THE ROLE OF PASSENGER LEUKOCYTES IN THE ANOMALOUS SURVIVAL OF NEONATAL SKIN GRAFTS IN MICE*

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In mice (1–3), as in other species (4, 5) including man (6), orthotopic skin transplants from genetically disparate fetal or neonatal donors often persist significantly longer than those from adults. Indeed, when *H-2* compatibility exists, such transplants may survive indefinitely, in many cases abrogating their hosts' capacity to reject subsequent adult tissues bearing the same transplantation antigens (1, 2, 7). For example, C57BL/6 female mice regularly reject *H-Y*–incompatible skin isografts from adult males (median survival time, 24.5 days), but $^{34}/_{46}$ (74%) accept those from newborn males (8). Of those females which tolerate neonatal male grafts, half are unresponsive to subsequent adult male grafts as well (8).

Even when stronger histoincompatibilities prevail, neonatal transplants exhibit preferential survival. Thus, skin homografts from infant C3H/HeJ mice may persist permanently on CBA/Ss recipients, and may render these hosts tolerant of simultaneous or subsequent adult skin homografts of similar origin (9).

Preliminary observations indicate that this privilege extended to neonatal skin homografts, including their ability to induce tolerance, may be related to the emigration of donor leukocytes from the graft vasculature (9). The experiments reported below were therefore designed to clarify the role of these "passenger" cells.

Materials and Methods

Animals.—The following isogenic strains of mice and their F_1 hybrids were used: CBA/Ss (CBA),[1] C3H/HeJ (C3H), A/Ss (A), and C57BL/6 (C57). CBA and C3H mice are both

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[1] *Abbreviations used in this paper:* A, strain A/Ss; C57, strain C57BL/6; CBA, strain

H-2^k (10) but differ by at least 11 other histocompatibility factors (11). Mice of strain A are *H-2*^{a} (10); those of strain C57, *H-2*^{b} (10). C3H mice were purchased from the Jackson Laboratories;[2] the others were derived from domestic sublines.

Grafting.—Full-thickness skin grafts measuring approximately 2.0 × 1.2 cm (comprising about one-half the integument of a newborn mouse) were obtained from fetal mice or from those less than 24 hr old. These grafts were transferred to adult males and virgin females of from 90 to 120 days of age according to procedures fully described elsewhere (12). Adult grafts of the same size and shape were taken from mice at least 90 days old. Male skin was not transplanted to female recipients except in C57 mice or as described below. Bandages were removed on the 8th–10th postoperative day and graft survival appraised daily thereafter. Animals which accepted fur-bearing grafts for more than 100 days were considered "tolerant."

Cell Suspensions.—Whole blood was drawn into heparinized[3] containers from the retro-orbital plexus of adult mice or from the heart of neonatal animals, and placed immediately into small Pyrex tubes[4] containing sodium citrate[5] and dextran.[6] Red blood cells were allowed to settle for 20–30 min after which the leukocyte-rich supernatant was removed. The erythrocyte fraction was resuspended in an equal volume of Hanks' balanced salt solution[7] (HBSS) containing citrate and dextran and allowed to sediment a second time to increase the yield of white cells. Leukocytes were then pooled, centrifuged at 800–900 rpm for 5 min, washed twice in HBSS, and then resuspended in a convenient volume of HBSS. With this method, it was possible to collect at least 5 million leukocytes from each adult mouse, and from 0.5 to 1 million leukocytes from each neonatal donor.

Cell suspensions were also prepared from the liver, spleen, lymph nodes, thymus, and bone marrow of adult mice, from the thymus and spleen of neonatal animals, and from the liver of fetal mice according to procedures described elsewhere (13).

Counting was performed with a hemacytometer under a phase-contrast microscope. Viability of cells within the suspensions, as determined by trypan blue exclusion, was always greater than 90%.

Cells were suspended in 0.5 ml of HBSS and injected intravenously into the lateral caudal vein, subcutaneously above the cervical vertebrae, or intraperitoneally.

Irradiation.—Performed as previously described (9).

Statistics.—Median survival times (MST) were computed by the method of Litchfield (14). Statistical significance was determined by chi-square analysis or by the *t* test using mean survival times and appropriate standard errors (15).

Tolerant Mice.—Strain A mice were rendered tolerant of C3H skin grafts by inoculating them intravenously with from 15 to 20 million (C3H × A)F_1 hybrid lymphocytes within 24 hr of birth (13, 16).

RESULTS

Control Data.—The survival times of neonatal and adult C3H skin grafts on male and female CBA recipients were reported previously (9). Both male and female CBA mice reject adult C3H skin grafts with equal promptitude (MST,

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CBA/Ss; C3H, strain C3H/HeJ; Con-A concanavalin A; HBSS, Hanks' balanced salt solution; MST, median survival time(s).

[2] Jackson Laboratories, Bar Harbor, Maine.

[3] Heparin; sodium heparin injection, Frank E. Lentz Wholesale Drug Co., Philadelphia, Pa.

[4] Bellco Glass Inc., Vineland, N. J.

[5] Sodium citrate solution 2.5%, Frank E. Lentz Co., Philadelphia, Pa.

[6] Dextran; mol wt 240,000; Pharmachem Corp., Bethlehem, Pa.

[7] Grand Island Biological Co., Grand Island, N. Y.

16.0 days), but they are not equally reactive toward neonatal C3H skin grafts. Whereas only 19% of the neonatal homografts are accepted by female CBA recipients, 78% of the infant to male grafts persist beyond the 100 day observation period. This latter response also occurs in CBA females when newborn grafts of F_1 hybrid origin are used. Thus, 77% of female CBA mice exposed to neonatal skin grafts from (C3H × CBA) F_1 mice are unresponsive to these infant homografts and to subsequent grafts from adult C3H animals as well (9). Nevertheless, in view of the sex difference of CBA mice in their reactivity to C3H newborn grafts, male CBA mice were employed primarily in the experiments reported below.

Importance of the Persistence of the Neonatal C3H or (C3H × CBA)F_1 Graft in Maintaining Tolerance of Adult C3H Skin in Unresponsive CBA Mice.—To determine whether continued exposure to neonatal grafts is essential for the

TABLE I

*Survival of Adult C3H Skin Grafts on Adult CBA Mice Exposed to Transient Neonatal Skin Transplants from C3H or (C3H × CBA) F_1 Hybrid Mice**

Donor	Recipient	Number	Distribution of GST‡ in days
C3H	CBA ♂♂	12	7 > 100, >87§, 36, 34, 2 × 9
F_1	CBA ♂♂	2	2 > 100
F_1	CBA ♀♀	8	8 > 100

* Recipients challenged with adult skin grafts 50 days after neonatal grafts of 100 days' standing removed.

‡ GST, graft survival times.

§ Animal died with graft intact.

induction of tolerance to adult C3H grafts in CBA mice, as is the case in C57 females with respect to *H-Y* (8), the following experiment was performed. 22 CBA mice bearing C3H or (C3H × CBA)F_1 neonatal grafts of 100 days' standing were anesthetized and the grafts carefully excised. After 50 days, each of these mice was rechallenged with an adult C3H skin graft. Whereas two of the recipients rejected their test grafts in accelerated fashion, and two rejected them after chronic reactions, 18 (82%) failed altogether to reject the adult transplants (Table I). Thus, in contrast to the results obtained with C57 mice (8), the continued presence of the neonatal graft is not requisite to the maintenance of unresponsiveness in the C3H-CBA strain combination.

Effect of Irradiation of the C3H Donor before Skin Grafting.—If the persistence of tolerance in the C3H to CBA strain combination is dependent upon the persistence of antigen, it remained necessary to determine the precise nature of the continuing antigenic stimulus. A likely explanation was that passenger leukocytes had emigrated from the blood vessels of the neonatal grafts and were persisting in the host. To determine if the unresponsiveness of CBA mice exposed to transient C3H neonatal grafts might be correlated with the survival of these

passenger leukocytes, C3H newborn mice were treated with 850 R whole body X-ray, a dose sufficient to destroy their lymphoid tissues (17, 18), just before their use as skin graft donors for a panel of adult male CBA mice. A panel of isogenic adult recipients were similarly challenged to determine the effects of irradiation per se upon the survival of these infant grafts.

Whereas all ($^{15}/_{15}$) of the isografts survived permanently and in excellent condition, only $^{1}/_{19}$ (5.3%) of the homografts survived for more than 100 days (Table II). This latter result differs significantly from the 78% permanent survival of unirradiated infant C3H grafts on adult CBA male hosts. Moreover, the one X-rayed graft which did persist, unlike those from unirradiated donors, failed to render its host unresponsive to a subsequent adult C3H graft.

In spite of these observations, it could be argued that the large X-ray dose

TABLE II

Survival of C3H Neonatal Skin Grafts after Irradiation of the Donor with 850 R X-Ray

Recipients		Days after transplantation									MST	SD
		0	10	12	14	16	18	20	28	>100		
CBA ♂♂	N*	19	14	12	9	8	6	4	1	1§	13.8	1.58
	%‡		74	63	47	42	32	21	5			
C3H ♂♂♀♀	N	15								15		

* N, number of original grafts surviving.

‡ %, percentage of original grafts surviving.

§ This animal rejected a subsequent adult C3H skin transplant.

acted synergistically with the normal immune response of the host to destroy the infant grafts, and that their demise was unrelated to the elimination of their leukocytic moiety. Indeed, skin transplants from 850 R irradiated adult C3H mice also fared significantly less well than similar grafts from untreated donors on CBA hosts. The MST of 17 such grafts was only 11.2 days compared to that of 16.0 days for grafts from unirradiated C3H animals ($P < 0.001$). This failure of irradiated grafts to fare as well as normal grafts may have resulted from the disruption of cells by X-irradiation and the consequent release of transplantation antigen in a highly immunogenic form. Also, healing-in difficulties may have contributed to their destruction (19, 20). The next series of experiments was therefore undertaken to test further the hypothesis that tolerance induction by the infant grafts is dependent upon their passenger leukocytes gaining access to the host.

Analysis of the Chimeric Status of CBA Males Rendered Tolerant with C3H Neonatal Skin Grafts.—If neonatal leukocytes of graft origin persist and proliferate in their CBA hosts, it should be possible to detect their presence. Accordingly, the following test was employed. Male CBA mice bearing infant

C3H skin grafts in impeccable condition for more than 100 days were sacrificed and their various lymphoid organs removed. Separate suspensions of at least 40 million cells were prepared from the thymus, lymph nodes, and spleen (13). Each of these suspensions was injected intraperitoneally into panels of adult CBA males and females. After 7 days, these hosts received skin homografts from adult C3H mice. Amost without exception, these skin grafts were acutely rejected, indicating that their host had been sensitized against C3H tissues by the cellular inocula prepared from the homograft-bearing isogenic donors. More specifically, the MST of the adult C3H skin grafts was 7.5 days on those CBA

TABLE III

Survival of Adult C3H Skin Grafts on Adult Male and Female CBA Mice Exposed to Isografts from CBA Males Bearing Neonatal C3H Skin Grafts >100 Days

Isograft tissue		Days after transplantation											MST	SD
		0	7	8	9	10	11	12	14	16	18	20		
LN*	N	8	5	3		1		0					7.5	1.27
	%		63	38		13								
Spl‡	N	11	8	2	1	0							7.5	1.12
	%		73	18	9									
Thy§	N	8	6	5	4	3	1	0					9.0	1.41
	%		75	63	50	38	13							
Skin	N	22	21	20	18	15	13	7	3		2	0	11.2	1.28
	%		95	91	82	68	59	32	14		9			

* LN, lymph nodes.
‡ Spl, spleen.
§ Thy, thymus.

mice which had been exposed to isologous splenic leukocytes from animals bearing C3H neonatal skin grafts, 7.5 days on those inoculated with lymph node cells from such animals, and 9.0 days on those which had received thymocytes (Table III). All of these results are very significantly different ($P < 0.001$) from the 16.0 day MST of adult C3H grafts on untreated CBA hosts.

Inasmuch as homograft immunity has been produced with skin isografts from "radiation chimeras" (21, 22) and from mice made immunologically tolerant by neonatal inoculation with allogeneic bone marrow or spleen cells (21), it remained to be seen whether skin isografts from CBA males bearing neonatal C3H transplants of 100 days' standing might also sensitize their isogenic hosts. Accordingly, skin from such CBA males was transplanted to 22 adult male and female CBA mice, each of which received a contralateral C3H adult skin homograft 7 days later. Whereas all of the skin isografts survived permanently, some exhibiting transient minor necrotic lesions, the MST of the homografts was

11.2 days, significantly disparate from that of 16.0 days computed for similar homografts on formerly "naive" CBA recipients ($P < 0.001$; see Table III).

These data support, but do not confirm, the interpretation that CBA males unresponsive to neonatal C3H skin grafts are cellular chimeras and that passenger leukocytes are involved in the induction of this unresponsiveness. For example, it could be argued that antigen is sequestered in these males, perhaps in such a way that its presentation to the secondary host via the transferred cells is immunogenic rather than tolerogenic.

The passive transfer of sensitized cells from "tolerant" animals (23) could likewise account for the accelerated reactions observed. Indeed, Mitchison (24) has suggested that tolerance and immunity may coexist, and our observation that CBA males sometimes accept skin grafts from C3H neonates while con-

TABLE IV

Survival of C57 Neonatal Skin Grafts on Adult C57 Females after Irradiation of the Donor with 850 R X-Ray

Donors		Days after transplantation								
		0	30	40	50	60	70	80	90	>100
C57 ♂♂	N	23	22	19	17	16			15	15*
	%		95	83	74	70			65	65
C57 ♀♀	N	18								18
	%									100

* Graft survival times (days) of adult C57 male skin grafts on 12 of these unresponsive females: 6 > 100, 36, 24, 23, 17, 13, 7.

currently rejecting contralateral grafts from C3H adults (9) is consistent with this proposal. Therefore, to investigate this possibility, i.e. to determine whether immunity to C3H skin grafts could be adoptively transferred by skin or thymic isografts from immune animals, 16 skin isografts of 2.0 × 1.2 cm and nine isologous thymus cell suspensions, each containing at least 40 million cells, were prepared from CBA males which had been challenged with and rejected newborn C3H grafts. These were transferred to 25 CBA's of both sexes. After 7 days, these mice received normal adult C3H skin grafts. The MST of 14.6 days and 14.5 days for recipients of skin and thymic isografts, respectively, were not significantly different from the MST of 16.0 days observed when untreated CBA hosts were challenged with similar grafts ($P > 0.2$). It does not seem likely, therefore, that the adoptive transfer of immunity was responsible for the results obtained.

Effect of Irradiation of the C57 Donor before Skin Grafting.—The fact that the persistence of a neonatal graft is not necessary to the maintenance of unresponsiveness in CBA mice, but is necessary in C57 females (8), suggests that the tolerogenic influence of the neonatal C57 male graft stems from the transplant

itself, and not from the leukocytes within it. Accordingly, it was anticipated that heavy irradiation of neonatal C57 male grafts would not interfere with their survival or their ability to induce tolerance. This prediction was substantiated by the following results.

Neonatal male C57 mice received 850 R whole body X-ray and were then used as skin graft donors for a panel of adult C57 females. Grafts from irradiated neonatal females served as controls. While all 18 of the female grafts survived for more than 100 days, $15/23$ (65%) of the infant male grafts also survived permanently (Table IV), a percentage consistent with the 74% survival of untreated neonatal male C57 isografts on adult females (8) ($P > 0.4$). Furthermore, these heavily irradiated infant male grafts did not lose their ability to protect subsequent adult male skin isografts. Thus, when 12 C57 females bearing irradiated neonatal male transplants for 100 days were challenged contralaterally with adult male skin isografts, six (50%) were found unresponsive to both newborn and adult grafts.

Analysis of the Chimeric Status of C57 Females Rendered Tolerant with Neonatal C57 Male Skin Isografts.—Since the ability of neonatal male skin isografts to induce tolerance of the Y-antigen in adult females depends apparently upon chronic exposure of these recipients to the grafts, rather than to the passenger cells contained within them, it was not anticipated that these female hosts would be demonstrably chimeric. Indeed, when aliquots of 20 million spleen cells from unresponsive C57 females are injected intravenously into newborn C57 females, and these latter recipients are subsequently challenged with adult male skin isografts, none accept the male transplants for more than 55 days (8). This assay is based upon the fact that contamination of the 20 million cells in the donor inoculum with as few as 12,500 male cells is sufficient to induce tolerance of *H-Y* (1). In fact, the MST of male grafts on these neonatally treated females is 29 days, not significantly disparate from the MST of similar grafts on untreated C57 females (8).

In the present study, separate suspensions containing at least 40 million cells were prepared from the lymph nodes, spleen, thymus, and bone marrow of female C57 mice bearing neonatal male skin isografts for more than 100 days. These suspensions were injected intraperitoneally into panels of isologous adult females which were challenged 7 days later with male skin grafts to determine whether they had been immunized against the Y-antigen, an observation which would imply that the cell suspensions with which these animals had been inoculated contained male cells. To learn if male cells might be found in the skin of a tolerant donor (21, 22), normal females were exposed to skin isografts from C57 females tolerant of newborn male skin, before receiving contralateral adult male test grafts.

The results of these tests, summarized in Table V, are consistent with the interpretation that neonatal male leukocytes either do not persist in detectable numbers in females unresponsive to infant male grafts, or that those which do

persist, having differentiated in a female hormonal milieu, fail to express the Y-antigen (25, 26). Indeed, none of the MST's in Table V is significantly different from the 24.5 day MST (8) of adult male skin grafts on formerly untreated females ($P > 0.1$).

Removal and Replacement of Leukocytes from Neonatal C3H Skin Grafts.—If the privilege afforded neonatal C3H skin grafts depends upon their passenger cell population, then replacement of this moiety with adult cells should adversely affect the survival of these grafts. Accordingly, 17-day-old embryonic skin isografts were transplanted to a panel of adult male C3H recipients. After

TABLE V

Survival of Adult Male C57 Skin Grafts on Adult Female C57 Mice Exposed to Isografts from C57 Females Bearing Neonatal Male C57 Skin Grafts >100 Days

Isograft tissue		Days after transplantation													MST	SD
		0	16	18	20	22	24	26	28	30	40	50	60	70		
LN	N	9			6	4		3	2	1	0				22.0	1.29
	%				67	44		33	22	11						
Spl	N	7	5			2			1	0					20.0	1.29
	%		71			29			14							
Thy	N	10			7	5	4	3	1		0				22.5	1.24
	%				70	50	40	30	10							
BM*	N	4					3	2			0				27.0	1.23
	%						75	50								
Skin	N	18	17		15	10	5	4	3	2	1			0	22.7	1.22
	%		94		83	56	28	22	17	11	6					

* BM, bone marrow.

4 days, i.e. when these transplants were about equivalent in age to newborn grafts, they were carefully excised, cleaned, and transferred to CBA males. Only $\frac{1}{24}$ (4.2%) of these "neonatal" skin homografts survived permanently (Table VI). Moreover, their MST approximated that of normal adult C3H skin grafts on similar hosts.

It would appear, therefore, that during the 4 day sojourn of these embryonic grafts on their isogenic adult hosts, vascular connections between graft and host were established (27–29), allowing fetal passenger cells to emigrate from the vessels of the graft, and permitting host cells to replace them. The following experimental observations confirm this fact.

17-day-old fetal strain A skin isografts were transplanted to adult strain A mice which had been rendered tolerant of C3H skin grafts by neonatal inocula-

tion with adult (C3H × A)F_1 lymphocytes. After 4 days on these intermediate chimeric hosts, the grafts were removed and transplanted to a panel of 17 normal strain A animals. 7 days later, these secondary hosts were challenged contralaterally with adult C3H skin grafts. The MST of these test grafts was 7.8 days, contrasting significantly ($P < 0.001$) with the MST of 10.7 days for adult C3H skin grafts on naive strain A recipients (9). Apparently, the C3H grafts were rejected in accelerated fashion because their hosts had been sensitized by adult leukocytes of (C3H × A)F_1 origin which the fetal isografts transported from their intermediate tolerant hosts.

With only three exceptions, the fetal isografts survived permanently. Many exhibited transient alopecia and minor necrotic lesions, however; (see references 29 and 30). 12 fetal strain A grafts transplanted to normal isogenic hosts, left in place 4 days, and then regrafted to new untreated strain A hosts, survived in excellent condition without exception.

Removal and Replacement of Leukocytes from Neonatal C57 Male Skin Grafts.—

TABLE VI

Survival of Neonatal C3H Skin Grafts on Adult Male CBA Mice after 4 Day Residence on Adult Male C3H Mice

	Days after transplantation									MST	SD
	0	10	12	14	16	18	20	24	>100		
N	24	23	21	16	11	3	2	1	1	15.1	1.23
%		96	88	67	46	13	8	4	4		

The foregoing data indicate that newborn passenger leukocytes are important to the persistence of neonatal C3H skin homografts on CBA recipients. To determine if the same is true of newborn C57 grafts, i.e. if newborn male C57 skin grafts carrying adult male leukocytes can survive on isologous female hosts, the following experiment was performed. Neonatal male skin was transplanted to adult C57 males, left in place for a period of 4 days, and then removed, trimmed, and regrafted to a panel of adult C57 female animals. A group of C57 females which received male skin from 4-day-old donors served as controls.

The results of these experiments (Table VII) indicate that, unlike the C3H-CBA situation, the exchange of adult leukocytes for neonatal cells does not prejudice the survival of newborn male skin isografts on C57 females. Thus, $^7/_{14}$ (50%) of the treated grafts were permanently accepted vs. $^7/_{12}$ (58%) of the untreated 4-day-old transplants ($P > 0.6$).

Direct Inoculation of C3H Leukocytes into CBA Hosts.—Because our observations are consistent with the interpretation that viable passenger cells are necessary for the induction of tolerance by neonatal grafts to adult skin transplants in the C3H-CBA strain combination, the following tests were undertaken to determine the ability of lymphoid cells alone to alter the subsequent reactivity

of their hosts to adult skin grafts. Panels of CBA males were inoculated with peripheral blood leukocytes, thymocytes, or spleen cells from adult or neonatal C3H mice. Other CBA males received C3H liver cells from 15-day fetuses, or from the dam bearing them. 50 days after inoculation, all of the recipients were challenged with adult C3H skin homografts.

Insofar as preliminary experiments with intravenous inoculations of 1 million or less thymocytes yielded equivocal results, this route of inoculation was abandoned and either 1 or 5 million cells were given subcutaneously. The results were provocative. Whereas the CBA males exposed to adult cells were almost invariably sensitized by them, those animals injected with homologous neonatal or fetal cells exhibited a wide range of response (Table VIII). Indeed, 1/16 (6%) of the hosts exposed originally to 5 million neonatal C3H thymocytes and 2/19 (11%) of those inoculated with 1 million neonatal peripheral blood leukocytes

TABLE VII

Survival of 4-Day-Old Male C57 Skin Grafts on Adult Female C57 Mice

Treatment of donor skin		Days after transplantation						
		0	20	30	40	50	75	>100
None	N	12		11	10	9	7	7
	%			92	83	75	58	58
4-day residence on adult male C57 mice	N	14	12		11	9	7	7
	%		86		79	64	50	50

subsequently accepted transplants of adult C3H skin, bearing these grafts in excellent condition for more than 100 days.

Nevertheless, these results indicate that, although infant C3H leukocytes may induce unresponsiveness in their CBA hosts when administered directly, they are far less efficient in this regard than when intact newborn C3H skin grafts serve as their vehicle. In fact, the majority of those animals which received neonatal or fetal inocula were sensitized.

This disparity between the tolerance-inducing ability of the skin graft and that of the "naked" leukocytes, may be related to the method by which passenger cells emigrate from the graft vasculature and gain entrance into the host. It may be related also to the number of cells which depart the transplant, or to the possibility that the newborn skin and the leukocytes within it act together to produce a state of unresponsiveness within the host. The questions of correct dosage and route of inoculation are currently under investigation.

Direct Inoculation of C57 Male Leukocytes into C57 Female Hosts.—In view of reports that fetal male C57 cells may render their isologous female hosts at least partially tolerant of the Y-antigen (31), and in view of our observations with the C3H-CBA system, we perfomed the following tests to determine whether fetal, neonatal, or adult male cells might affect the reactivity of adult female C57

recipients towards skin grafts from adult male donors. As in the previous series of experiments, C57 adult females received subcutaneous inoculations of either 1, 5, or 6 million cells from the peripheral blood and thymus of newborn or adult C57 males. Other females received either fetal or adult male liver cells. 50 days after injection, all of the recipients were challenged with skin isografts from adult male C57 animals. The results of these tests are shown in Table IX.

TABLE VIII

Survival of Adult C3H Skin Transplants on Adult CBA Males Injected Subcutaneously with Cells from Fetal, Neonatal, or Adult C3H Mice 50 Days before Grafting

Type cells injected	No. of cells injected $\times 10^6$		Days after transplantation													MST	SD
			0	8	10	12	14	16	18	20	22	24	26	28	30	>100	
Neonatal thymus	5	N	16		8	6	3		1							1	11.0	1.32
		%			50	38	19		6							6		
Adult thymus	5	N	11		3	1			0								10.0	1.10
		%			27	9												
Neonatal PBL*	1	N	19		12	8	7		4		3		2			2	12.0	1.56
		%			63	42	37		21		16		11			11		
Adult PBL	1	N	12	11	1	0											9.1	1.07
		%		92	8													
14 day fetal liver	1	N	6		0												10.0	1.00
		%																
Adult liver	1	N	6		1	0											9.4	1.07
		%			17													
Neonatal spleen	1	N	12		6	5		4	1	0							10.0	2.72
		%			50	42		33	8									

* PBL, peripheral blood leukocytes.

Without exception, the test grafts were rejected by those females previously exposed to inocula of adult male cells and by those injected with newborn male cells. Indeed, the MST of these test grafts (Table IX) was, in each case, significantly disparate ($P < 0.005$) from the 24.5 day MST (8) of similar grafts on naive females. Thus, neonatal male leukocytes injected subcutaneously, in the quantities shown, not only fail to render their hosts unresponsive, but in fact sensitize them against the Y-antigen. Preliminary experiments with low doses of neonatal male thymocytes injected intravenously indicate that this route of inoculation is no more satisfactory, from the standpoint of tolerance induction, than the subcutaneous mode of injection.

DISCUSSION

Whereas current reports have established that passenger leukocytes contribute to the immunogenicity of the skin grafts bearing them (21, 22, 29, 30, 32, 33), it is evident from the present study that passenger antigens (most probably in the form of leukocytes) contribute also to the induction of tolerance by neonatal skin homografts. Thus, infant C3H homografts whose leukocyte populations have been eliminated by X-irradiation or by transient sojourn on an intermediate adult host of the same strain, do not display prolonged survival on CBA

TABLE IX

Survival of Adult Male C57 Skin Transplants on C57 Females Injected Subcutaneously with Cells From Fetal, Neonatal, or Adult Male C57 Mice 50 Days before Grafting

Type cells injected	No. of cells injected $\times 10^6$		Days after transplantation														MST	SD
			0	10	12	14	16	18	20	22	24	26	28	30	32	34		
Neonatal thymus	5	N	8	7	2	1	0										11.0	1.15
		%		87	25	13												
Adult thymus	5	N	11	5	2	0											10.0	1.11
		%		45	18													
Neonatal PBL	1	N	20	17	15	14	10		9	5	3				2	0	16.3	1.55
		%		85	75	70	50		45	25	15							
Adult PBL	1	N	12	10	8	6		4		2		1		0			14.5	1.54
		%		83	67	50		33		17		8						
16 day fetal liver	6	N	12	9	0												10.3	1.05
		%		75														
Adult liver	6	N	6		4	2	1	0									13.2	1.21
		%			67	33	17											

mice. Moreover, the continued presence of the neonatal C3H grafts is not necessary to maintain tolerance in their CBA hosts, an observation consonant with the demonstration that CBA males bearing long-term neonatal C3H skin grafts are leukocyte chimeras. Indeed, whereas injections of adult C3H leukocytes almost invariably sensitize their hosts, and have not, in the dosages employed, prolonged the life of subsequent test grafts, single subcutaneous inoculations of 1 million peripheral blood leukocytes or 5 million thymocytes from newborn C3H donors occasionally render their adult male CBA recipients unresponsive to adult C3H skin grafts transplanted 50 days later.

Neonatal C3H grafts which precede those from C3H adults are more successful in the induction of unresponsiveness to the latter than neonatal C3H grafts

transplanted concomitantly with those of adult origin (9). Thus, proliferation of the emigrating donor leukocytes may contribute to the establishment of tolerance.

In the antigenically weaker C57 system, passenger leukocytes are not implicated in the anomalous survival of *H-Y*–incompatible newborn skin isografts. Thus, in contrast to our observations with the C3H-CBA strain combination, intravenous or subcutaneous injections of isologous newborn male cells into adult females, in the quantities tested, fail to prolong the life of subsequent adult male transplants. Moreover, male grafts from 850 R irradiated neonates survive just as well as those from unirradiated donors on adult C57 females, as do those newborn grafts which have presumably lost their passenger cells after 4 days' residence on intermediate adult male hosts. Furthermore, unlike tolerance induced in CBA mice by infant skin grafts, tolerance in C57 females is abolished by removal of the neonatal graft (8).

Examination of C57 females bearing long-term isografts from newborn males indicates that the various lymphoid compartments of these recipients contain no detectable male cells. While this failure to demonstrate chimerism could be attributed to the fact that any passenger cells which had emigrated from these transplants subsequently differentiated in a female milieu, i.e. in an environment known to repress the expression of the Y-antigen (25, 26), it is noteworthy that attempts to confer tolerance by grafting newborn female C57 mice with isologous adult male skin have also been unsuccessful (34). The unresponsiveness to the Y-antigen which results from exposure to infant male skin resembles that induced by multiparity (1) since females made tolerant by these methods are not, in either case, demonstrably chimeric (1, 8).

These findings support the interpretation that, in the C57 system, it is the newborn male skin graft per se which alters the response of the female host, perhaps through its intense proliferative capacity and consequent ability to "overgrow" the weak level of sensitivity invoked. In view of the growth capacity and reparative ability of neonatal skin, it is plausible that infant male C57 isografts may provide the same stimulus as massive adult male skin isografts which are also accepted by C57 adult females (35). However this argument fails to explain why neonatal male C57 grafts transplanted singly survive better than those transplanted concomitantly with adult male grafts (8).

C57 females exposed simultaneously to C57 neonatal and adult male skin transplants frequently slough the adult grafts but fail to reject the infant grafts (8), even though both presumably express the same Y-antigen (though perhaps in different degrees, see reference 36). This fact, together with the demonstration that neonatal C57 male grafts occasionally survive indefinitely on specifically immunized hosts (8), suggests that immunological enhancement (37, 38) may be important in the persistence of these infant grafts.

C3H infant grafts also occasionally survive on CBA males while contralateral

adult C3H grafts are destroyed (9). They may also persist on sensitized hosts (9). It is conceivable, therefore, that these transplants, as well, are enhanced. However, preliminary attempts to extend the survival of C3H neonatal skin grafts on CBA females with sera from CBA males bearing C3H mammary tumors or long-term infant C3H skin grafts, or from CBA males exposed to repeated intraperitoneal injections of C3H spleen and lymph node cells, have been unsuccessful. Perhaps, enhancing CBA antibodies, if they exist, are rapidly adsorbed in vivo by the neonatal C3H homografts.

A possible relationship exists between the expression of common antigens by tumor and embryonic or newborn cells (39–44), the susceptibility of tumors (37) and infant grafts (45) to immunologic enhancement, and the ability of each, as a homograft, to prolong the life of subsequent adult transplants (46–48) (see references 49 and 50). It is also provocative that fetal cells and neoplastic cells treated with ethylenediaminetetraacetate (EDTA) are agglutinated by concanavalin A (Con-A), a jack-bean globulin which binds carbohydrate, whereas normal adult cells are agglutinated by Con-A only after trypsinization (51). Thus, Con-A receptor sites are masked in normal adult cells, but not in fetal or tumor cells (51).

These observations are consistent with the interpretation that the topography of the fetal, neonatal, or tumor cell membrane is unlike that of a normal isologous adult cell membrane. As a result of this topographical difference, their transplantation antigens may not be similarly accessible to host immunocyte receptor molecules, and this disparity may be crucial in determining whether tolerance or immunity occurs.

SUMMARY

The anomalous survival of neonatal C3H skin grafts on CBA mice is correlated with the emigration of passenger leukocytes from the graft vasculature. Thus, newborn homografts whose leukocyte populations are eliminated by X-irradiation or by transient sojourn on an intermediate adult C3H host, do not display prolonged survival. Moreover, the continued presence of the newborn grafts is not requisite to the maintenance of the unresponsive state, an observation consonant with the demonstration that CBA mice bearing long-term neonatal C3H skin grafts are leukocyte chimeras.

In contrast, neonatal male C57 skin grafts may persist on C57 females after heavy irradiation of the donor, or after passage on an intermediate adult male host. In addition, tolerance is broken by removal of long-persistant newborn grafts from hitherto unresponsive females, and chimerism is not detectable in female C57 mice tolerant of infant male isografts.

Finally, leukocytes of neonatal C3H origin, inoculated subcutaneously into CBA males, may occasionally render these animals unresponsive to subsequent adult C3H skin homografts, whereas those taken from infant C57 males usually

sensitize their adult female hosts. Thus, passenger leukocytes are implicated in the extended survival of C3H neonatal homografts on CBA recipients, but not in the persistence of *H-Y*–incompatible neonatal skin isografts on C57 females.

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