellular reactions against freeze-dried grafts (Chalmers 1959) and frozen allografts (Bonfiglio, Jeter and Smith 1955) have been reported as being less intense than those against fresh allografts.

In the present study composite xenograft-autografts were used, and these were provided with a supply of bone marrow cells which may include osteogenic precursors. In the case of the treated composite xenograft-autografts there can be no question of new bone being laid down by the graft cells. In the case of the fresh bone, grafted without marrow, the osteocytes of the graft looked dead as early as two weeks after grafting; by this time there was already considerable cellular reaction around the graft and the population of this tissue included plasma cells. Antibody was detectable in the serum of a high proportion of rats receiving such fresh, washed cancellous xenografts. Thus there was a brisk host reaction, and graft cells were killed before they had an opportunity to lay down new bone.

In the studies reported by Salama and his colleagues (1973) it was found that the degree of plasma cell and lymphocyte infiltration in the partially deproteinised Kiel bone and deproteinised Oswestry bone xenograft and composite xenograft-autografts was considerably lower than that found in the other types of graft.

There is some evidence of a correlation between the immunological response and new bone formation in composite xenograft-autografts in the findings presented above. Thus the grafts eliciting the lowest degree of antibody production during the first two weeks were of the Oswestry bone, which also gave the highest levels of new bone formation. However, it is of interest that, in the later phases after grafting, antibody was elicited by Oswestry grafts in

most recipients and yet the new bone score continued to rise between the second and sixth week. Thus it may be suggested that an immune response late after grafting, when osteogenesis is well established, has little effect on the graft. In contrast an early immune response such as was seen in recipients of decalcified bone, may lead to impairment of new bone formation.

SUMMARY

1. The humoral immune response of rats against sheep iliac bone grafts has been examined.
2. Fresh, marrow-containing grafts elicited a brisk and sustained antibody response. Attempts to wash out the marrow were not uniformly successful in removing cellular antigens from the grafts.
3. Decalcifying and freezing bone grafts at -20 degrees Celsius do not impair immunogenicity to any significant extent. Immunogenicity was found to be reduced in grafts subjected to freeze-drying.
4. Deproteinised Oswestry bone grafts and Kiel grafts gave rise to antibody production in a few recipients, and in the case of the former this response did not occur until after six or seven weeks from grafting.
5. The highest degree of osteogenesis in composite bone xenograft-autografts was found by Salama and colleagues (1973) to occur in Oswestry bone grafts. It is suggested that osteogenesis in xenografts may be impaired by an immune response.

We wish to thank Mr R. G. Burwell for his helpful advice, Miss M. Hart and Mr D. J. Sayers for their skilled technical assistance, and Mrs R. Whetter for preparation of the manuscript.

This work was supported by grants from the National Fund for Research into Crippling Diseases and the Medical Research Council.

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