Further Study on Chimerism in Tolerant Mice.* (31475)

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In our previous reports chimerism in tolerant animals was quantitated by two techniques(1,2). Using the T6 chromosome marker technique a high percentage of donor cells was detected in lymphoid tissues of tolerant mice in parent to parent combinations(2). On the other hand, by the differential cytotoxicity test, only a small proportion of donor-type cells was found in spleens of parental animals tolerating F_1 hybrid skin grafts(1). The latter findings confirmed similar results reported by others(3,4).

In the present report we would like to describe studies on chimerism in the F_1 hybrid into parent combination by use of the T6 chromosome marker technique.

Materials and methods. Inbred mice of the A and T6 strains and (A × T6)F_1 hybrids from the local colony were used. The T6 mice were inbred by brother-sister mating since being received from C. E. Ford in 1957 as outcrossed mice homozygous for the T6 translocation chromosome. They are histocompatible among themselves but are not histocompatible with the A strain. Cells of the T6 strain have two T6 chromosomes; cells of the (A × T6)F_1 hybrid have one; cells of the A strain have none.

Tolerance was induced by injecting newborn animals intraperitoneally with 40 $\times 10^6$ spleen cells within the first 24 hours of life. Test skin grafts were transplanted 6 to 8 weeks after birth, according to the technique already reported(5). Preparation of cell suspensions, the method for chromosome preparation and the irradiation procedure were described elsewhere(6).

Results and discussion. Skin grafts transplanted among mice of the T6 strain were always retained in autograft-like conditions for more than 200 days. The same was true for grafts transplanted from T6 and A parental strains to (A × T6)F_1 hybrids. Approximate mean survival time of skin grafts exchanged between the two parental strains and the F_1 hybrids were as follows: A → T6 = 10 days (14 mice); T6 → A = 10 days (15 mice); (A × T6)F_1 → A = 11 days (10 mice).

Induction of tolerance. Forty-seven newborn strain A mice were injected intraperitoneally with 40 × 10^6 (A × T6)F_1 spleen cells. Of these 37 were grafted 6 to 8 weeks after birth with (A × T6)F_1 skin. The results are shown in Table I.

Female A strain recipients are somewhat less susceptible to the induction of tolerance than are males; there were no females in the group of long-term tolerant mice. This relative refractoriness of female mice to tolerance induction was previously described and discussed(1).

Eleven males displayed a long lasting but incomplete tolerance, *i.e.*, grafts were affected by a chronic rejection reaction. This reaction continued throughout the observation period, showing at times acute phases that would destroy part of the graft. Grossly unaffected portions of the graft may appear normal and show a moderate hair growth. Histology disclosed a mild infiltration of the graft bed with mononuclear cells. Under affected portion of the graft more intensive infiltrates were found. It is difficult to explain why an essentially chronic rejection shows occasional acute outbursts involving only portions of the graft. It might be that the antigens released from the graft during its partial destruction could temporarily reestablish the level of the antigens necessary for the maintenance of tolerance, thus averting temporarily a complete rejection.

To determine whether similar or more violent rejection reactions would affect subsequent grafts from the same source, second-set (A × T6)F_1 grafts were transplanted to the other side of the chest of 7 short-term tolerant ani-

* This investigation has been supported by research and training grants from Nat. Cancer Inst., CA-03367, T4-CA-05021, and K6-CA-14,219.

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TABLE I. Induction of Tolerance in Strain A Mice to (A × T6)F_1 Skin Grafts.*

	Female		Male	
	No.	%	No.	%
Long-term tolerant (>55 days)	0	0	11	48
Short-term tolerant (15–55 ")	10	71	10	43
Nontolerant (<14 ")	4	29	2	9
Total tested	14	100	23	100

* Strain A mice were injected intraperitoneally with 40×10^6 (A × T6)F_1 spleen cells within 24 hr of birth.

mals that had completely rejected the first graft, and of 11 long-term tolerant recipients still bearing first-set grafts undergoing chronic rejection (Table II).

In mice that rejected the first graft after a short period of tolerance, the second graft survived for a time interval very similar to that of the first one. This indicates that loss of a temporarily tolerated skin graft is not necessarily synonymous with complete loss of such tolerance, and is not necessarily followed by second-set immunity. Residual low level lymphoid chimerism (see later) might be responsible for this failure of complete reactivation of immunological ability.

Second-set grafts in long-term tolerant mice survived longer than 60 days, but they were affected by a chronic rejection reaction, very similar to the one appearing in the first graft. Therefore, the second graft revealed a continuation of incomplete tolerance. Since a freshly transplanted second-set graft also survived for a long time, persistence of the first graft cannot be ascribed to a greater resistance of an established graft to rejection, as compared to a newly set graft.

Chimerism in tolerant mice. We hoped that a study of chimerism in long-term incompletely tolerant animals might provide useful information regarding the nature of the partial tolerance. For this purpose 3 long-term tolerant males were sacrificed for chromosome analysis 116 days after transplantation of the first skin graft. At that time each of them carried 2 (A × T6)F_1 skin grafts that were preserved for more than 50% of their original size. One hundred, 101 and 109 metaphases were read. The number of donor type mitoses in the spleen was 1, 4, and 2, respectively. The very low content of donor cells may explain why the tolerance is not complete. This result is at variance with those obtained by Nakic *et al* (personal communication). These authors found that CBA/T6 mice rendered incompletely tolerant by neonatal inoculation of larger numbers of (A × CBA/T6)F_1 spleen cells contain up to 60% of donor type dividing cells in their spleen.

TABLE II. Survival Times of the First- and Second-Set (A × T6)F_1 Skin Grafts on Incompletely Tolerant Strain A Animals.*

No. of animals	Sex	Survival of 1st graft (days)	Survival of 2nd graft (days)†
7	♀	15, 15, 15, 22, 32	15, 15, 16, 20, 31
	♂	19, 55	27, 55
11	♂	>60	>60

* Second-set skin grafts were transplanted 52-62 days after transplantation of the first one.

† Survival times for the second grafts are given in order corresponding to those for the first grafts.

Spleens from 2 animals that retained the test skin graft for 35 days and then rejected it were also analyzed for content of T6 marker chromosome. The analysis was performed 33 days after the complete rejection of the graft. In one animal 1 out of 64 metaphases was of donor type, while in the other 7 out of 103 were of donor type. The presence of residual low level lymphoid chimerism in mice after rejection of a temporarily tolerated skin graft is in accordance with published observations(1,3).

Four among the nontolerant A mice were sacrificed 17 and 18 days after rejection of their graft (in less than 14 days), and their spleens examined for T6 positive (donor) cells. Forty mitoses were scored in each spleen and no donor dividing cells were found.

Chimerism in animals tolerant for over 2 years. It has been shown in this laboratory that mice of 3 inbred strain to inbred strain

TABLE III. Mortality of (A × T6)F_1 Hybrids Irradiated with 500 r and Injected with Lymphoid Cells from Control or Incompletely Tolerant A Mice.

Host*	Donor	Cell dose (viable)	Mortality	
			No. dead/No. injected	Time period
(A × T6)F_1	None	—	0/10	—
	(A × T6)F_1	50×10^6	0/10	—
	Normal A	15×10^6	10/10	6–28
	" "	30×10^6	10/10	10–26
	Long-term incompletely tolerant A	30×10^6	4/ 4	25–38
	Idem	30×10^6	4/ 4	19–26
	"	30×10^6	4/ 4	19–78

* After irradiation hosts were injected with antibiotics for one week to prevent imminent Pseudomonas infection (see reference No. 10).

combinations, rendered tolerant to skin homografts by intravenous plus intraperitoneal injection of spleen cells at birth, contained more than 80% of donor type cells in their spleens, thymuses, and mesenteric lymph nodes(2). Two strain A mice rendered tolerant of (A × T6)F_1 skin grafts survived for over 2 years still bearing fully tolerated skin grafts. They were sacrificed and tested for lymphoid chimerism 26 months after skin grafting. These mice showed no evidence of skin homograft rejection at any time. Each of them showed a very low level of donor lymphoid cells at time of sacrifice. The number of donor cells found per total cells scored in spleen and mesenteric lymph node respectively were 0/105 and 1/45 for one mouse and 2/24 and 0/2 for the second mouse. Such long survival of skin homografts in spite of very low levels of donor lymphoid chimerism may relate to adaptation of long surviving grafts(7). The possible role of replacement of the graft's vascular endothelium and intima by circulating host cells in long lasting grafts has been discussed elsewhere(8). The declining immunological reactivity of older animals(9), and antigens from the skin graft itself may also play a role in perpetuating a previously established immunological tolerance.

Functional testing of lymphoid system of incompletely tolerant mice. Since tolerance of A mice injected with (A × T6)F_1 cells was incomplete and rejection crises were often seen, it seemed important to evaluate the functional status of the lymphoid tissues of these long-term incompletely tolerant mice. Lymph node and spleen cells from 3 such tolerant animals were harvested separately and injected intravenously into 3 separate groups of 4 adult (A × T6)F_1 recipients irradiated with 500 r. The donors of the lymphoid cells were sacrificed 130 days after transplantation of the first skin graft. At this time each donor had 2 skin grafts preserved for approximately 40 to 80% of the original size. The groups of animals involved and the results obtained are presented in Table III.

Lymphoid cells from each of the 3 strain A animals bearing 2 partially preserved (A × T6)F_1 skin grafts were able to cause death of sublethally irradiated F_1 recipients. The clinical picture and morphological findings in dying (A × T6)F_1 mice suggest that they succumbed to homologous disease. As compared with cells from untreated strain A donors, lymphoid cells from incompletely tolerant A mice appeared to be less effective in producing mortality in F_1 recipients. It is, however, impossible to decide whether lymphoid cells from incompletely tolerant donors are individually less reactive, or the number of reacting cells in the suspension is low. The results of this functional test provide the basis for explaining the nature of incomplete tolerance in our animals.

Summary. By inoculation of 40×10^6 (A × T6)F_1 spleen cells intraperitoneally into newborn A strain mice incomplete tolerance of varying duration was established to skin grafts of the spleen donor strain. Three long-term incompletely tolerant A mice were cellular lymphoid chimeras with 2.3% of T6

positive donor cells in their spleens when sacrificed at 116 days after skin grafting. Lymphoid cells (30×10^6) from 3 comparable animals sacrificed at 130 days produced fatal homologous disease when injected into sublethally irradiated (A $\times$ T6)F_1 hybrids. The F_1 hybrids succumbed after a longer latent period (19-78 days) than after injection of lymphoid cells (30×10^6) from control strain A mice (10-26 days).

Seven strain A mice with short-term tolerance (15 to 55 days), did not immediately regain their immunological reactivity. A second test graft of the same origin placed after rejection of the first graft survived for periods remarkably similar to those for the first graft. When 2 comparable short-term tolerant mice were tested for chimerism, each of them contained a small percentage of donor dividing cells in the spleen at 33 days after skin graft rejection (7/103 and 1/64 respectively).

Four nontolerant mice (graft rejected within 14 days) did not display any detectable lymphoid chimerism (0/40 for each) when tested shortly after the rejection of the test graft.

In 2 strain A mice with complete tolerance to (A $\times$ T6)F_1 skin homografts for 26 months as a result of intravenous and intraperitoneal injection of F_1 spleen cells at birth, the graft remained fully preserved in spite of very low levels of lymphoid chimerism at the time of sacrifice.

We are grateful indeed to the Department of Physics, M.D. Anderson Hospital and Tumor Institute for providing us with the irradiation source. The excellent technical assistance of Mr. J. Session, Mrs. M. B. Davis and Mrs. B. Priest is gratefully acknowledged.

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Received July 13, 1966. P.S.E.B.M., 1966, v123.

Observations on the Decomposition of Hemin by Fatty Acid Hydroperoxides.* (31476)

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The catalytic properties of hemoglobin and hemin in the oxidation of unsaturated fat, first observed by Robinson(1) in 1924, have since been the subject of extensive investigation(2). In aqueous emulsions of unsaturated fatty acids this catalytic action is exerted at the water-lipid interface in the presence of oxygen with subsequent decomposition of hemin(3,4). No hemin catalysis is observed in a homogeneous solution of linoleic acid in glacial acetic acid, dioxane or pyridine (4). It is not clear whether the destruction of hemin in a heterogenous medium is caused by free radicals formed in the process of peroxidation or by the action of preformed hydroperoxides. The absence of hemincatalyzed methyl linoleate oxidation in glacial acetic acid suggests that this medium is suitable for the study of hemin-hydroperoxide reactions

* This research was supported by a grant from the USPHS (NB-04201).