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The Induction of Tolerance by Skin Homografts on Newborn Rats

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SUMMARY

IMMUNOLOGICAL tolerance can be produced in a small proportion of rats grafted at birth with foreign homologous skin. In spite of the regional origin of the antigenic stimulus provided by such a homograft, the tolerance which it produces is certainly systemic, because it extends to homografts transplanted later in life to a different part of the body. Evidence of a regional component in the tolerance produced by a skin homograft transplanted at birth was not obtained.

Extirpation of a skin homograft one month after its transplantation at birth weakens the state of tolerance that prevails two months after birth, but does not abolish it completely.

It is likely but not certain that skin homografts transplanted at birth produce tolerance of thyroid homografts transplanted from the same donors two months after birth.

INTRODUCTION

It has been known for many years that a certain small proportion of skin homografts transplanted to newborn mammals and birds may survive for months (Danforth and Foster, 1929; Reed and Sander, 1937; Cannon and Longmire, 1952; Rawles, 1955). Had the grafting been postponed until a few weeks after birth—in chicks, two weeks—then the homografts would have been destroyed within about twelve days of their transplantation. Their anomalously long survival cannot be attributed *merely* to the immunologically undeveloped state of newborn animals, because some of the grafts survived far into the period during which the ability to reject foreign tissue would normally have matured. We shall show that the phenomenon is simply a special form of immunological tolerance in the sense defined by Billingham, Brent and Medawar (1956): the skin homograft elicits tolerance and then enjoys the consequences of having done so.

The grafting of skin, particularly of adult skin, upon newborn animals is a laborious and inefficient method of producing tolerance as such, but it has one special advantage: a skin homograft provides what is predominantly a *regional* antigenic stimulus, in the sense that the antigenic matter issuing from it impinges mainly upon the regional lymph nodes (Mitchison, 1954; Billingham *et al.*, 1954). (Splenic cells, when injected intravenously or intraperitoneally, are widely disseminated throughout the lymphoid tissues of the host: Mitchison, 1956; Billingham and Brent, 1957.) The experiments described in this paper represent a first attempt to take advantage of this property in the analysis of tolerance. We have tried to answer the following questions:

(*a*) Can tolerance be in fact produced by the grafting of adult rat skin upon newborn rats? The case for supposing that it might do so rests upon the demonstration by Woodruff and Simpson (1955) and Woodruff and Sparrow (1958) that, in rats of the strains we

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have ourselves used, the period of life during which the introduction of foreign homologous cells can cause tolerance rather than immunity extends for more than a week beyond birth. There should therefore be ample time for a skin homograft to establish vascular connections with its host before this 'adaptive period' begins to wane.

(*b*) If so, is the tolerance so produced regional or systemic—i.e. is it in force merely or mainly within the lymphatic territory of the skin homograft which produced the state of tolerance, or does it extend to other parts of the body as well? It is at least theoretically possible that a skin homograft on a newborn animal should subvert only the lymphoid tissue upon which its antigenic output is mainly concentrated. We have tested this hypothesis by inducing tolerance with a skin homograft transplanted to the right side of the body at birth, and following this first graft with a second graft, transplanted from the same donor to the left side of the body, eight weeks after birth.

(*c*) Does any tolerance that may be produced by a skin homograft endure after the homograft itself has been removed—i.e. does tolerance of foreign homologous cells depend upon the continued presence of those cells and the continuous output of antigenic matter from them? We have tried to answer this question by grafting newborn rats with homografts of adult skin, and then dividing them into two groups, in one of which the homografts were widely and deeply excised four weeks after birth. All the rats were given second homografts from their respective donors eight weeks after birth, and the behaviour of the two groups of second homografts was then compared.

(*d*) Can skin homografts on newborn rats produce tolerance of tissues other than skin, e.g. the thyroid? The injection of splenic cells will certainly produce tolerance of thyroid tissue in rats (Woodruff and Sparrow, 1958) and of adrenal cortical tissue in mice (Medawar and Russell, 1958), and there is other evidence of the lack of organ-specificity in the antigens responsible for transplantation immunity. We expected, therefore, that any tolerance which might be produced by grafting newborn rats with skin from an adult donor would extend to grafts of the same donor's thyroid gland.

METHODS

The subjects of these experiments were rats belonging to two closed and partly inbred colonies: an albino strain (recipients) and a hooded strain (donors). The donor strain—chosen as such to make possible the use of grafts with coloured hair—was not sufficiently uniform to justify the assumption that any tolerance produced by a skin graft from one of them would extend to skin taken from another. In each experiment, therefore, the donor of the first, or tolerance-conferring, graft had of necessity to be the donor of the second graft transplanted eight weeks later.

The technique of grafting adult skin upon newborn rats was as follows. Skin bearing black hair (but in the interphase of a hair growth cycle) was removed from four month old female donors, either by means of a dermatome or by cutting extremely thin grafts freehand. Circular disks 5 mm. in diameter were punched from the excised skin and fitted into defects prepared on the right side of the chest of newborn albinos by removing skin down to the level of the panniculus carnosus. The grafts were very effectively held in place by an overlapping disk of thin, flexible adhesive plaster, the graft itself being protected from the plaster by a small centrally placed circle of filter paper. All the newborn rats in any one litter, to a total of eight, received one skin homograft each from the same donor.

The rats were weaned at four weeks. No graft which had healed into place soundly had broken down, but we retained for use only those which were supple and pink, with well-defined margins, and with no trace of scabbing. More than 90 per cent of the grafts had grown black hairs (the thinnest grafts cut freehand sometimes failed to do so). The grafts were scored for their degree of survival eight weeks after birth and thereafter at fortnightly intervals.

The second grafts, transplanted to the left side of the chest when the recipients were eight weeks old, were 2 × 2 cm. squares, cut with a dermatome and sewn into place by the method of Woodruff and Simpson (1955). Dressings were removed after ten days and the grafts were scored for their degree of survival 2, 3, 4, 6, 8, 10 and 12 weeks after their transplantation.

When the experiment called for it (Groups 2 and 5 below), the first skin homograft was excised together with at least 4 mm. of normal skin around it, and with the whole thickness of the panniculus carnosus below.

The thyroid grafts consisted of their donor's entire thyroid gland, cut into about ten pieces and grafted as a clump immediately below the femoral vessels in the groin of thyroidectomized recipients. The status of the grafts was assessed at monthly intervals after grafting by administering 5 microcuries of ^{131}I intraperitoneally and counting over the graft with a scintillation counter 8, 24, 48 and 72 hours after the administration of the iodine, as described by Woodruff and Sparrow (1958). When the test was negative or dubiously positive the animal was killed and a search was made for any sign of surviving thyroid tissue. On each occasion a count was also done in the neck region to find out whether or not there was any significant quantity of regenerating thyroid tissue in the neck.

Seventy-two grafted rats belonging to fifteen litters were accepted for experimental work. The animals within each litter were allotted by a strict process of randomization to five experimental groups, though for reasons made clear above only one rat in each litter could be made the recipient of a thyroid homograft. The groups were as follows:

Group 1.—First grafts left undisturbed; second grafts from the same donors transplanted to the opposite side of the body eight weeks after birth.

Group 2.—First grafts excised four weeks after birth; otherwise as Group 1.

Group 3.—First grafts left in position; no later treatment.

Group 4.—First grafts left undisturbed; thyroidectomy and transplantation of thyroid homografts from the original skin donor eight weeks after birth.

Group 5.—First grafts excised four weeks after birth; otherwise as Group 4.

RESULTS

BEHAVIOUR OF FIRST SKIN HOMOGRAFTS

Histological examination of twenty-five homografts which had been transplanted at birth and removed four weeks later (Group 2) revealed the presence of newly formed hairs and sebaceous glands in all. In thirteen there was little or no cellular reaction of any kind; in eight there was a clearly perceptible round cell and/or fibroblastic reaction, and in four others the reaction was decidedly more severe. (Chronic inflammatory reactions of this kind, falling short of the intensity of that which accompanies the acute breakdown of homografts in normal animals, is a familiar sign of incomplete tolerance: Anderson, Billingham, Lampkin and Medawar, 1951; Woodruff and Simpson, 1955;

Billingham *et al.*, 1956.) Comparison between the histological appearance of the grafts and their outward appearance before they were removed showed that the weaker kind of inflammatory reaction does not reveal itself to the naked eye. 'Naked-eye scores' tend therefore to err on the side of optimism.

Eight weeks after birth, thirty-two out of thirty-three first homografts were still alive (Groups 1 and 3; Table 1), but only nineteen were classified as perfect, i.e. supple, uncontracted, and (after clipping away the hairs) of a fresh pink colour. A further nine were outwardly normal except for some measure of contraction, and in two others breakdown was evidently in progress, though not complete.

TABLE 1

SURVIVAL OF SKIN HOMOGRAFTS TRANSPLANTED TO NEWBORN RATS

The rats in Group 3 received no other treatment; those in Group 1 received second homografts from the same donor, transplanted to the opposite side of the body eight weeks after birth

	Surviving grafts after					
	4 *weeks*	8 *weeks*	10 *weeks*	12 *weeks*	14 *weeks*	16 *weeks*
Group 3	6/6	5/6	4/6	2/6	1/6	1/6
Group 1	27/27	27/27	12/27	5/27	4/27	4/27
Total	33/33	32/33	16/33	7/33	5/33	5/33

Table 1 summarizes the later history of first homografts. The number of surviving homografts fell steeply after the eighth week, until by the fourth month after birth only five in thirty-three survived; of these, only two could be classified as perfect grafts by naked-eye appearance. Although the number of animals in Group 3 is smaller than that which we now realize would have been desirable, it is clear from Table 1 that the final number of surviving homografts, and the rate at which that final number is achieved, is much the same in animals which do (Group 1) or do not (Group 3) receive second homografts eight weeks after birth. There can, then, be no important regional component in tolerance. If tolerance were wholly or almost wholly confined to the lymphatic territory of the first homografts, then the second homografts, by activating a different and *ex hypothesi* 'virgin' group of nodes, should have provoked an active immunity and should therefore have made the first homografts of Group 1 break down very much more rapidly than the first homografts of Group 3. No such distinction is apparent in the data.

BEHAVIOUR OF SECOND HOMOGRAFTS

The behaviour of the second homografts in Groups 1 and 2, i.e. of grafts transplanted to the opposite side of the body eight weeks after birth, is summarized in Table 2.

It is clear that the first homografts have elicited a minor but quite definite degree of tolerance on behalf of the second homografts, for the experience of M.F.A.W. with several hundred skin homografts of the same size, transplanted in the same way to normal rats of the same strain and of the same age, has shown that only one in ten can be expected to survive as long as fourteen days, and none at all to the end of the third week (Table 2, last row). In our present experiments, 15/33 grafts survived to the end of the third week

TABLE 2

SURVIVAL OF SECOND SKIN HOMOGRAFTS TRANSPLANTED EIGHT WEEKS AFTER BIRTH TO RATS WHICH HAD RECEIVED FIRST SKIN HOMOGRAFTS FROM THE SAME DONORS AT BIRTH

In Group 1 the first homografts were left undisturbed throughout the experiment. In Group 2 the first homografts were radically excised four weeks after their transplantation

	Surviving grafts after						
	2 *weeks*	3 *weeks*	4 *weeks*	6 *weeks*	8 *weeks*	10 *weeks*	12 *weeks*
Group 1	12/27	9/27	9/27	7/27	6/27	6/27	6/27
Group 2	12/26	6/26	5/26	2/26	1/26	1/26	1/26
Total	24/53	15/53	14/53	9/53	7/53	7/53	7/53
Normal expectation*	5/53	0/53	0/53	0/53	0/53	0/53	0/53

* In untreated eight-week old rats receiving homografts of the same size from the same donors.

and, of these, seven were still alive at the end of the third month—three of them, to outward appearance, perfect grafts. It follows from the design of this experiment that the tolerance induced by the first homografts was systemic, for it extended to second homografts drained by a different system of lymphatics and communicating with a different group of nodes.

The radical excision of the first homograft after four weeks (Group 2), though it has significantly reduced the likelihood of securing or maintaining tolerance, has not abolished it completely. Indeed, one of the three grafts judged perfect at the end of three months belonged to an animal of Group 2.

Table 3 is a record of the degree of survival of first and second homografts on all the animals of Group 1 in which *either* graft enjoyed any degree of survival at the tenth week from birth (i.e. two weeks after transplanting the second homograft). In theory (Medawar, 1945) first and second homografts should break down almost simultaneously; the fact that the second homograft often seemed to outlive the first homograft cannot be taken too seriously, because it is almost impossible to apply the same criteria of degree of survival to grafts of different areas, thicknesses, and lengths of standing. On the other hand, the fact that the first homograft never outlived the second is clear evidence that it had not contrived to 'adapt' itself to its host during the two extra months in which it had an opportunity to do so.

THYROID HOMOGRAFTS

The evidence that skin homografts at birth can produce tolerance of thyroid homografts transplanted eight weeks later is not quite conclusive (Table 4; Groups 4 and 5). Six out of the thirteen rats in Groups 4 and 5 were judged to have surviving thyroid homografts a month after transplantation. Three of the six owe their classification to histological evidence, the ^{131}I test having being dubiously positive; had these three been allowed to run on it is possible that, like the other three, they would have declared themselves positive at the end of the fourth month. One thyroid homograft among nine controls, grafted to untreated hosts, was also functional after four months (data borrowed from Woodruff and Sparrow, 1958). (The occasional survival of thyroid homografts in

TABLE 3

CLASSIFICATION OF THE DEGREE OF SURVIVAL OF FIRST HOMOGRAFTS AND (*italic* TYPE) SECOND HOMOGRAFTS IN RATS OF GROUP I, INCLUDING ALL ANIMALS IN WHICH EITHER GRAFT SHOWED ANY DEGREE OF SURVIVAL TWO WEEKS AFTER THE TRANSPLANTATION OF THE SECOND GRAFT

A = normal grafts; B = grafts with minor signs of homograft reaction; C = homograft reaction clearly in progress but not yet complete; D = total breakdown. (The numbers in the left column refer to recipients)

First homograft: *Second homograft:*		*Weeks after transplantation*						
		4	8	10 *2*	11 *3*	12 *4*	14 *6*	16 *8*
517	1st: *2nd:*	A	A	A *A*	A *A*	A *A*	A *A*	A *A*
535	1st: *2nd:*	A	A	A *C*	 *D*	D		
583	1st: *2nd:*	A	C	C *A*	 *A*	D *B*	 *C*	 *D*
591	1st: *2nd:*	A	A	D *A*	 *A*	 *B*	 *D*	
604	1st: *2nd:*	B	A	D *C*	 *D*			
607	1st: *2nd:*	B	A	D *C*	 *D*			
618	1st: *2nd:*	A	B	C *D*		D		
627	1st: *2nd:*	B	B	C *A*	 *B*	C *B*	D *B*	 *C*
634	1st: *2nd:*	A	A	A *A*	A *A*	A *A*	A *A*	A *A*
636	1st: *2nd:*	A	A	D *A*	 *B*	 *C*	 *D*	
748	1st: *2nd:*	B	B	B *D*	D			
752	1st: *2nd:*	A	A	A *A*	 *A*	B *B*	B *C*	C *C*
754	1st: *2nd:*	A	B	B *D*		D		
803	1st: *2nd:*	A	A	C *A*	 *A*	C *B*	C *B*	C *B*
805	1st: *2nd:*	A	A	C *A*	 *B*	D *B*	 *B*	 *B*
810	1st: *2nd:*	A	A	C *D*		D		

untreated guinea-pigs had already been noticed by Woodruff and Woodruff, 1950.) Taken at their face value, the difference between the proportions of surviving thyroid homografts in normal and supposedly tolerant rats a month after grafting (1/9 and 6/13 respectively) might be expected to occur by luck alone about once in every twelve or thirteen such sets of trials. The excision of the first homograft (Group 5) seems to have made no difference.

TABLE 4

SURVIVAL OF THYROID HOMOGRAFTS IN RATS WHICH HAD RECEIVED SKIN HOMOGRAFTS AT BIRTH FROM THE THYROID DONOR

In Group 4 the skin homografts were left undisturbed throughout the experiment. In Group 5 the skin homografts were radically excised four weeks after their transplantation. Functional survival of the grafts was assessed by their power to concentrate ^{131}I, checked by histological examination in three rats of the four-week group

	Survival of thyroid homografts			
	4 weeks	*8 weeks*	*12 weeks*	*16 weeks*
Group 4	2/6	1/5	1/5	1/5
Group 5	4/7	2/5	2/5	2/5
Total	6/13	3/10	3/10	3/10
Untreated rats*	1/9	1/9	1/9	1/9
Tolerance induced by splenic cells*	8/9	8/9	8/9	8/9

* From the data of Woodruff and Sparrow (1958).

DISCUSSION

Three of the four questions put in the Introduction can now be answered with reasonable confidence. (*a*) The grafting of adult skin homografts to newborn rats can produce immunological tolerance, as revealed not so much by their own prolonged survival—for that might conceivably have been due to some kind of antigenic adaptation of the grafts (Cannon, Weber and Longmire, 1954; Weber, Cannon and Longmire, 1954)—as by the abnormally prolonged survival of second homografts transplanted from the same donors eight weeks later.

Further, (*b*), the tolerance produced by skin homografts transplanted at birth must be very largely systemic, for the second grafts which revealed its presence lay in an entirely different lymphatic territory. Unless the regional nodes serving the second homografts had been partially unreactive to donor antigens, no state of tolerance could have been revealed. Conversely, any regional component in the tolerance produced by a skin homograft must be very small, because first homografts broke down no more quickly on animals which received second homografts than on animals which did not. First grafts on the latter should have survived much longer if they had established a privileged position for themselves in their own lymphatic territory. Thirdly, (*c*), it is not unconditionally necessary for the first homograft, that which produced tolerance, to be continuously present for the state of tolerance to be maintained. Its excision one month after its own transplantation, and one month before the transplantation of the second graft, lowered

the degree of tolerance ultimately obtained but did not abolish it completely. To the fourth question, (*d*), no final answer can be given: it is likely that skin homografts on newborn animals produce tolerance of thyroid homografts, but the case has not been established beyond reasonable doubt.

How far are we justified in regarding a skin homograft as regional antigenic stimulus? The evidence that it is so in adults is very strong (Mitchison, 1954; Billingham *et al.*, 1954), but the distribution of lymphatic territories in newborn rats may be less strictly parochial than in adults, and cells or antigenic matter issuing from the homograft might therefore get a much more than merely local distribution. As a counter-argument, it may however be urged that there is still likely to be a big difference of degree between the responses of the regional and the more distant lymphoid centres to antigenic matter issuing from skin homografts on newborn mice; yet, if our earlier reasoning is correct, the contralateral lymph nodes serving the second homografts were no better qualified to respond to the antigenic stimulus than the nodes serving the first homografts. Our results can be explained by the hypothesis that a state of tolerance induced in one set of regional lymphoid centres is somehow propagated to other lymphoid centres, with the effect that they too become unresponsive to a later challenge by the appropriate antigenic stimulus. The idea is made easier to credit by Gowans's demonstration (1957 and unpublished work) that lymphocytes are circulating cells, with its corollary that lymphocytes manufactured in one lymphoid centre will rapidly and at random percolate through all the others; but the mechanism of the transformation, if anything of the kind occurs, is completely mysterious. The problem has been discussed by Medawar (1957).

Not all the cells in the dermis of a skin homograft can be regarded as 'fixed'; some may escape; and this qualifies the interpretation of the experiment in which the skin homograft producing tolerance was widely and deeply excised a month before the second grafts were transplanted. But although it would be idle to pretend that *all* the foreign cells in the body had been removed with the first homograft, nearly all of them must have been. The original homograft produces such feeble tolerance even when it is left in place that if the maintenance of tolerance did indeed depend upon the continuous emission of antigenic matter, then the reduction of its output to perhaps less than 1 per cent of its original value, after the extirpation of its principal source, would be expected to cause tolerance to disappear completely. In fact it did not do so.

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