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Retarded Immunological Recovery in Sublethally X-Irradiated Mice by Additional Thymic Exposure. Reversal with Injected Marrow Cells* (33787)

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The pronounced inhibition of immune responses following sublethal X-irradiation and the requirement of an intact thymus for the subsequent recovery of immune competence are well documented [see reviews by Taliaferro (1), and Miller (2)]. Recent reports have indicated that transplanted cells of the thymus interact synergistically with cells present in the bone marrow to elicit immunocompetence in X-irradiated animals (3–5). *In vitro* observations showed that thymus–marrow cell combinations can reactivate immunological competence of irradiated spleen tissue (6). In the irradiated animal, therefore, recovery of the immune capacity might be dependent not only on recovery of thymic function but also on the recovery of the immunologic stem cells of the marrow. In the present study, the effect of these 2 parameters was investigated by re-exposing the thymus of sublethally whole-body X-irradiated mice to high doses of radiation in order to further delay thymic recovery, and by augmenting the pool of marrow cells in other similarly treated animals. The immunological capability of the mice was then measured at various time intervals. The results show: (i) the residual reticular-epithelial cells of the thymus in sublethally irradiated mice are functionally radiosensitive; (ii) that both the recovery of this thymic function and the supply of marrow-derived immunogenic precursor cells are important determinants of the recovery of immunocompetence in sublethally irradiated mice.

Methods. Adult (3–4 month old) (C57LXA/He)F_1 hybrid mice bred at this Laboratory, were exposed to 500 rad whole-body X-radiation in perforated Lucite tubes placed on a rotating table. The radiation factors were as follows: 250 kVp, 15 mA, 0.5 mm Cu and 1 mm Al filters, 28 rad/min dose rate, and 40 cm TSD. Within 1 hr some of these mice were anesthetized (sodium pentobarbital) and taped into ⅛ in. thick lead boxes (i.d., 7 × 11 × 2 cm) in such a manner that a circular hole in the lead, 1.2 cm in diameter, was located over the thymus. This was done by palpating the first 3–4 segments of the sternum through the hole. The relationship of the irradiated area and the sternum are shown in Fig. 1. The shielded mice were then re-

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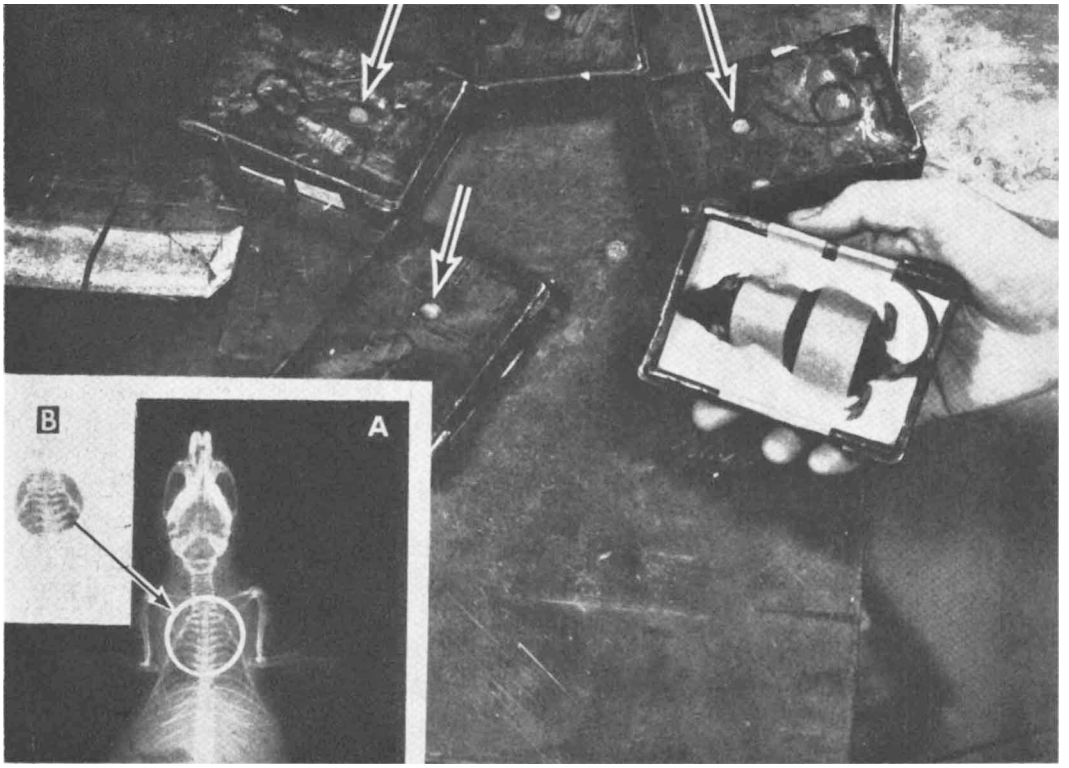

FIG. 1. View of the lead shielding arrangement showing 4 shields each with a circular opening (arrows) for thymic irradiation. The fifth shield has been inverted to show the placement of the anesthetized mouse. The X-radiation source is centered directly above the mice. Inserts: Radiographs (ventral aspect) of the unshielded mouse (A), and of the thymic region of the otherwise shielded animal (B).

exposed in groups of 5 on a rotating lead-covered platform to 500, 1000, or 2000 rad at a dose rate of 115 rad/min (TSD = 20.75 in.). It is estimated that the shielded tissues of the mice received under these conditions an additional dose (through scattering) of 1.5% of the thymus dose, i.e., 30 rad in the case of the 2000-rad groups. Other mice were surgically thymectomized 2–4 weeks prior to whole-body irradiation as controls.

Following irradiation, 10×10^6 viable (by the criterion of eosin exclusions) syngeneic bone marrow cells contained in 0.2 ml of TC 199 (Difco) plus 10% fetal calf serum were injected iv. The marrow was obtained by forcing the suspending media through the femur and tibia shafts with a syringe and needle.

Three tests of immune competence were carried out. Two of these measured the ability of the mice to elaborate antibody-producing cells, and the third was a measure of a cell-mediated immune response, i.e., the homograft reaction.

1. Hemolysin plaque-forming cells (PFC) were enumerated in the spleen by the Jerne technique (7) using Hege's modifications (8). The mice were challenged at 6, 30, or 60–65 days following irradiation with 400×10^6 washed ($3\times$ in saline) sheep red blood cells (RBC) injected iv. Four days later analyses were performed on the spleens of at least 3 individual mice. Three or more plates were made of aliquots from each spleen, the aliquots containing 2.5×10^5 to 10^7 nucleated cells depending on the anticipated activity. The mean number of PFC per spleen $\pm$ SD for the individual mice were calculated from the average PFC count of each group of 3 plates.

TABLE I. Plaque-Forming Cells in Mouse Spleen Following 500 Rad Whole-Body X-Irradiation plus Additional Irradiation of the Thymus.[a]

Treatment	Isogenic marrow (no. of cells injected)	PFC/spleen (± SD) when challenged at (days): 6	30	60–65
500 rad whole body	None	18 ± 1	7300 ± 3100	76,800 ± 18,100
	10^7	339 ± 220	18,900 ± 7200	102,800 ± 10,100
+ 500 rad to thymus	None	46 ± 31	6500 ± 3500	61,800 ± 12,400
	10^7	714 ± 491	30,600 ± 500	127,300 ± 80,200
+ 1000 rad to thymus	None	12 ± 2	1000 ± 400	84,700 ± 34,800
	10^7	809 ± 92	18,300 ± 5700	163,400 ± 73,200
+ 2000 rad to thymus	None	21 ± 14	750 ± 160	67,600 ± 43,700
	10^7	162 ± 72	11,700 ± 6000	236,700 ± 87,900
Prior thymectomy + 500 rad whole body	None	17 ± 2	440 ± 180	1000 ± 200
	10^7	33 ± 13	1600 ± 800	5200 ± 1600

[a] Normal PFC per spleen = 187,600 ± 99,000.

2. The assay for antigen reactive cells (ARC) was carried out by the method of Playfair (9). ARC have been defined by Miller (2) as cells which respond to antigen by proliferation and differentiation to give rise to antibody producing cells. The test mice were sacrificed at various times following irradiation and an aliquot of each spleen suspension containing 1/4 to 1/50 of the spleen cell content injected into at least 3 heavily irradiated (900 rad) intermediate syngeneic hosts. The intermediate hosts were then injected iv with 400×10^6 washed sheep RBC. Eight days later, the spleens of the intermediate hosts were analyzed for areas of hemolysin production by cutting each spleen into 26–30 fragments and placing the fragments in sequential order in an agar (1.4%)-containing petri dish. The fragments were then overlayed with agar (0.7%) containing sheep RBC, and the hemolysin producing areas developed as described by Playfair (9). Since the maximum ARC which could be counted with confidence in each spleen was only 3, several intermediate hosts were used for each donor. The number of ARC was calculated from the combined total aliquots of each donor spleen injected into the hosts and the total number of active areas produced. In cases where no ARC were found, limiting values were calculated by assuming that 1 or less ARC was contained in the total spleen inoculum, while in cases where confluent active areas occurred, limits were calculated assuming that 3 or more areas per intermediate host spleen were present. The values are expressed as the number of ARC detected per donor spleen. The ARC values thus determined represent only 15% of the total ARC present in the donor spleen, according to Kennedy (10).

3. The homograft response to tail skin grafts was studied by the method of Bailey and Usama (11). Three allogeneic donor strains of varying histocompatability were used: C57Bl/6J donors (H-2b), which differ from the host LAF_1 mice (H-2a+b) in the "weaker" non-H-2 isoantigens; C3H/HeJ donors (H-2k), which in our hands appears to be less antigenic than other strains differing at the H-2 locus; and DBA/2J mice (H-2d), the most incompatible donor among the three. The mice were grafted 1 day after irradiation in one experiment or at various times up to 35 days in another. The fate of the grafts was followed 3 times weekly using a dissecting microscope (10× magnification). The criteria of rejection have been reported previously (12). The p values and confidence intervals were calculated using Student's t test.

Results. Plaque-forming cells. When the mice were challenged 6 days after irradiation, there were 12 to 46 PFC/spleen in all groups not receiving marrow (Table I). These cells are probably the radioresistant background PFC observed by Hege (8). In

the corresponding mice injected with marrow, the PFC values for the surgically thymectomized group was higher than the uninjected group, though not significantly ($p = 0.08$). However, in the thymic irradiated marrow recipients PFC levels were considerably higher ($p = 0.015$ to 0.03) than in any of the corresponding groups which did not receive marrow cells.

At 30-days postirradiation, the level of PFC per spleen in the 500 rad only group (no marrow) was only 7300. However, with increasing doses of radiation to the thymus, the number of PFC decreased, and only 750 PFC/spleen were present in the group receiving 2000 rad to the thymus. This decrease is significant ($p<0.01$). In fact the number of PFC found in the most heavily irradiated group approximated that of the surgically thymectomized mice (440 PFC/spleen).

The injection of marrow cells significantly increased the splenic PFC levels at 30 days to 11,700 or more in all groups except for the thymectomized, which showed only 1600 PFC/spleen. The increase from 750 PFC in the group receiving 2000 rad to the thymus to 11,700 PFC following marrow cell infusion is highly significant ($p<0.001$). It is apparent that the injection of marrow cells reversed the effect of the additional thymic irradiation.

At 60–65 days, splenic PFC values were approaching the normal range (187,000 ± 99,000) in all groups not given marrow, except for those which had been thymectomized (1000). In the marrow-injected mice the PFC values were in the normal range, excepting again the thymectomized group (5200 PFC/spleen). In an additional control group no whole-body irradiation was given, but the mice were either surgically thymectomized or the thymus only was exposed to 2000 rad. These animals showed no deviation from the normal splenic PFC level 6–14 days after treatment.

Antigen reactive cells. The results of the ARC assays were not as definitive as for PFC but they followed the same pattern. At 6 days postirradiation (Table II) few or none

TABLE II. Antigen-Reactive Cells in Mouse Spleen Following 500 Rad Whole-Body X-Irradiation plus Additional Irradiation of the Thymus.[a]

Treatment	Isogenic marrow (no. of cells)	Observed[b] ARC/spleen (range) (days): 1	6	15	30–40	65–70	84
500 rad whole body	None	<1.5	<2	<10–22	2–44	<8.6–69	>130–192
	10^7	<1.9–1.9	<6.0	<2.7–9.8	16–53	99–203	—
+ 500 rad to thymus	None	—	<2	<12	6–31	—	99–>133
	10^7	—	<6.0	—	42–65	>116	—
+ 1000 rad to thymus	None	—	<3	<10	7–19	—	73–>126
	10^7	—	<6–6.9	—	18–32	89–171	—
+ 2000 rad to thymus	None	—	<2–2	<9	3–22	—	83–>98
	10^7	—	<7	—	<8–34	24–187	—
Prior thymectomy + 500 rad whole body	None	—	<2	<8	<3–9	—	11–16
	10^7		<8.5–11	—	<8–36	56–58	—

[a] Nonirradiated range per spleen = 69–195.
[b] Representing an estimated 15% of the total ARC per spleen (10).

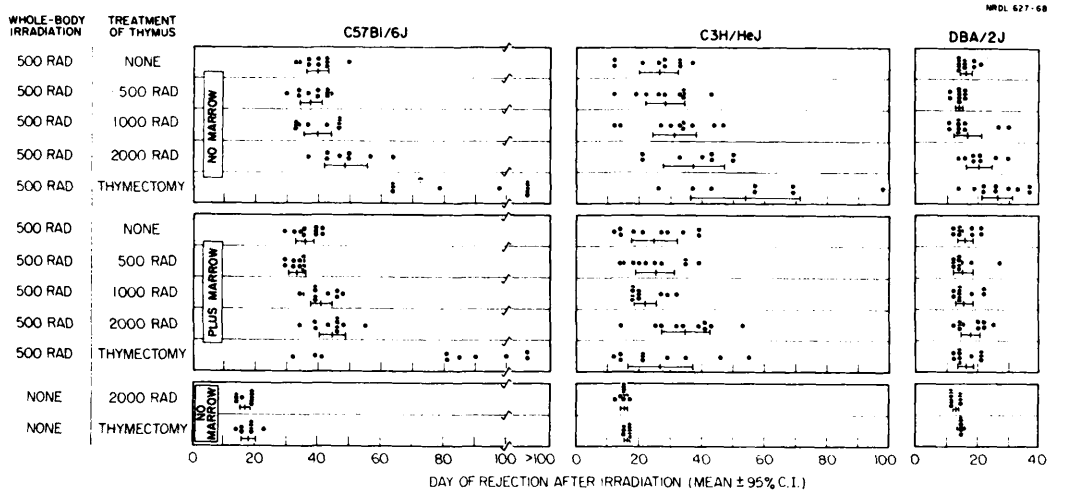

FIG. 2. Skin homograft rejection times in groups of sublethally X-irradiated (500 rad) LAF_1 mice whose thymus had been either surgically extirpated or exposed to additional localized radiation. Syngeneic bone marrow cells (10^7) were injected into some groups just after exposure and all mice were grafted 1 day later.

of these cells were found in the spleens in any of the respective groups, either with or without injected marrow. At 30–40 days in mice not given marrow, the range of ARC was highest in the 500 rad only group, and decreased with irradiation of the thymus to levels approaching those of the thymectomized controls. The range of ARC values was in general higher when marrow was given. The significant increase in PFC at 30 days in the group receiving 2000 rad to the thymus and marrow cells was not as evident with ARC. With the exception of the thymectomized mice, ARC values in all groups approximated the normal range by about 70 days.

Homograft response. The fate of C57Bl/6 skin homografts engrafted 1 day postirradiation is shown in Fig. 2. This is the least antigenic graft and it showed the greatest prolongation of mean survival time (mst). Controls, thymectomized or thymus only irradiated, reject this graft in 17 days. Thymectomized mice which received marrow had 5 out of 10 "takes" for greater than 100 days (2 over 200 days); while in thymectomized mice not given marrow, 2 out of 10 maintained this graft for more than 200 days. As the dose of radiation to the thymus increased, the rejection time also increased though not significantly. Bone marrow cells decreased the rejection time but the difference was not statistically significant.

Figure 2 also shows the mst for C3H grafts. All thymectomized mice rejected these grafts with a mst of 54 days. The administration of marrow cells resulted in earlier graft rejection in the thymectomized group, i.e., mst = 27 days. With increasing doses of radiation to the thymus there was again a trend towards prolonged rejection times. The injection of bone marrow cells tended to decrease rejection times. These differences were not, however, statistically significant. Essentially the same patterns of graft rejection were found with the most antigenically disparate DBA/2 homografts (Fig. 2). The mst of C3H and DBA/2 grafts on unirradiated thymectomized or thymus only irradiated control mice was 13 and 12 days, respectively.

Thus, these results failed to show that recovery of the homograft response was affected significantly by additional thymus irradiation and by marrow cell therapy in 500 rad whole-body X-irradiated mice. However, it should be emphasized that the rejection time in mice grafted 1 day postirradia-

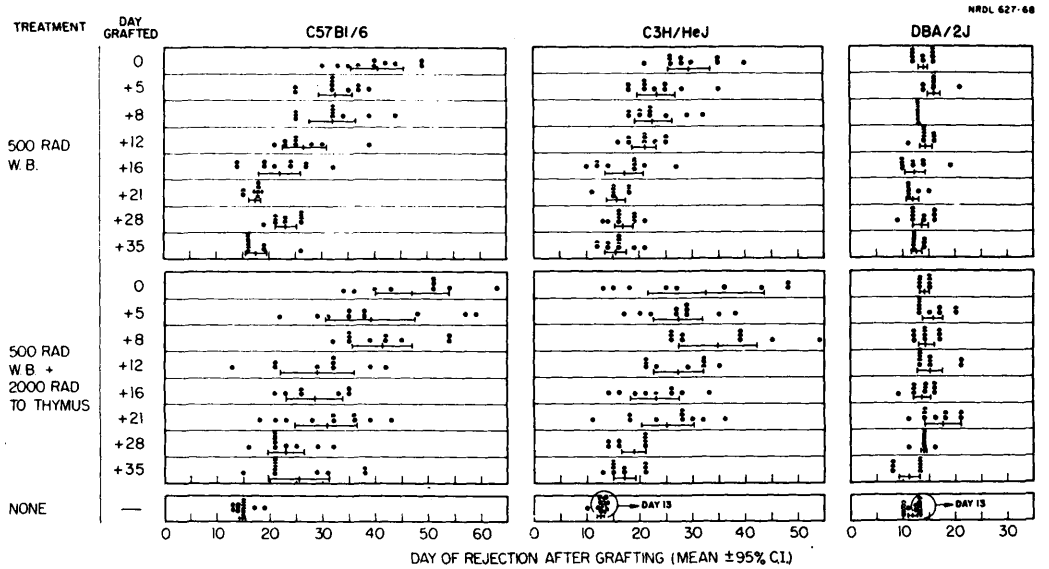

FIG. 3. Skin homograft rejection times in sublethally X-irradiated (500 rad) LAF_1 mice grafted at various time intervals following exposure. In one group of mice the thymus was locally exposed to an additional dose of 2000 rad.

tion measures only the total recovery time, and does not precisely delineate when recovery might have started. Therefore, a second set of experiments were carried out in which whole-body irradiated (500 rad) and whole-body plus thymus irradiated (500 rad + 2000 rad) mice were skin grafted at various times following irradiation (Fig. 3). With the C57Bl/6 homografts on mice which had recieved 500 rad whole-body irradiation only, the time of rejection shortened progressively until near normal mst was attained in those mice grafted 35-days postirradiation. The same results were seen with the C3H homografts; while with the DBA/2 homograft, no significant prolongation of graft rejection was observed even in the group grafted just after irradiation. When the mice received whole-body plus thymus irradiation, the mst values for C57Bl/6 and C3H homografts were all prolonged, but were not significantly different from the values in the nonthymic irradiated groups. Syngeneic grafts which were engrafted along with the allogeneic grafts on all recipients showed a 98% survival.

Discussion. The early inhibitory effect of X-irradiation on the immune response known for many years is certainly evident in the present experiments. Splenic PFC were depleted by a factor of 10^{-4} 6 days after 500 rad. ARC were essentially not detectable at that time. The homograft rejection time for non-H-2 (C57Bl/6) homografts was prolonged by 25–30 days. Recovery to normal levels required at least 60 days for the hemolysin plaque-producing cells, and approximately 35 days for the homograft response. In addition, these experiments illustrate the well established dependency on thymus for the recovery of the immune system following irradiation (2). PFC, ARC as well as homograft reactivity to non-H-2 allografts were all depressed in thymectomized irradiated (500 rad) adult mice for more than 60 days, in contrast to intact irradiated controls.

In a histological study of the irradiated thymus, Trowell (13) showed that virtually all thymocytes are destroyed by an X-ray dose of 500 R leaving a residual stroma of thymus reticular epithelial cells. These latter cells were found to be resistant to doses as high as 5000 R. Dukor (14) found that thymus grafts which had been exposed to 2060 R *in vitro* showed evidence of lymphopoietic regeneration at 11 days; and Engeset and Schooley (15) have recently reported that

regeneration in the X-irradiated (400 R) portion of a partially shielded thymus begins between 3 and 4 days and is complete by 7–8 days. Cross (16) has estimated that the *in situ* thymus is already functioning by 13 days following 850 R of whole-body X-irradiation. By measuring the ability of *in vitro* irradiated thymus grafts to restore immune competence to neonatally thymectomized mice, Miller (17) showed that the irradiated thymus was functioning within 1–2 weeks after 500 R, but after 2000 R was still functionally depressed at 3 weeks. Thus, the histologically radioresistant recticular epithelial cells of the thymus are apparently functionally radiosensitive (17).

The present experiments confirm this concept. Our data show that additional doses of radiation (up to 2000 rad) to the thymus further retards the functional activity of this tissue. For example, at 30 days following 500 rad whole-body X-irradiation, PFC levels in the spleen were rising and were about 5% of the normal. However, in the mice exposed to increasing additional doses of thymic radiation, the degree of recovery was inversely related to the total dose; and in those mice in which the thymus received a total dose of 2500 rad, the splenic PFC levels were not different from those in surgically thymectomized 500 rad irradiated mice (Table I). Similar, though not conclusive evidence from the ARC and the homograft responses was also noted. Thus, the reticular epithelial cells of the thymus are functionally radiosensitive and recovery of the immune capabilities of the sublethally irradiated mouse is dependent, at least in part, on recovery of the function of these cells.

The recovery of the thymus appears to be not the only limiting factor, however. As shown in Table I, the injection of 10^7 bone marrow cells into the irradiated mice significantly reversed the inhibiting effect of radiation on the thymus, so apparent at 30 days. Thus, it appears that even though thymic function is depressed, the irradiated thymus can still interact in some manner with marrow cells, presumably with the immunogenic precursor cells. In the sublethally irradiated animal the number of such marrow cells available is obviously limited and their recovery must therefore also limit the recovery of immune competence.

The present findings are consistent with recent *in vivo* evidence for a synergistic interaction of marrow and thymus cells (3–5). Also, Globerson (6) has shown by *in vitro* studies that recovery of immunocompetence in spleen tissue following high doses of X-radiation is afforded by addition to the culture of a combination of thymus and bone marrow cells. The mechanism involved in this functional interaction is at present unknown, but immunocompetence is thought to be induced by the thymus, perhaps by a hormone (2, 6), reacting in some way with immunogenic precursor cells derived from the bone marrow (18). On the other hand, Radovich (19) has proposed another mechanism of cellular interaction—that bone marrow cells nonspecifically affect the localization or proliferation of antibody-forming cells in the spleen. The present results obtained with injected bone marrow cells could be explained by this mechanism also.

If marrow–thymus cell interaction occurs, then it may occur soon after irradiation, even with a highly irradiated thymus. The results at 6 days (Table I) show higher PFC values in all groups, with the possible exception of the thymectomized group. This increase is probably not due to the transfer of thymus independent PFC or ARC (20) present in the marrow inoculum, since there was only a slight increase in the PFC values for the thymectomized group. If this is so, then the higher values obtained when marrow was injected represent the effect of a thymus–marrow synergism similar to that seen by Claman (3), except that in this case the thymus has been exposed *in situ* to radiation doses up to 2500 rad. However, more work is needed to substantiate this finding for it should be noted that in other studies, grafts of *in vitro* irradiated (500 R) thymus failed to exhibit functional capability within 1 week, and after 2000 R such grafts exhibited only slight recovery by 3 weeks (17). Also, Claman (3) has shown that thymus–marrow cell interac-

tion does not occur within 5 days when grafts of *in vivo* irradiated (500 R) thymus are transplanted with unirradiated marrow cells in his test system. The present data do show that thymic function eventually recovers after *in situ* exposure to as much as 2500 rad.

The same general pattern of recovery was found with the antigen-reactive cell, the immediate precursor of PFC. However, because of the wide variability in the data, definitive conclusions cannot be drawn. It should be noted that both the PFC and the ARC are thymus independent, but that their precursors are thymus dependent (5). It follows, therefore, that the effect of surgical or "radiation" thymectomy must be expressed on immunocompetent precursor cells prior to their maturation to the ARC stage.

The results with the homograft rejection system are inconclusive, but do show some trends. It has been shown in the past (2) that the thymus is involved in restoration of this form of immune response and this fact is apparent here in the surgically thymectomized mice. The greater dependence on the thymus for rejection of the more antigenically compatible non-H-2 homograft (C57B1/6) is also well established (21). Despite the obvious dependency of the homograft reaction on the thymus neither statistically prolonged rejection times nor delayed recovery of the homograft response were apparent, even after an additional dose of 2000 rad to the thymus. However, the fairly consistent trends showing prolongation of the least disparate grafts (i.e., C57B1/6) with increasing doses of radiation to the thymus (Fig. 2) and also a slight delay in the recovery time for the homograft reaction (Fig. 3) suggest at least that the ability of the thymus to restore cell-mediated responses is also radiosensitive. In addition, the tendency for reversal of the effect of thymic irradiation observed in the mice receiving marrow cells would suggest that the functional marrow–thymus cell interaction so apparent in the PFC test system, is also involved in recovery of the homograft response.

Miller (17) reported that thymic grafts irradiated *in vitro* with 2000 R were cytologically fully regenerated by 3 weeks yet were deficient in their functional capacity. Similarly, the thymus of mice exposed to a total of 2500 rad in the present experiments had apparently recovered to normal size by 30 days but was functionally deficient. Histopathological studies on the thymus and other lymphoid tissue from the mice whose thymus had been locally irradiated and/or received marrow inoculation and additional investigations on the nature of the functional interactions between heavily irradiated reticular-epithelial thymus cells and nonirradiated bone marrow cells are being carried out.

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Constant Monitoring of Plasma Luteinizing Hormone by Radioimmunoassay in Individual Rats Following Injection of Hypothalamic Extract* (33788)

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The presence of an LH releasing factor (LRF) in hypothalamic extracts from several species has been demonstrated primarily by means of a bioassay based on the phenomenon of ovarian ascorbic acid depletion (OAAD) in response to LH (1–3). Both the specificity of the assay and the inability of LRF to act directly upon the ovary of the assay animal have been questioned (4–6). Results are further complicated since LRF may act directly on the pituitary of the OAAD assay animals when they are used to measure LH in serum from experimental rats. Finally, the experimental approach to studies of LRF activity have been limited by the sensitivity of bioassays, which are not adequate to measure serum LH concentrations in individual rats.

The present results were obtained with radioimmunoassay for rat LH (7) which does not appear to measure LRF. The collection of blood through a carotid–aortic cannula eliminated both the problems of stress and the need for anesthesia. By utilizing whole blood in the radioimmunoassay, more than 30 sequential measurements of serum LH concentrations were obtained from each rat.

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[2] Career Development Awardee of the National Institute of Child Health and Human Development.

Materials and Methods. Hypothalamic extracts were prepared by homogenization of rat hypothalamic tissue in 0.1 *N* HCl (5 hypothalami/ml) followed by centrifugation. The supernatant fluid was lyophilized and dissolved in phosphate buffered saline (PBS) just prior to use. Extracts of brain tissue from the cerebral hemispheres, including cerebral cortex, were similarly prepared and will be referred to as cortical extracts.

Mature Holtzman female rats, approximately 300 g in weight and castrated at least 1 month earlier, were anesthetized with ether and cannulated via the left carotid artery. The structure of the cannula is shown in Fig. 1. Following the operation, the rats were injected subcutaneously with estrogen (50 μg of estradiol valerate) and progesterone (25 mg) in peanut oil. Three days later the animals were heparinized and connected, via the cannula, to a peristaltic pump set to remove approximately 12 μl of blood/min. Blood was pumped directly into 100 μl Hamilton syringes (with the plunger removed) until approximately 70 μl of blood had been collected. The desired volume of blood, usually 60 μl, was added to the appropriate volume of PBS with 1% lyophilized egg white (Sigma) in an assay tube. The final volume of serum plus PBS-1% egg white equaled 500 μl. In most cases hematocrits were not measured, but were assumed to approximate 50%. The diluted blood samples were stored at 4° until