

ALLOGENEIC THYMUS GRAFTS AND THE RESTORATION OF  
IMMUNE FUNCTION IN IRRADIATED THYMECTOMIZED  
MICE\*, ‡

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In the course of studying the recovery (1) of hemolytic plaque-forming cells in the spleen of lethally-irradiated thymectomized mice, we observed that allogeneic thymus grafts, unlike syngeneic ones, did not support this recovery. This failure was contrary to expectations based on published experiments (2-5), and a detailed investigation was therefore undertaken to elucidate the question. In particular, it seemed important to determine whether the immunological deficiencies of the thymus-deprived animal could be divided into those restored by thymus homografts and those not restored by this maneuver, a finding which would support a dual mechanism of thymus action. Furthermore, such an investigation bears on the important and still unanswered question of humoral vs. cellular means of thymus action. The present experiments concern restoration of a variety of immune functions, both cellular and humoral, in irradiated thymectomized mice grafted with thymuses from several allogeneic strains.

*Materials and Methods*

*Animal.*—Inbred mice of various strains were obtained from the Jackson Laboratories, Bar Harbor, Maine, and during experiments were maintained in an isolated caging system (Carworth Farms, New City, N. Y.) with acidified drinking water (addition of 5 ml of 12 N HCl per 12 liters water to produce a pH of 2.5). Pregnant mice (12-14 days) were obtained from the same laboratory.

*Experimental Design.*—The details were similar to those in previous publications (1, 6, 7). Adult mice (female, 8-10 wk old) were thymectomized, lethally irradiated (875 R, 280 kv, 1.4 mm Cu, 67 R/min), and injected intravenously with 4-5 million marrow cells obtained from the femurs of animals of the same strain and sex. Thymectomy was done within the week prior to irradiation, and marrow grafting in the 4 hr after irradiation. Thymus grafting was performed with the technique of East and Parrott (8); the day after irradiation a single whole

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newborn (less than 36 hr old) thymus was placed under the kidney capsule. (In several experiments, mentioned separately in the text, the time of thymectomy or of thymus grafting was delayed). The usual experiment contained 3 groups of 16 animals, irradiated control, thymectomy control, and thymectomy with thymus graft. Mortality for the experimental period was quite variable from experiment to experiment, and deaths tended to occur within particular isolated cages. Overall only about one-half the animals survived the entire experiment, but there was no excess mortality in any experimental group, and no disorder was seen that resembled the wasting disease of thymectomized newborns. Only data from surviving animals were employed.

30 days after irradiation animals received full thickness skin grafts by conventional grafting technique (9). The plaster cast was removed on the 10th or 11th day, and grafts were scored visually on alternate days for the next 2 wk and weekly thereafter. In those experiments in which the animals' spleens were plaqued, the skin graft could only be followed for 50 days, in those subject to graft vs. host assay for 100 days, and in the others for 140 days. Thus all immunological parameters were not studied in a single experiment. All animals were subject to postmortem examination and animals with residual thymus were discarded from consideration. (However, it should be remarked that the thymus of normal CBA mice 140 days after lethal irradiation may be a small unimpressive structure weighing only a few milligrams, and that therefore a thymic remnant could be missed). Thymus grafts were examined and weighed at postmortem.

*Hemolytic Plaque Assay.*—The method of Jerne et al. (10) as previously modified (6) was employed to determine the number of hemolysin-producing spleen cells. Animals were challenged with 0.2 ml of 10% sheep cells ( $5 \times 10^8$  cells) 75 days after irradiation, and were sacrificed 4 days later. An appropriate sample of sieved spleen was incubated with sheep erythrocytes in Gey's solution which was gelled by the addition of Agarose. The gelled suspension was incubated for 2 hr at 37°C, 1 ml of 1:10 guinea pig serum was added, and the suspension incubated for an additional hr to develop the direct (19S) plaques.

*Graft-Vs.-Host Assay.*—A published method was used (11). Newborn F<sub>1</sub> mice of appropriate genotype (CBA  $\times$  DBA/2 or CBA  $\times$  A) were injected when less than 24 hr old. Sieved spleen cells (Medium 199) from three animals of each experimental group (irradiation control, thymectomy control, and thymectomy plus thymus graft) were pooled and injected intraperitoneally into appropriately marked newborns. Five to six newborns were employed to assay each spleen cell pool, and blank animals received only Medium 199. Animals were sacrificed 9 days after injection and spleen and body weight determined.

*Hemagglutinin and Hemolysin.*—Animals were challenged intravenously with 0.2 ml of 10% sheep cells and bled from a tail vein 7 days later. Sera were assayed with fresh sheep cells, with and without the addition of complement, employing the Microtiter apparatus and two-fold dilutions (12).

*Calculation of Per Cent Restoration of Graft Rejection.*—Because of the large number of skin and thymus graft combinations employed, a simple calculation was devised to express restoration in each skin/thymus combination by a single per cent figure. Grafts rejected in less than 17 days are assigned an arbitrary value of 1, those rejected in 17–25 days a value of 2, those rejected in 26–50 days a value of 3, and those surviving more than 50 days a value of 4. (Where grafts are followed for 100 days, a value of 5 is assigned to each graft surviving more than 100 days). The calculation is made separately for each skin/thymus combination by multiplying the number of animals whose grafts were rejected during a particular time period by the appropriate value and summing these products for each group (irradiated control, thymectomy control, and thymectomy with thymus graft) in the particular combination. The sums are then corrected for the varying numbers of animals in each group: the sum for the irradiated control group is multiplied by the ratio of animals in that group to the number of thymectomy

controls; the thymectomy graft sum is multiplied by the ratio of animals in that group to the number of thymectomy controls; the sum for the thymectomy control group remains unmodified. The potential restoration of graft rejection is defined as the sum for the thymectomy control group minus the corrected sum for the irradiation control group, and the observed restoration for the graft defined as the sum for the thymectomy control group minus the corrected sum for the thymectomy thymus graft group. By dividing the figure for observed (thymus graft) restoration by the figure for potential restoration and multiplying by 100, the resulting number is an estimate (in per cent) of restoration of that particular skin homograft rejection by that thymus graft. These calculations have been made for the data in Table I and the results are presented in Table II.

#### RESULTS

*Skin Grafting in CBA Mice.*—Table I presents data on skin homograft survival in unoperated and control thymectomized CBA mice, and in thymectomized animals bearing various thymus grafts under the kidney capsule.

Without thymus grafting (Table I), the prolongation of skin graft survival in irradiated thymectomized CBA mice is quite variable. When the incompatibility lies outside the H-2 locus (AKR, C57BR/cd, and C3H) skin grafts are accepted permanently, but with H-2 incompatibility this is not the case. The results with DBA/2 skin are typical, with only 10 of 39 grafts retained beyond 100 days and 4 grafts rejected normally. This great spread of rejection time is also seen in thymectomized CBA mice with C57BL, BALB/c, DBA/1, and SJL skin. Strain A skin is exceptional among the H-2 incompatible combinations, and like non-H-2 homografts, it is permanently retained by thymectomized CBA mice. It is evident that A skin also enjoys a longer survival time in the intact controls than do the other H-2 incompatible skin grafts (14.2 days as compared with 11.5–12.6 days for the others).

The data from Table I on the restorative capacity of various thymus grafts on skin homograft rejection has been summarized in Table II. Certain conclusions are evident. Isogenic thymus (CBA) completely restores skin graft rejection, and allogeneic H-2 *compatible* thymus (AKR) restores graft rejection even of skin with minor histocompatibility differences (with the exception of skin from the thymus donor).

The results from grafting thymuses differing at the H-2 locus are more complex. C57BL thymus completely restores the rejection of skin from this strain, while A thymus similarly restores the rejection of A skin (Table II). Indeed, by examining the data in Table I for mean rejection times, it is clear that A and C57BL thymus grafts lead to more rapid rejection of the respective skin than is seen in nonthymectomized controls. However, there appears to be only slight restoration of the rejection of third party homografts (13–17%) with the exception of DBA/2 and BALB/c (both H-2<sup>b</sup>) where animals are restored to approximately 75% of normal. H-2 incompatible thymus homografts do not restore rejection of skin differing at minor (non-H-2) histocompatibility loci (AKR).

TABLE I  
*Skin and Thymus Grafting in Normal and Thymectomized CBA Mice*

Skin graft	Thymectomy	Thymus graft	Number of mice with skin graft survival of					Average survival
			<17 days	17-25 days	26-50 days	>50 or 51-100 days	>100 days	
C57BL	-	-	33	0	0	0		12.1 ± 0.9
C57BL	+	-	4	12	5	10		
C57BL	+	A	14	6	3	9		
A	-	-	27	0	0	0		14.2 ± 0.7
A	+	-	1	3	2	26		
A	+	A	20	1	2	3		11.4* ± 0.9
C57BL	-	-	27	1	0	0		12.2 ± 0.6
C57BL	+	-	1	4	10	20		
C57BL	+	C57BL	23	0	0	0		10.7 ± 1.3
C57BL	+	AKR	16	1	0	0		
C57BL	+	CBA	8	0	0	0		
C57BL	Late†	-	1	4	5	2		
A	-	-	28	0	0	0		14.2 ± 1.6
A	+	-	0	1	2	20		
A	+	C57BL	3	0	2	15		
DBA/2	-	-	29	0	0	0	0	12.6 ± 1.4
DBA/2	+	-	4	6	11	8	10	
DBA/2	+	C57BL	25	3	0	6	0	
BALB/c	-	-	27	0	0	0		11.8 ± 1.9
BALB/c	+	-	5	5	4	20		
BALB/c	+	C57BL	32	4	3	3		
BALB/c	+	CBA	7	0	0	0		
DBA/1	-	-	20	0	0	0		11.5 ± 1.0
DBA/1	+	-	1	2	3	11		
DBA/1	+	C57BL	1	5	5	7		
SJL	-	-	29	0	0	0		12.2 ± 1.6
SJL	+	-	5	3	9	7		
SJL	+	C57BL	11	3	6	10		
AKR	-	-	7	7	0	0	0	16.2
AKR	+	-	0	0	0	0	16	
AKR	+	C57BL	0	0	0	0	5	
AKR	+	AKR	0	0	0	0	14	

TABLE I—*Concluded*

Skin graft	Thymectomy	Thymus graft	Number of mice with skin graft survival of					Average survival
			<17 days	17-25 days	26-50 days	>50 or 51-100 days	>100 days	
AKR§	—	—	3	0	0	0	0	11.7
AKR§	+	—	0	1	1	0	5	
AKR§	+	AKR	1	1	0	0	4	
C57BR/cd	—	—	3	5	1	0	0	19.1
C57BR/cd	+	—	1	0	0	1	10	
C57BR/cd	+	AKR	2	8	0	0	0	17.9
C3H	—	—	0	6	3	0	0	24.4
C3H	+	—	0	0	0	0	10	
C3H	+	AKR	1	7	1	1	0	26.2

\* Average excludes 5 grafts rejected beyond 25 days.

‡ Thymectomy delayed 14 days after irradiation and bone marrow grafting.

§ Thymus grafting delayed 70 days after irradiation and bone marrow grafting.

TABLE II

*Restoration\* of Skin Graft Rejection by Thymus Grafting in CBA Mice (H-2<sup>k</sup>)*

Strain of donor skin	H-2	Strain of donor thymus			
		C57BL	A	AKR	CBA
C57BL	b	101	27	99	101
A	a	13	80		
DBA/2	d	74			
BALB/c	d	72			
DBA/1	q	17			
SJL	s	14			
AKR	k	0		0	
C57BR/cd	k			106	
C3H	k			103	

\* Results expressed as the per cent of thymectomy impairment restored by thymus graft. Derived from data in Table I by calculations described in the text.

*Skin Grafting in C57BL Mice.*—Lethal irradiation and bone marrow grafting do not significantly delay rejection of H-2 incompatible homografts in the immunologically more reactive C57BL mouse (Table III). To demonstrate significant prolongation of homograft rejection in thymectomized animals of this strain, it is necessary to graft from strains such as the 129 (Table III) which differs at a locus other than the H-2. This markedly limits the studies that can

be done in C57BL mice, and for this reason thymus grafting experiments were not undertaken in this strain.

*Graft-Vs.-Host Assays.*—Graft-vs.-host assays are consistent with the skin graft work in demonstrating the immunological reactivity of thymectomized

TABLE III  
*Skin Grafting in Normal and Thymectomized C57BL Mice*

Source of skin	Thymectomy	Number of mice with skin graft survival of					Average survival
		<17 days	17-25 days	26-50 days	51-100 days	>100 days	
C57BR/cd	—	7	0	0	0	0	12.0 ± 0.6
	+	4	1	0	0	0	
CBA	—	5	0	0	0	0	
	+	5	0	0	0	0	
BALB/c	—	5	0	0	0	0	
	+	5	0	0	0	0	
129/J	—	8	0	0	0	0	11.8 ± 0.6
	+	0	2	2	1	3	

TABLE IV  
*Graft-Vs.-Host Assay\* in Normal and Thymectomized CBA Mice with and without Thymus Grafts*

Assay strain	Source of spleen cells				
	Irradiated control	Irradiated thymectomized	Irradiated thymectomized C57BL thymus	Irradiated thymectomized A thymus	Blank
DBA/2 × CBA	6.2 ± 2.0	4.6 ± 1.1	6.0 ± 2.1		4.6 ± 0.6
DBA/2 × CBA	6.5 ± 1.1	4.8 ± 0.9		5.7 ± 2.2	4.8 ± 1.8
A × CBA	6.7 ± 1.3	5.7 ± 0.8	6.1 ± 0.9		5.9 ± 0.8

\* Ratio of spleen weight (milligram) to body weight (gram). Each number is the average of 30-48 animals ± standard deviation.

CBA mice with or without allogeneic thymus grafts (Table IV). This assay detects little reactivity in thymectomized irradiated animals, but a restoration of reactivity towards DBA is observed after either C57BL or A thymus grafting. There is also probably some restoration of reactivity towards A mice after C57BL thymus grafting, but the data are not conclusive.

*Hemolysins, Hemagglutinins, Hemolytic Plaques, and Lymphocyte Counts in Normal and Thymectomized CBA Mice with and without Thymus Grafts.*—The

data in Table V indicate a marked depression of both hemolysins and hemagglutinins in irradiated thymectomized mice and no significant restoration after grafting with either C57BL or A thymuses. (The possible exception is the several animals showing  $\log_2$  antibody titers of 8 or above after grafting.) Similarly, Table VI demonstrates that allogeneic thymus grafts incompatible at the H-2 locus (A and C57BL) do not restore the marked depletion of hemolytic

TABLE V  
*Antibody Levels in Normal and Thymectomized CBA Mice with and without Thymus Grafts*

	Thymectomy	Thymus graft	Number of mice with $\log_2$ antibody titers of									
			<3	3	4	5	6	7	8	9	10	>10
Hemagglutinin	-	-	3	3	0	3	4	16	39	28	14	6
	+	-	86	13	10	9	2	1	0	0	0	0
	+	C57BL	61	6	7	2	2	1	2	2	0	1
	+	A	24	3	4	1	1	1	1	0	0	0
Hemolysin	-	-	0	0	1	5	3	17	19	21	13	15
	+	-	60	19	12	4	3	2	0	0	0	1
	+	C57BL	49	7	6	2	0	2	0	2	1	1
	+	A	17	3	3	0	1	0	1	0	1	1

TABLE VI  
*Hemolytic Plaque Response in Normal and Thymectomized CBA Mice with and without Thymus Homografts*

Thymectomy	Thymus graft	Weight of thymus graft	Plaques/spleen	Standard deviation	Number of animals
		mg			
-	-		38,400	$\pm$ 25,100	65
+	-		1,500	$\pm$ 1,700	43
+	C57BL	0	1,300	$\pm$ 1,600	59
+	A	0	1,750	$\pm$ 900	12
+	AKR	59 $\pm$ 21	36,300	$\pm$ 31,800	22

plaque-forming cells in the spleen of irradiated thymectomized mice. Thymus allografts compatible at this locus (AKR) do restore such animals. It should be noted that while A and C57BL thymus grafts are identifiable only as minute scars on the kidney capsule (6 wk after thymus grafting), the H-2 compatible AKR thymus allograft achieves a weight comparable to that of a thymus isograft (cf. reference 1, Table VI).

There is also no evidence that C57BL thymus allografts restore the depressed lymphocyte count observed in irradiated thymectomized CBA mice. 10 wk after irradiation and thymectomy, the values observed in groups of 24 animals

are: intact controls  $5950 \pm 2300$ ; thymectomy controls  $3500 \pm 1300$ ; and thymectomy and C57BL thymus graft  $3200 \pm 1800$ .

#### DISCUSSION

The extensive immunological impairment of thymectomized irradiated adult CBA mice reported here is consistent with earlier observations (13, 14). Thus almost complete depression of graft-vs.-host activity and hemolytic plaque-forming ability of the spleen, hemolysin and hemagglutinin formation, and rejection of skin homografts with minor (non-H-2) genetic incompatibility were demonstrated. However, lymphocyte counts were but moderately depressed, and rejection time for skin with major (H-2) incompatibility was remarkably erratic with rejection times for most H-2 incompatible strains bridging the entire spectrum from normal rejection to permanent acceptance. (Strain A skin was exceptional with permanent acceptance observed in thymectomized CBA mice and unusually long survival, for H-2 incompatible skin, in nonthymectomized animals.) This erratic rejection is reminiscent of the sporadic defect observed by Humphrey et al. (15) in the ability of neonatally thymectomized mice to form various antibodies. These authors suggested that the presence or absence of response to a particular antigen was dependent upon whether or not seeding by the thymus had taken place in the newborn prior to thymectomy. We have no alternate explanation, but the proposal of Humphrey et al. seems unlikely in the thymectomized irradiated adult mouse in which complete thymectomy is easily performed and in which lethal irradiation obliterates the immunologically responsive cells.

Our results with C57BL animals clarifies the inconsistency of earlier work on thymus function in this strain (14). It is clear that even after thymectomy and irradiation the C57BL mouse possesses sufficient immunological reactivity to reject H-2 incompatible skin. Homografts compatible at the H-2 locus must be used to demonstrate convincingly thymus function in these animals.

The central question we set out to answer in this investigation is whether a thymus homograft, despite its own rejection, is able to restore some or all of the immunological deficit of the thymectomized animal. Such restoration would provide strong evidence for humoral mediation of thymus activity.

Thymus homografts with H-2 compatibility do not answer this question, since such grafts are permanently accepted. Specific immunological tolerance can be demonstrated by survival of the thymus and of subsequent skin grafted from the same strain. Animals restored with such H-2 compatible thymus grafts reject third party (H-2 compatible) skin grafts normally, and produce a normal complement of plaque-forming spleen cells in response to antigenic stimulation. The effectiveness of the H-2 compatible thymus graft is emphasized by its ability to restore immune responsiveness when the thymus is grafted even 10 wk after thymectomy and irradiation.



This induction of specific tolerance to H-2 compatible skin homografts with thymus deserves comment. Does this observation substantiate the view of Isakovic, Smith, and Waksman (16) that the thymus determines immunological tolerance? In this connection it would be important to establish whether the host tissues are populated by host lymphoid cells or by lymphoid cells of the genotype of the grafted thymus. Unfortunately, graft-vs.-host assays can not be applied to such instances where the incompatibility lies outside the H-2 locus (11), and the available chromosome marker study gives inconclusive results (3): cells of both host and thymus donor genotype are found in lymph nodes and spleen. Thus it remains plausible that the tolerance induced by H-2 compatible thymus homografts represents a conventional tolerance mechanism in which developing lymphoid cells are presented with large amounts of histocompatibility antigen.

With the crucial H-2 *incompatible* thymus graft, interpretation of results is complex because of overlapping of the H-2 alleles. When the thymus graft is followed by a skin graft of the same strain, accelerated skin rejection indicates that the thymus graft has immunized the recipient. Clearly, such rejection of skin from the same strain as the thymus graft can be explained by immunization without any restoration of immune function. When third party skin grafts are applied the results are variable; slight to moderate restoration is seen with various H-2 incompatible strains. Interpretation of experiments employing CBA recipients is ambiguous because all available thymus-skin combinations (including the ones used in these experiments) allow overlapping of part of the H-2 locus between thymus and skin donor. For example, all the strains studied, except the CBA, possess the 6 and 28 components of the H-2 locus (17). (Strain A recipients might permit this immunogenetic difficulty to be circumvented). Thus, immunization cannot be excluded as an explanation for restoration of third party skin graft rejection in CBA mice. The same arguments apply to restoration of graft-vs.-host activity since the histocompatibility relationships are the same.

We have not observed the return of immune function other than rejection of H-2 incompatible skin and graft-vs.-host activity after grafting with H-2 incompatible thymuses. There is no restoration of rejection of H-2 compatible skin homografts, or of hemolytic plaque-forming spleen cells, hemolysins, hemagglutinins, or peripheral lymphocyte counts. This contrasts sharply with H-2 compatible thymus grafts, where survival of the graft is associated with return of all these immune functions. We are therefore led to conclude that the reconstitution observed with H-2 incompatible thymus grafts can be satisfactorily ascribed to immunization by the thymus graft and does not necessarily indicate either immune restoration or humoral thymus function. It is evident from these experiments that thymus grafts placed beneath the kidney capsule serve as an effective means of immunization. The proliferating graft presents large amounts of histocompatibility antigen to the recipient's lymphoid system,

and depending upon the histocompatibility differences between host and graft, there results either tolerance or immunization.

Thymus homografts have been used in several earlier investigations. The study of Globerson and Feldman in mice (4) suffers from the same interpretive difficulty discussed above, i.e., since third party skin grafts were not employed the thymus graft may immunize rather than restore. Miller, Leuchars, Cross, and Dukor (2), and later Leuchars, Cross, and Dukor (3) have done studies in mice similar to our own but less extensive, with similar skin grafting results. However, these authors observed some restoration of hemolysin and hemagglutinin response, a discrepancy that is difficult to explain since some of the present work was repeated with express attention to their experimental details. Another pertinent investigation is that of Law (5) who employed xenogeneic (rat) thymus grafts in mice. Law reports restitution both of third party (mouse) skin graft rejection and of hemolysin and hemagglutinin formation. The results with rat thymus xenografts are also difficult to reconcile with the present work, but it should be pointed out that other workers (18) have been unable to restore immune function using the rat/mouse system.

In balance then, the case for significant immunological restoration by thymus homografts (with H-2 incompatibility) remains to be established. Clearly, the present studies do not prove that such restoration does not occur, nor do they disprove the existence of a thymic hormone. However, this investigation does demonstrate that immunization of the recipient by the strategically placed thymus graft is a significant event and can satisfactorily explain the degree and parameters of immune recovery seen in the CBA mouse.

#### SUMMARY

Irradiated and thymectomized CBA mice are markedly depressed in several immunological parameters (skin homograft rejection, graft-vs.-host activity and hemolytic plaque-forming cells of the spleen, hemolysin and hemagglutinin formation, and peripheral lymphocyte counts). In the present experiments the ability of homografts of neonatal thymus placed beneath the kidney capsule to restore immunological capacity of such animals was studied.

Thymus homografts which share the same H-2 locus with the CBA mouse were permanently tolerated and immunological restoration was complete. Skin from the thymus donor was specifically retained, but third party skin with even minor (non-H-2) incompatibility was normally rejected and hemolytic plaque-forming cells of the spleen were restored.

Thymus homografts which differ at the H-2 locus were promptly rejected and led to accelerated rejection of skin subsequently grafted from the thymus donor. With such H-2 incompatible thymus grafts, third party skin with minor histoincompatibility was retained while there was slight to moderate restoration of rejection of skin with major (H-2) incompatibility. Graft-vs.-host activity was

restored, but there was no return of plaque-forming spleen cells, hemolysins, hemagglutinins, or peripheral lymphocyte counts.

In view of the cross-reactivity at the H-2 locus in CBA mice between thymus and third party skin donors, it was felt that restoration of skin rejection and graft-vs.-host activity could be adequately explained on the basis of immunization by the thymus graft and did not require the postulation of true immune restoration or a thymus hormone.

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