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Transplantation Immunity in the Isologous Mouse Radiation Chimaera

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Summary. The survival of skin homo- and heterografts on isologous CBA mouse chimaeras has been investigated. Homografts usually persist for considerably longer than on normal unirradiated mice. Immunization of the host against the appropriate foreign antigens before irradiation neither reduces nor increases the duration of this persistence. When an irradiated non-immune host is restored with bone marrow from an immunized donor, a measure of immunity is transferred.

If adult spleen cells from normal or immunized donors are added to the restorative inoculum, strongly antigenic foreign skins are shed with something like normal rapidity, but weakly antigenic skins may be retained for 100 days or more, and even indefinitely.

Heterografts do not enjoy a span of survival comparable with that of homografts.

These findings are discussed, and it is concluded that two factors are of importance in the prolongation of graft survival: (1) A weakening of the mechanism by which antigens are recognized as foreign, (2) an overall central depression of the immune response.

INTRODUCTION

It is now firmly established that lethally X-irradiated mice may be induced to recover by the intravenous injection after irradiation of foreign haematopoietic tissue. Several workers have shown by various methods that recovery is largely, if not entirely, due to recolonization of the host's radiation-damaged haematopoietic tissues by the normal cells injected (Lindsley, Odell and Tausche, 1955; Ford, Hamerton, Barnes and Loutit, 1956; Mitchison, 1956; Vos, Davids, Weyzen and van Bekkum, 1956; Makinodan, 1956; Nowell, Cole, Habermeyer and Roan, 1956). The recovered animals are thus chimaeras; that is to say, they consist of a mixture of cells from different origins. They may be *isologous* (when the host and donor belong to the same highly inbred strain), *homologous* (when they belong to different strains of the same species), or *heterologous* (when they belong to different species — for example mouse/rat). The tissues which are most effective in inducing recovery in the mouse are adult bone marrow, infant spleen and foetal liver, all of which give 80-100 per cent initial recovery in this laboratory. The evidence of Ford, Hamerton, Barnes and Loutit (1956), Ford, Ilbery and Loutit (1957), Gengozian, Urso, Congdon, Conger and Makinodan (1957), and Brocades Zaalberg and van Bekkum (1959), indicates that the injected cells repopulate not only the bone marrow, but also the spleen, thymus and lymph nodes. There is therefore a high probability that the injected material will take over at least a part of the animal's immunological functions. This probability is confirmed by the work of Grabar, Courcon, Ilbery, Loutit and Merrill (1957), who showed by immuno-electrophoresis that the gamma globulins in irradiated mice restored

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with rat bone marrow were of rat type (*see also* Weyzen and Vos, 1957). Indirect confirmation is also provided by the fact that 'secondary disease' in homologous chimaeras, attributable to an immunological reaction by the injected tissue against the host, is less severe when a donor tissue theoretically capable of exhibiting actively acquired tolerance in the sense of Billingham, Brent and Medawar (1953) — i.e. foetal liver – is used (Barnes, Ilbery and Loutit, 1958; Uphoff, 1958).

The immunological characteristics of radiation chimaeras have been studied by the grafting of skin (Main and Prehn, 1955, 1957; Trentin, 1956a and b; Brocades Zaalberg, Vos and van Bekkum, 1957; Barnes, Ford, Ilbery and Loutit, 1958; Barnes and Loutit, 1959) and of strain-specific tumours (Barnes, Ford, Ilbery, Koller and Loutit, 1957; Ilbery, Koller and Loutit, 1958). The above work has shown that homologous chimaeras will regularly accept grafts from donor-strain, host-strain, and the F_1-hybrid between them; similarly, mice restored with F_1 marrow will accept grafts from both parent strains as well as the hybrid.

Most of the skin-grafting work has been done with mice given homologous or F_1-hybrid marrow, and relatively little attention has been paid to the isologous chimaera. Main and Prehn (1955) noted that BALB/C skin-grafts on DBA/2 mice restored with DBA/2 marrow survived for a longer period than would be expected of skin homografts on unirradiated mice, and two such grafts were retained permanently. BALB/C and DBA/2 have the same antigens at the important H-2 histocompatibility locus (Snell, 1958), and the two strains may therefore react only weakly with each other. Trentin's (1956a) results also suggest a slightly prolonged (22–40 days) retention of homografts by 11 A/A* chimaeras, although the grafts were applied no less than 5 months after irradiation. These results show that isologous chimaeras are at least temporarily unable to mount an effective response to some foreign murine antigens, and that the capacity to respond may even be permanently impaired. Ilbery *et al.* (1958) found that Sarcoma I of Strain A mice killed 33 per cent of CBA/CBA chimaeras when inoculated 46 days after irradiation, while the mortality in normal unirradiated CBA was only 3 per cent. Tumour transplantation, however, has limitations as a test of immunological reactivity on account of the invasive and lethal nature of the graft, which may overwhelm its host in spite of an incipient immune response.

We therefore felt that it would be of interest, especially in view of possible applications to human surgery, to acquire more data on the survival of skin homografts of various degrees of incompatibility on isologous chimaeras. Moreover, we hoped that by pre-immunizing the irradiated host or the donor of haematopoietic tissue against one or other of the future skin donors, we might throw some light upon the mechanism of homograft rejection in chimaeras.

STOCKS AND METHODS

STOCKS

The test animals in all experiments were male mice of strain CBA, 3–5 months old at the time of irradiation. Male mice of strains CBA, C3H, C57BL and A were used as donors of skin. All the mice were bred from the Harwell stock by our colleagues T. C.

* To describe chimaeras we use the convention, P/Q where P = host and Q = donor. Thus CBA immune to A/CBA would denote a CBA mouse immunized against strain A, lethally irradiated and restored with normal CBA haematopoietic tissue.

Carter, M. F. Lyon and R. J. S. Phillips. The strains have been maintained by strict sib-mating with frequent re-selection of lines. Male albino rats of the Harwell colony were the donors of rat skin; they too have been sib-mated for many generations.

X-IRRADIATION

Mice were irradiated in boxes of five by the standard technique of this laboratory (Corp, 1957). In almost all cases they received a mean dose of 1007 rads ± 3 per cent (250 Kv, 14 mA, HVL 1.2 mm. Cu) at a dose-rate of 71.6 rads/minute. In the earlier experiments the nominal dose was 950 rads, which had previously been found to be the 30-day LD 98-100 for CBA mice in this laboratory; latterly it was discovered that an incorrectly calibrated dosimeter had caused doses given during a period between 1957 and 1958 to be underestimated by 6 per cent. The actual dose received by all the mice in this series was the same, approximately 1007 rads.

SUSPENSIONS FOR POST-IRRADIATION THERAPY

Bone marrow was obtained from the shafts of two femurs of adult mice (3–5 months old). After removal of the epiphyses, the bones were flushed through with 0.85 per cent NaCl by means of a syringe and size 14 needle. Macroscopic particles of marrow were broken up by drawing them several times through the needle, and the resulting suspension was immediately injected by the tail-vein into five mice; each mouse received approximately 2×10^6–5×10^6 nucleated cells.

Livers were obtained from 14–18 days' foetuses and mushed in saline with an electrically operated mincer. Large particles were allowed to settle by gravity and the remaining suspension was diluted so as to contain 5×10^6–20×10^6 cells in the 0.2 ml. given intravenously to each mouse.

Suspensions of adult mouse spleen were obtained in the same way as those of foetal liver. For animals receiving bone marrow plus spleen, the usual amount of bone marrow was flushed out with a spleen-suspension containing approximately 5×10^7 cells in 0.2 ml.

IMMUNIZATION

Mice were immunized against foreign mouse or rat antigens by three injections of adult spleen cells suspended in saline, at intervals of a fortnight — the first intravenous, the second intraperitoneal, the third subcutaneous. About 10^7 cells were injected on each occasion. Immunized mice were irradiated or used as donors of bone marrow or spleen 10–14 days after the final injection.

SKIN GRAFTING

The method used was essentially that of Billingham and Medawar (1951). Full-thickness pinch-grafts about 1 cm. across were taken from the back of the donor after hair had been removed with an electric clipper. They were then stripped of underlying fat and muscle and applied to areas of appropriate shape and size on the host's flank, bared down to the panniculus carnosus. The dressing consisted of muslin impregnated with soft paraffin, topped with a piece of cellophane and bound around with plaster of Paris. The

dressing was removed after 10–14 days. Grafting was performed under Nembutal anaesthesia. Care was taken to avoid areas of skin which were in an active phase of growth, both when taking grafts and when preparing the beds on the host.

Some difficulty was experienced in determining satisfactory criteria for graft-survival in these experiments; in most cases the classical picture of an inflammatory homograft reaction was not seen, and grafts not infrequently persisted as well-demarcated bare patches for several weeks after hair had been grown and lost again. This was particularly characteristic of C3H grafts, although not confined to them. The visible shedding process with C57BL and A grafts was generally rather more rapid, total scabbing often preceding total loss of hair. In the case of rat grafts something approaching a normal inflammatory reaction was seen, with haemorrhage and early scabbing. Scoring was further complicated by the poor physical condition of some of the recipient animals. A loss of total body-weight of up to 25 per cent occurs in irradiated marrow-injected CBA mice during the first 10 days after irradiation, associated with a severe temporary anaemia. A proportion of grafts applied on the day of irradiation undoubtedly failed to survive for these reasons. A loosening of the plaster jacket round the wasting animal was a particularly frequent source of trouble. The incidence of failures in the period up to 20 days after irradiation was as high among isologous CBA as among homologous grafts. Gross infection of the grafts was uncommon, but a dusting of Cicatrin (Calmic), an antibiotic powder, was often given when the dressings were removed.

We eventually adopted the following criteria for assessing the survival of grafts:

1. Grafts which did not survive more than 20 days were considered as possible technical (or otherwise non-immunological) failures.
2. Only grafts which grew at least a modicum of new hair were scored as initial successes.
3. Rejection of a graft was considered to have begun when the first signs of scabbing and/or thinning of hair was observed; it was considered to be complete when either the whole graft area was a hard scab, or the last hair had been lost, whichever happened earlier. (No graft which lost hair in this way was observed to grow more subsequently.)

We are conscious that these criteria are to some extent unsatisfactory — the first because it fails to lay down a clear rule for distinguishing between technical failures and possible early immune reactions; the second because rapidity of hair-growth is well known to vary according to the physiological state of the donor skin and of the host at the time of grafting. Their general effect, however, is to give the lowest possible number of initial successes, and the earliest possible dates of shedding.

Plates I-III illustrate the gradual shedding of one of the longest-surviving C57BL grafts.

EXPERIMENTAL DETAILS AND RESULTS

EXPERIMENT I

In this experiment the test animals were normal CBA males, irradiated with 1007 rads and given normal CBA adult bone marrow. The majority were skin-grafted on the day of irradiation, but smaller groups were grafted 1, 3, 7, 8, 14, 28, 35 and 37 days after irradiation. Each mouse received either a homograft, or a homograft and an isograft, or two different homografts on the right flank. Control unirradiated CBA mice were also homografted, twenty with C57BL skin and fifteen with C3H skin.

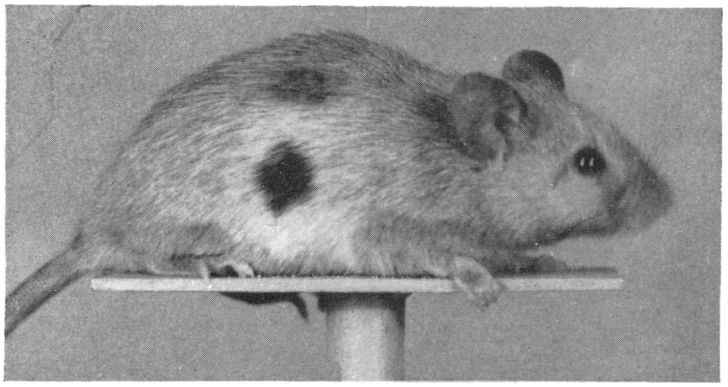

PLATE I. CBA/CBA (bone marrow) chimaera from Experiment 1, with intact and hairy C57BL (ventral) and C3H (dorsal) skin grafts.
Photographed 59 days after grafting.

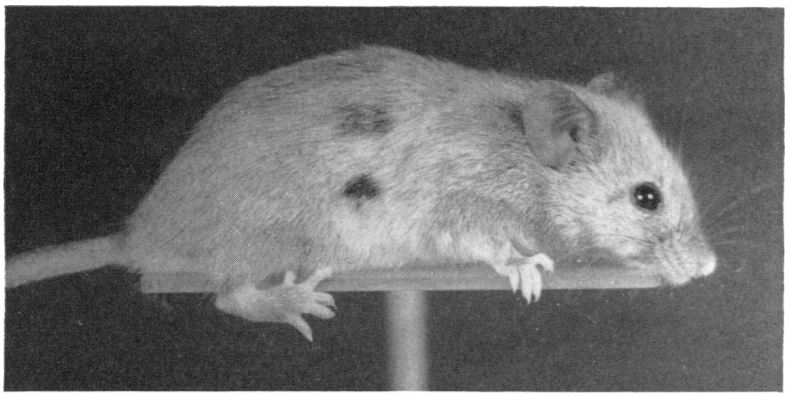

PLATE II. The same animal as above, photographed 93 days after grafting. The C57 BL graft has lost much of its hair and is breaking down. The C3H graft is unchanged.

PLATE III. The same animal as above, photographed 107 days after grafting. Breakdown of the C57 BL graft is complete. The C3H graft is unchanged.

Results

All thirty-five control homografts on unirradiated mice were shed in less than 20 days. (This contrasts with results reported by Werder, Hardin and Morgan (1959), when 39 per cent of full-thickness C3H grafts survived in CBA mice for 30 days or more.) Isografts of CBA skin on the same mice survived indefinitely. The results of grafting the irradiated

TABLE 1

SURVIVAL OF SKIN ISO- AND HOMOGRAFTS, APPLIED AT VARIOUS INTERVALS AFTER IRRADIATION, ON CBA MICE LETHALLY IRRADIATED AND RESTORED WITH BONE MARROW FROM NORMAL ADULT CBA DONORS

Time of skin-grafting after irradiation (days)	*Origin of grafts*	*No. of mice grafted*	*Initial successes*	*Earliest sign of shedding*	*Last graft wholly shed*	*Mean start of shedding*	*Mean end of shedding*	*Intact at time of writing*
				(Days after grafting)				
0	CBA	14	8	—	—	—	—	8(360 days)
	C57BL	22	14	30	112*	55	62	0
	C3H	6	6‡	112	—	—	—	3(360 days)
	A	9	7	43	60	49	54	0
3	C57BL	6	5	30	75	47	57	0
7–8	CBA	4	3	—	—	—	—	3(360 days)
	C57BL	7	5	28	72	43	48	0
14	CBA	5	5	—	—	—	—	5(360 days)
	C57BL	9	6	23	105†	42	51	0
	C3H	4	4	60	—	—	—	2(200 days)
28	CBA	3	3	—	—	—	—	3(360 days)
	C57BL	3	0	—	24	—	—	0
35–37	C3H	9	9	26	—	—	—	3(160 days)
	C57BL	14	0	—	<20	—	—	0

* Shedding begun 8 days previously.
† Shedding begun 15 days previously.
‡ Two of these mice died at 30–40 days after grafting with grafts intact.

mice are given in Table 1. The large proportion of CBA grafts applied on the day of irradiation which failed to survive 20 days, indicates the extent to which non-immunological factors were at work; all CBA grafts which were intact at 20 days survived indefinitely. On the other hand the 100 per cent early shedding of C57BL grafts applied on days 35–37 was probably a largely immunological phenomenon; technical failure of this magnitude is improbable both because the hosts had recovered and gained weight after the irradiation, and because C3H grafts on the same animals were retained for long periods. The shedding-time, expressed as days after *irradiation*, is comparable with that of grafts applied on day 0, but less variable.

Three facts emerge from these results:

1. Skin homografts survive considerably longer on recently formed isologous CBA chimaeras than on unirradiated CBA mice.

2. The length of survival is related to the degree of genetical similarity between graft and host. Our C3H and CBA mice have the same antigens at the H-2 locus (Snell, 1958; Gorer, 1958), and C3H skin may survive for a year and upwards on the isologous CBA chimaera. Strains A and C57BL differ from CBA at the H-2 locus and most grafts from

them are retained for between 40 and 80 days, never more than 120 days. Where C3H and C57BL grafts are applied to the same animal under the conditions of this experiment, the C57BL always provokes a more rapid reaction than does the C3H.

3. The mean shedding-time, expressed as days after irradiation, of grafts applied at intervals up to 5 weeks is close to that of grafts applied on the day of irradiation (Fig. 1).

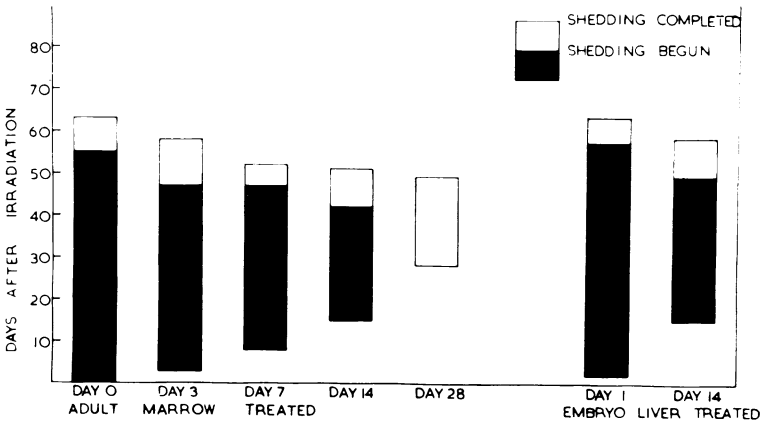

FIG. 1. Mean survival of C57BL skin-grafts, applied 0, 3, 7, 14 and 28 days after irradiation, on CBA mice lethally irradiated and restored with normal CBA adult bone marrow or foetal liver.

TABLE 2

SURVIVAL OF SKIN HOMOGRAFTS, APPLIED AT VARIOUS INTERVALS AFTER IRRADIATION, ON CBA MICE LETHALLY IRRADIATED AND RESTORED WITH CBA FOETAL LIVER

Time of skin-grafting after irradiation (days)	*Origin of grafts*	*No. of mice grafted*	*Initial successes*	*Earliest sign of shedding*	*Last graft wholly shed*	*Mean start of shedding*	*Mean end of shedding*	*Intact at time of writing*
				(Days after grafting)				
1	C3H	7	5	40	—	—	—	1 (200 days)
	C57BL	7	7	44	80	56	63	
14	C3H	7	7	42	—	—	—	4 (240 days)
	C57BL	7	6	21	63	35	43	0
47	C3H	2	1	85	100	(85)	(100)	0
	C57BL	3	0	—	—	—	—	—

EXPERIMENT 2

The mice in this experiment were seventeen normal CBA irradiated with 1007 rads and given isologous foetal liver from 16–18 day embryos. It is known that mice exposed *in utero* to foreign murine tissue antigens may thereafter be permanently or temporarily tolerant of those antigens (Billingham, Brent and Medawar, 1953). Tolerance is attributed to the maturation of the immune system in the presence of the antigens, which are thereafter not recognized as foreign. It seemed to us possible that, through a similar mechanism, irradiated animals restored with foetal liver might retain immediately applied homografts for longer than those treated with adult bone marrow. Each mouse was grafted with C3H

and C57BL skin, 1, 14 or 47 days after irradiation. The results, given in Table 2, indicate that homograft survival is as long as, but no longer than, in animals given adult marrow (Figs. 1, 2 and 4).

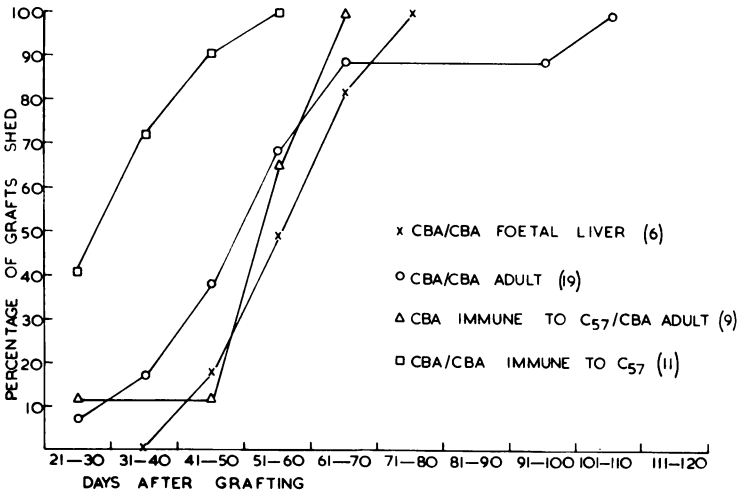

FIG. 2. Cumulative shedding of initially successful C57BL skin grafts applied on the day of irradiation. The hosts were CBA mice (normal or pre-immunized), lethally irradiated and restored with bone marrow from normal or pre-immunized CBA donors or with normal CBA foetal liver. The numbers in brackets represent the number of initially successful grafts in each group. The time of shedding is taken as the 10-day period which includes the time mid-way between the beginning and end of shedding.

TABLE 3

SURVIVAL OF SKIN HOMOGRAFTS, APPLIED ON THE DAY OF IRRADIATION, ON PRE-IMMUNIZED CBA MICE LETHALLY IRRADIATED AND RESTORED WITH BONE MARROW FROM NORMAL ADULT CBA DONORS

Host	*Origin of grafts*	*No. of mice grafted*	*Initial successes*	*Earliest sign of shedding*	*Last graft wholly shed*	*Mean start of shedding*	*Mean end of shedding*	*Intact at time of writing*
				(*Days after grafting*)				
CBA imm. to C57BL	C57BL	9	9	21	70	52	60	0
	C3H	6	4	20	—	—	—	3(240 days)
	A	3	0	—	—	—	—	—
CBA imm. to A	A	10	7	20	120*	52	62	0
	C57BL	5	5	50	70	56	65	0
	C3H	5	5†	110	—	—	—	3(155 days)
CBA imm. to C3H	C3H	9	7	72	—	—	—	3(155 days)
	C57BL	5	3	24	63	43	53	0
	A	4	1	105	112	(105)	(112)	0

* Shedding begun 4 days previously.
† One of these mice died with intact graft at 40 days.

One mouse grafted after 1 day shed its C3H graft before the C57BL after retaining it intact for 43 days; in other cases the C57BL skin provoked a more rapid reaction, as in Experiment 1. We are unable to explain the exception on immunological grounds.

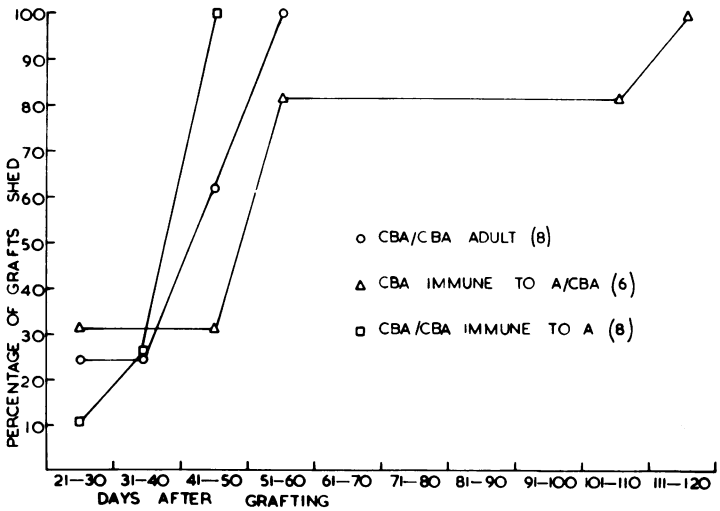

FIG. 3. Cumulative shedding of initially successful A skin grafts applied on the day of irradiation. The hosts were CBA mice (normal or pre-immunized), lethally irradiated and restored with bone marrow from normal or pre-immunized CBA donors. The numbers in brackets represent the number of initially successful grafts in each group. For note on the time of shedding, see legend to Fig. 2.

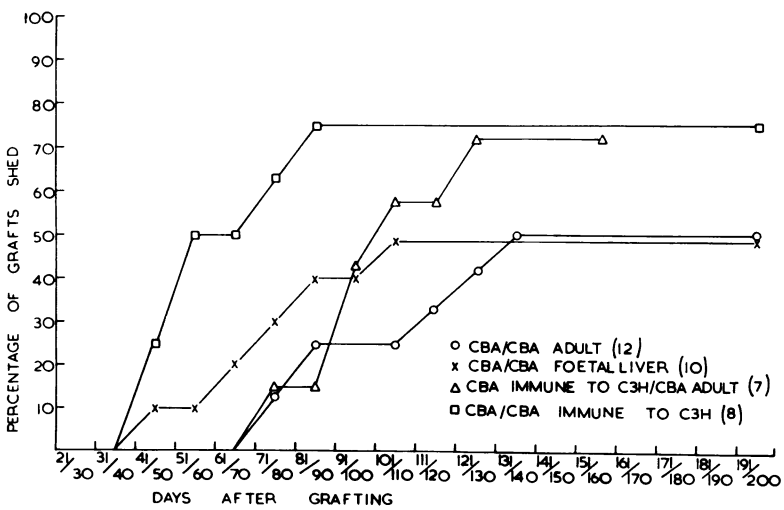

FIG. 4. Cumulative shedding of initially successful C3H skin-grafts applied on the day of irradiation. The hosts were CBA mice (normal or pre-immunized), lethally irradiated and restored with bone marrow from normal or pre-immunized CBA donors or with normal CBA foetal liver. The numbers in brackets represent the numbers of intially successful grafts in each group. For note on the time of shedding, see legend to Fig. 2.

EXPERIMENT 3

In this experiment twenty-eight mice were pre-immunized against one of the strains A, C57BL or C3H during the 6 weeks before they received 1007 rads; they were restored with normal adult CBA bone marrow. After irradiation they were grafted on the same day with skin from the strain to which they had been immunized and with one other foreign skin. We anticipated that the host's power to produce a cellular response to the graft-antigens would be annihilated or greatly impaired by the radiation, but that humoral isoantibodies would continue to be present in the serum. Results of this experiment are given in Table 3. These give no evidence that the rejection of homografts has been either accelerated or slowed by the presumed presence of circulating antibody (*see* Figs. 2-4).

TABLE 4

SURVIVAL OF SKIN HOMOGRAFTS, APPLIED ON THE DAY OF IRRADIATION, ON NORMAL CBA MICE LETHALLY IRRADIATED AND RESTORED WITH BONE MARROW FROM PRE-IMMUNIZED ADULT CBA DONORS

Marrow donor	*Origin of grafts*	*No. of mice grafted*	*Initial successes*	*Earliest sign of shedding*	*Last graft wholly shed*	*Mean start of shedding*	*Mean end of shedding*	*Intact at time of writing*
				(*Days after grafting*)				
CBA imm. to C57BL	C57BL	17	12	20	65	31	41	0
	C3H	11	7*	84	—	—	—	3(280 days)
	A	6	4	31	60	33	46	0
CBA imm. to A	A	13	8	20	48	37	44	0
	C57BL	8	7	34	55	40	46	0
	C3H	5	1	70	120	(70)	(120)	0
CBA imm. to C3H	C3H	13	10†	46	—	—	—	2(176 days)
	C57BL	9	7	30	71	47	53	0
	A	5	2	29	124‡	63	80	0

* Two of these mice died with intact grafts at 38–39 days.
† One of these mice died with intact graft at 47 days.
‡ Shedding begun 26 days previously.

EXPERIMENT 4

This was the reciprocal of Experiment 3, normal CBA mice being irradiated and restored with bone marrow from CBA pre-immunized against one of the strains A, C57BL, or C3H. Altogether forty-five mice were grafted with two foreign skins, one being from the strain to which the marrow donor was immune. In this situation the possibility existed that the injected marrow cells from the immunized donors would elaborate immunologically competent tissue with greater specific potency, and elaborate it perhaps more rapidly, than would marrow from a normal donor. The results are presented in Table 4.

Taken as a whole these results, when compared with those of Experiment 1, show that mice receiving marrow from an immunized donor shed skin-grafts from the immunizing strain sooner than do mice receiving normal marrow (*see* Figs. 2–4). This fact emerges most clearly in the groups given marrow from donors immune to C57BL (Fig. 2).

It is suggestive that out of seven mice in this experiment which were restored with bone marrow 'immune to C3H' and then successfully grafted with both C3H and C57BL skin, two shed the C3H graft before the C57BL. This was not to be expected except in the

presence of a specific immunity to C3H, since C57BL contains the more powerful antigens. However, the same phenomenon was seen in one mouse out of fourteen which had been restored with material from a non-immunized donor (Experiments 1 and 2).

EXPERIMENT 5

A number of mice in Experiments 1, 2 and 4 retained intact and hairy C3H grafts for 240 days or more. The aim of this experiment was to determine whether this retention was due to some process of adaptation to the foreign environment on the part of the graft (involving loss or suppression of antigenicity), or to absence or suppression of response (for whatever cause) on the part of the chimaerical host. If the former alternative were true, one would expect new grafts of C3H skin to be rejected. Rejection of the new graft, and perhaps also of the old, would also be expected if the old had been the object of a low-grade immune reaction which was not outwardly discernible. Eight mice with grafts of long standing were regrafted with C3H skin by the usual technique. All eight new grafts were accepted, and at the time of writing (90 days later) seven out of eight are growing luxuriant hair. The eighth has thin hair only, and the old graft on the same mouse is represented by an atrophic patch of hairless skin. This mouse is wasted and has been ailing since it was regrafted. Nutritional rather than immunological factors may be at work here. The old grafts on the other seven mice went through an initial period of depilation, but rapidly regrew their hair; all appear healthy at present.

These results indicate that prolonged survival of a C3H skin-graft is not the prerogative of that particular graft, but is accorded also to subsequent grafts from the same strain.

EXPERIMENT 6

In all the foregoing experiments adult bone marrow or foetal liver was used as the restorative tissue. These tissues contain little or no mature lymphatic material. It seemed probable that the inclusion of mature lymphatic cells in the inoculum, particularly if they came from immunized donors, would hasten the rejection of homografts. To test this hypothesis, we restored eighteen irradiated mice with a suspension containing about 5×10^7 adult spleen cells in addition to the usual amount of bone marrow. The donors were normal or pre-immunized CBA mice. Grafts were applied on the day of irradiation.

Results are given in Table 5.

TABLE 5

SURVIVAL OF SKIN HOMOGRAFTS, APPLIED ON THE DAY OF IRRADIATION, ON NORMAL CBA MICE LETHALLY IRRADIATED AND RESTORED WITH MIXED BONE MARROW AND SPLEEN FROM NORMAL OR PRE-IMMUNIZED ADULT CBA DONORS

Donor of bone marrow and spleen	*Origin of grafts*	*No. of mice grafted*	*Initial successes*	*Times of shedding (days after grafting)*	*Intact at time of writing*
Normal CBA	C3H	4	3	60–70 (1)	2(180 days)
	C57BL	4	0	All <20	0
CBA imm. to C57BL	C57BL	5	0	All <20	0
	A	5	0	All <20	0
CBA imm. to A	A	4	0	All <20	0
	C57BL	4	0	All <20	0
CBA imm. to C3H	C3H	5	2	28–70 (1)	1(180 days)
	C57BL	5	0	All <20	0

The fact that all C57BL and A grafts were shed in less than 30 days is in accordance with our hypothesis. The long retention of one-third of the C3H grafts was unexpected, despite the relatively close genetic affinity of C3H to CBA.

EXPERIMENT 7

As was noted earlier, the shedding of C3H grafts in the above experiments was often a very gradual process. If one assumes that it was brought about by an immunological reaction, the reaction was but feeble.

The present experiment was designed to show whether, after shedding one graft, animals gave anything like a 'second-set response' to a second.

Five mice were selected which had shed their C3H grafts slowly between 1 and 3 months after grafting. To each of these a fresh C3H graft was applied approximately 2 months after the breakdown of the first was complete.

The history of the mice and the state of the new grafts at the time of writing are given in Table 6.

TABLE 6

RESULTS OF RE-GRAFTING 5 CBA/CBA CHIMAERAS WITH C3H SKIN APPROXIMATELY 2 MONTHS AFTER THEY HAD COMPLETELY SHED PREVIOUS C3H GRAFTS

Mouse	*Restorative haematopoietic tissues*	*Shedding-time of old graft*	*Comments on new graft (6 weeks after grafting)*
1	Bone marrow only from donor immune to C3H	6–10 weeks	Completely shed in <20 days
2	,,	10–12 weeks	,,
3	,,	6–8 weeks	Graft intact. No scabbing, but hair-growth retarded and very patchy
4	Bone marrow and spleen from donor immune to C3H	4–10 weeks	Graft intact. No scabbing, but hair-growth retarded and patchy
5	Bone marrow and spleen from non-immune donor	6–10 weeks	Graft intact. No scabbing, but only half of graft growing new hair

Only two of the five mice gave anything like normal responses to the fresh homografts. The other three grafts seem to be the objects of a niggling reaction, similar to that which caused the ultimate breakdown of earlier C3H grafts on the same mice.

These few results suggest that heightened sensitivity to C3H antigens, if it was ever present after the shedding of the original graft, was in some cases of short duration. Alternatively the hosts, though 'sensitized', were still immunologically so enfeebled as to be unable to mount an effective reaction.

EXPERIMENT 8 — HETEROGRAFTS

Experiments 1–7 were concerned with homografts of mouse skin. In Experiment 8 we extended our findings to cover heterografts of rat skin under similar conditions. There is no anatomical or physiological, as opposed to specifically immunological, bar to the successful grafting of rat skin on to mice. It is our experience that, on the CBA mouse/rat

(Harwell strain) chimaera, Harwell rat skin takes well and grows a good crop of characteristic rat hair. In the present experiment thirty-seven normal or pre-immunized CBA mice were irradiated, restored with marrow, or marrow and spleen, from normal or pre-immunized CBA donors, and grafted with rat skin the same day.

None of the grafts was retained for longer than 30 days, and only four looked reasonably intact at 20 days (all on non-immune mice given marrow only from non-immune donors). None grew new hair. In most cases the grafts were inflamed and hard by the fourteenth day. These results contrast sharply with those for homografts. It appears that even the recently formed chimaera is able to respond rapidly to a tissue so foreign as rat.

In two small experiments skin from hamsters or chickens was grafted on to normal CBA/CBA chimaeras; these grafts were rejected if anything more rapidly than those of rat skin.

DISCUSSION

IMMUNOLOGICAL REACTIVITY OF THE ISOLOGOUS CHIMAERA

The immune response to foreign antigens may be expressed in two ways, by the production of extracellular antibodies, which circulate in the plasma, or of activated cells. The properties of the humoral antibodies, e.g. agglutinins, precipitins, lysins, have been studied for generations to elucidate the body's defence mechanisms against infective agents and their products. The role of the activated cells has been identified more recently, and the means whereby they act is still obscure. However this may be, activated cells seem to be the effective mechanism of immunity in delayed hypersensitivity (e.g. to old tuberculin) and in the homograft reaction to foreign tissue.

Gengozian *et al.* (1958) have investigated the competence of radiation chimaeras to produce humoral antibodies. They have concluded that the more foreign the antigen the stronger the response. They have also shown that the isologous chimaera is more efficient than the homologous, which in turn is more efficient than the heterologous. Furthermore they found that this immunological competence takes time to be attained.

Studies in this laboratory have been concerned with the capacity of radiation chimaeras to respond to tissue-grafts. Our experience is that the isologous chimaera CBA/CBA regains the capacity of normal animals to reject grafts of foreign tumour only slowly over the first few months of its chimaerical existence (Barnes *et al.*, 1957; Ilbery *et al.*, 1958). When it has been established 6 months or more it rapidly rejects foreign grafts of normal skin of strain A (Barnes and Loutit, 1959).

The present investigation covers the reactivity of the recently established isologous chimaera to foreign skin-grafts. The results of Experiments 1 and 2 showed that, as with foreign tumour so with foreign skin, there was a prolongation of the period of initial acceptance. The foreign tumour, by virtue of its malignancy, had been able to kill the chimaerical host before sufficient immunity developed. The foreign skin merely remains as a normally functioning homograft apparently until the mechanism of immunity has been restored sufficiently to cause the graft to be shed. When skin which was markedly foreign to CBA (such as C57BL and A) had been applied, it remained anatomically intact and growing hair for about 2 months. It then slowly lost its hair and became scabby, which we take to be the manifestation of an indolent immune response. When the skin graft was from a C3H mouse, historically closely related to, and of the same H-2 phenotype as CBA, the skin was retained even longer and often apparently indefinitely. This was initially interpreted (Loutit; in press) as a phenomenon of actively acquired tolerance,

which was defined as representing 'the specific and systemic failure of the mechanism of the immunological response which is brought about by exposing the mechanism to antigenic stimuli when it is functionally immature'. The definition of Billingham, Brent and Medawar (1956b) limited the condition to induction during embryonic or neonatal life.

ACTIVELY ACQUIRED TOLERANCE

Now that further observations are available, this postulate, that actively acquired tolerance has developed in the regenerating and donor-derived lymphatic tissue of the radiation chimaera, should be re-examined.

We may first consider Experiment 2 (CBA/CBA embryo liver, grafted with C57BL and C3H skins). Assuming that the capacity of the host to respond to foreign murine antigens has been destroyed — an assumption which is justified by the well-established fact that foreign murine cells can proliferate in the lethally irradiated mouse and survive indefinitely (Ford, Ilbery and Loutit, 1957) — the injected tissue and its progeny will be solely responsible for any cellular immune response that may occur. In this case the injected material is foetal, and it might therefore be expected, on exposure to foreign murine antigens, to develop not immunity but tolerance. Table 2 indicates that one of the animals grafted on the day after irradiation retained its C3H skin for more than 200 days; this conforms to conditions which would be acceptable to many as favouring immunological tolerance. However, the same long and possibly permanent persistence of C3H grafts was seen in four animals when grafting was delayed for 14 days after irradiation. It is just possible to believe that the lymphatic tissue will still be in a condition to exhibit tolerance 14 days after the institution of chimaerism and so explain prolonged (though in the case of C57BL less prolonged) survival of skin homografts applied at this time. But it seems less likely that it could still exhibit tolerance after 47 days; yet one C3H graft of three applied at this time was accepted for 85–100 days.

The results of Experiment 1 (CBA/CBA adult marrow) could be explained in a similar way, if one assumed that normal adult marrow would behave immunologically like embryo tissue; this would probably involve its containing no mature lymphatic cells capable of survival and proliferation. Even were the assumption correct, there is still the difficulty of explaining in terms of tolerance the long survival of some C3H grafts applied 35 days after irradiation (Table 1).

Experiment 4 (CBA/CBA marrow from immune donor) also gave results dissonant with an hypothesis of acquired tolerance. For example, CBA mice given marrow from a donor immune to C57BL shed their C57BL grafts more quickly than those given marrow from a normal donor (Fig. 2) — though still much more slowly than do non-irradiated CBA mice. The same is true with grafts of strain C3H on mice given marrow 'immune to C3H' (Fig. 4) and probably also with A grafts in the parallel situation (Fig. 3). This means that some immunity is carried over in the marrow inoculum to the irradiated host. The necessary corollary to this is that at least one cell which is both sensitized and capable of proliferation is present in the inoculum; more probably there are many. In such conditions, it is difficult to see how the prolonged survival of the homografts can be attributable to tolerance, which depends on the lymphatic tissue being immature and non-reactive; except on a hypothesis following Burnet (1959) involving marked clonal selection for 'tolerant' clones, with elimination of 'immune' clones.

There are two further reasons why the long survival of homografts cannot be attributed to tolerance in the sense defined above. Firstly, two out of four C3H grafts survived for

more than 180 days on mice restored with bone marrow and 50 million adult spleen cells from a normal donor; moreover, one out of five survived for a similar period on mice treated with marrow and spleen from a donor sensitized to C3H.

Secondly, in Experiment 7, three out of five mice which had slowly shed C3H grafts failed to give a normally vigorous response to similar grafts applied 2 months later. This is not the behaviour one would expect of animals in which a previously existing state of tolerance had been abolished. The results of this experiment point rather to a general enfeeblement of the immune mechanism towards weak antigens.

ADAPTATION

Another possibility must be considered in relation to the C3H grafts; namely that those which survive for 100 days or so may become adapted to the foreign environment in such a way as no longer to constitute an effective antigenic force. Results of Cannon, Weber and Longmire (1954) and of Weber, Cannon and Longmire (1954) were attributed to adaptation of grafted chicken skin, though Billingham *et al.* (1956b) have argued that they were more probably due to tolerance. The pattern of C3H graft survival (Fig. 4) lends some support to such an interpretation: in each group the minority of grafts which survive for upwards of 100 days are retained indefinitely. The possible mechanisms by which adaptation might be effected need not be discussed, since Experiment 5 showed that adaptation was not primarily, if at all, responsible for the long-surviving grafts. If adaptation had been responsible a second C3H graft applied long after the first would presumably have been shed; but with one exception the mice all accepted their second graft, the exception being a sick animal. The results of this experiment are also of interest in revealing that the mice could not respond to C3H antigens even under the fresh stimulus of a second graft.

OTHER MODIFICATIONS OF IMMUNITY

Barnes *et al.* (1957) considered that some mechanism like 'immunological paralysis' (Felton, 1949) might account for the long survival of some homologous chimaeras which survived the secondary disease (graft-versus-host immunological reaction). The conditions in the present experiments do not include the excess of antigen which obtains in homologous chimaeras. There, the whole of the host is antigen to the newly forming lymphoid tissue. Here, only a small piece of skin is antigen.

The 'enhancing' effect of humoral antibody on the growth of homologous grafted tumours (Casey, Meyers and Drysdale, 1948; Kaliss, 1957) is now well authenticated. In the present experiments, only Experiment 3, where pre-immunized mice were subsequently homografted, provided conditions which are likely to have been (temporarily) suitable for 'enhancement'. In other experiments it could only be important if the chimaera's ability to produce enhancing antibodies recovered before it was able to give other types of immune response (i.e. cytotoxic and cellular). Survival of grafts in Experiment 3 was no longer than in Experiments 1 and 2. Moreover, many of our grafts persisted for times far in excess of those reported for enhanced skin homografts in mice by Billingham, Brent and Medawar (1956a).

MECHANISMS OF HOMOGRAFT-REJECTION IN CHIMAERAS

None of the generally discussed modifications of the processes of immunity seems alone to account for all the results described. It is worth while to summarize what can be fitted into conventional theory and what must be at present left to speculation.

Firstly, we assume that in Experiment 3 the pre-immunized animals have circulating humoral antibodies. They should have had them by theory; and similar animals investigated some years ago in this laboratory certainly had (Barnes and Loutit, 1956). In spite of these presumed antibodies specific for antigens in the skin homograft, the grafts took and flourished. The grafts might have stimulated further production of soluble antibody, since the secondary responses of such antibody-production are much less affected than primary responses, presumably because they are derived from relatively radio-resistant plasmatoid cells. The observation of acceptance and retention of the grafts for a period of many weeks is in accord with the view that humoral immunity is not by itself sufficient to bring about the breakdown of grafts of solid tissue; a cellular response is required. Lymphoid tissue is the generally accepted source of the activated cells. When lymphoid tissue is heavily irradiated, the reticulo-endothelial network survives, but the lymphocytic derivatives are destroyed. This fits in with the concept that it is the lymphocytic derivatives which are the mobile activated agents.

The re-formation of the lymphocytic elements of lymphoid tissue takes some time (Congdon and Urso, 1957) even when lymphoid precursors in infant spleen are given as therapy (Barnes and Loutit, 1956). As noted earlier, the cytological evidence indicates that the reforming lymphatic tissue is derived from the donated cells. The results of the present experiments also suggest that skin homografts were rejected not by a resurgence of the irradiated host's immune mechanism but by activation of lymphatic material elaborated from the injected haematopoietic tissue. When in Experiment 4 animals were restored with bone marrow from immune donors, some acceleration of the homograft response was noted. When lymphoid as well as myeloid tissue was given as treatment the recovery of the homograft response was usually still further quickened.

Foreign skin grafts in our experiments were rejected in descending order of antigenicity — first inter-species, then intra-species with H-2 phenotypes different from the host (i.e. H-2^a, H-2^b), and finally intra-species with the same H-2 phenotype (H-2^k). The fact that rat grafts are shed with a more or less typical, and not greatly delayed inflammatory reaction shows that the chimaera can deal with a foreign graft of strongly antigenic character within a maximum of 30 days, and in most cases considerably sooner.

There are two important features about the homograft responses which we have seen in our experiments. Firstly, they are subnormal. Secondly, they are *differentially* subnormal; the closely related skin was shed more slowly than the more distantly related. (This difference in the speed of response to grafts of different strains is seen also in unirradiated mice, but it is much greater in the chimaera.) There are three distinct ways in which this second feature may be interpreted:

(*a*) There may be an impairment of the chimaera's immunological recognition mechanism, so that the weaker the antigen the more likely it is to escape detection as 'foreign'.

(*b*) Strong antigens may give a bigger stimulus to lymphatic proliferation and maturation than do weaker ones and so, in the chimaera, evoke an earlier and larger response.

(*c*) The grafts may vary in their sensitivity to a given level of immune response: the greater the genetic disparity between graft and host, the more susceptible may the graft be to destruction.

It is quite likely that all these three possibilities may be important. Support for the first is provided by the results of Experiment 4 (normal hosts restored with marrow from

immunized donors). Homografts from the strain to which the marrow donor was immune were shed rather more quickly than similar homografts in Experiments 1 and 2 (non-immune host/non-immune donor). It may be presumed that in this situation a certain number of 'immune cells' were transferred to the host animals; the proliferation of these would render a recognition mechanism in the new host unnecessary, and a faster reaction would be expected.

The first possibility does not itself account for all the results with C3H skin. In none of the experiments, even those in which adult spleen cells were administered, were 100 per cent of C3H skin-grafts shed, although what shedding there was generally took place earlier in Experiments 4 and 6 than in other experiments. It appears, therefore, that, although the provision of 'immune cells' speeded the rejection of some C3H grafts, other such grafts were able to survive indefinitely notwithstanding their having presumably been 'recognized' as foreign. A proportion even survive in the presence of mature sensitized lymphoid cells (Experiment 6). Moreover in Experiment 7, mice which had shed (though slowly) their initial C3H grafts, again failed to give a reaction of anything approaching normal speed when re-grafted with similar skin.

The evidence suggests that the chimaera is deficient not only in its recognition of antigens, but in its capacity to mount an effective response once they have been recognized. It seems that the weaker the antigenicity of a graft, the higher the level of immunological competence which is required to shed it. The CBA/CBA chimaera may in some cases permanently fail to attain the required level. Whether this is because it is simply unable to mobilize an adequate number of lymphoid cells, or because some other factor is missing, we do not know. Animals which retain their foreign grafts appear no less healthy than those which reject them, so there is probably no deficiency of bacterial immunity.

CONCLUSIONS

We have reviewed possible explanations for the prolonged survival of skin homografts reported in this paper. More work of a serological and histological nature is needed to explain our findings. Meanwhile, two factors appear to us to be principally involved:

1. A weakening of the mechanism by which antigens are 'recognized'.
2. An overall central depression of the immune system. This may result from some inadequacy in quantity or quality of the immunologically reactive cells derived from the donor (presumably lymphoid cells) and, if the host's system plays any supporting role, damage to that system which can not be functionally restored by bone marrow or spleen therapy.

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