THE THYMUS AND RECOVERY OF THE SHEEP ERYTHROCYTE RESPONSE IN IRRADIATED MICE*‡

By ALAN C. AISENBERG, M.D., and CAROLINE DAVIS

(From the John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Massachusetts 02114)

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The thymus has been recognized to play an essential role in immunological responsiveness for 6 yr, yet despite considerable investigation the essential nature of its action, be it cellular or humoral, remains elusive. Part of the uncertainty and controversy in the thymus literature undoubtedly arises because many techniques employed to measure immune impairment after thymectomy and subsequent restoration have been either qualitative or semiquantitative and may obscure the underlying mechanisms.

We have found that the plaque spleen assay developed by Jerne is quite satisfactory for analyzing precisely the cellular kinetics of drug-induced tolerance to sheep erythrocytes (1, 2). In the present investigation we have used the same technique to study the recovery of immunological responsiveness of lethally irradiated CBA mice. Recovery of this hemolysin response is largely thymus-dependent and the system is amenable to a number of experimental manipulations.

Methods

Female CBA mice (Jackson Laboratories, Bar Harbor, Maine) were 7–10 wk old at the time of irradiation. Lethally irradiated animals received 875 r (280 kv, 1.4 mm Cu, 67 r/min) followed within 4 hr by the intravenous injection of 4–5 $\times$ 10^6 bone marrow cells obtained from the femurs of 10 to 20-wk-old mice of the same sex and strain. Sublethally irradiated mice received 525 r without bone marrow. Unless otherwise specified, adult animals were thymectomized 1–4 days before irradiation by a method previously described (3). Thymus grafting was done with the technique of East and Parrott (4), placing a single whole CBA or C57BL thymus from an animal 12–48 hr of age under the kidney capsule through a flank incision. Thymus grafts were removed either by simple excision or by nephrectomy (5).

Immunological responsiveness was evaluated by the technique of Jerne (6, 7), as previously modified (1), which determines the number of 19S hemolysin-producing spleen cells. Animals were challenged with 0.2 ml of 10% sheep cells (5 $\times$ 10^8 cells) intravenously 4 days before sacrifice. An appropriate aliquot of sieved spleen was incubated with sheep erythrocytes in

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Gey's solution which was gelled by the addition of Agarose (SeaKem brand obtained from Bausche & Lomb Incorporated, Rochester, N. Y.). The gelled suspension was incubated for 2 hr at 37°, 1 ml of 1:10 guinea pig serum was added, and the suspension incubated for an additional hour to develop the direct (19S) plaques. The data presented in the figure and tables are the arithmetic mean of the direct plaques of the separately plated spleens.

All animals were subject to postmortem examination, and data from thymectomized animals with residual thymus were discarded. Similarly data from operated or nonoperated animals weighing less than 20 g or with spleen cell counts of less than 100 million (5 wk or more after irradiation) were discarded. Such animals usually have pneumonia and respond erratically to sheep cell injection. It is our impression that mortality after lethal irradiation is reduced by the use of acidified drinking water (addition of 5 ml of 12 N HCl per 12 liters of water to produce a pH of 2.5).

TABLE I

Plaque-Forming Spleen Cells and Total Spleen Cells at Various Times after Lethal Irradiation (and Bone Marrow) without and with Antigenic Challenge

Time after radiation	Antigenic challenge	Control			Irradiated			Irradiated, thymectomized		
		Plaques per spleen	Cells per spleen $\times 10^6$	Plaques per 10^6 cells	Plaques per spleen	Cells per spleen $\times 10^6$	Plaques per 10^6 cells	Plaques per spleen	Cells per spleen $\times 10^6$	Plaques per 10^6 cells
2 days	−	*47*	221	0.21	*6*	13	0.43	*2*	7	0.26
10 days	−	*45*	155	0.29	*30*	98	0.31	*21*	90	0.23
5 wk	−	*53*	153	0.35	*44*	118	0.37	*67*	120	0.56
	+	257,000	238	1080	19,600	168	117	146	122	1.2
10 wk	−	*44*	135	0.32	*45*	156	0.31	*39*	137	0.30
	+	113,000	245	464	52,400	208	252	2,480	159	15.0
20 wk	−	*56*	179	0.64				*65*	142	0.92
	+	128,000	210	611	44,900	165	272	2,200	138	15.9

RESULTS

Recovery from Lethal Irradiation in Normal and Thymectomized Mice.— Fig. 1 records the recovery of immune responsiveness in normal and thymectomized CBA mice who received 875 r followed by bone marrow administration. The total hemolytic plaques of the spleen 4 days after intravenous challenge have been plotted. 2½ wk after irradiation both operated and unoperated animals are unresponsive. 5 wk after X-ray the unoperated animals (open circles) show a partial return of immunological reactivity which reaches a maximum 10 wk after irradiation and then slowly declines. The maximum recovery of the unoperated animals reaches a level of about 50% that of non-

irradiated animals, and this percentage remains constant during the later decline.

Thymectomized irradiated animals recover very slightly the ability to form hemolysin over the 30 wk of observation (open triangles): the level of plaque-

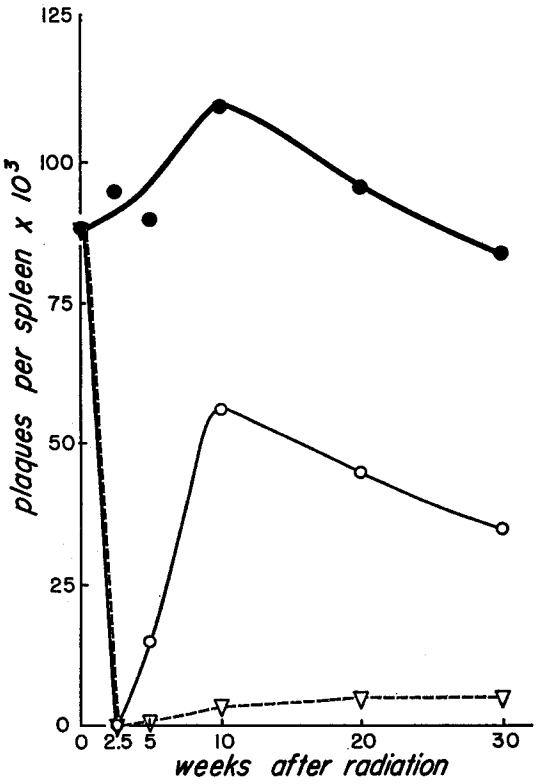

FIG. 1. Recovery of plaque response of normal and thymectomized mice after lethal irradiation and bone marrow restoration. ●——●, unirradiated, intact controls; ○——○, irradiated, intact animals; ▽---▽, irradiated, thymectomized animals. Each point is the arithmetic mean of 12 animals separately plaqued.

forming cells at all time points is less than one-twentieth that of intact, irradiated animals.

Recovery of Plaque-Forming Cells of the Unstimulated Spleen (Base Line Plaques) and Total Spleen Cells.—In Table I the number of plaque-forming cells and total spleen cells (with and without antigenic stimulation) are reported for normal, normal irradiated, and thymectomized irradiated animals. Challenged mice were not plaqued for the 2 and 10 day points since there is no recovery of responsiveness at these early times, but the later times (5, 10,

and 20 wk) were studied in both challenged and unchallenged animals. Two points of interest can be made from these data. First, the unstimulated plaque-forming cells (numbers in italics) recover at the same rate in thymectomized irradiated, and intact irradiated mice despite the absence of significant immunological recovery (plaques after antigen) in the operated animals. Thus it will be noted that the number of base line plaques (plaques without antigenic stimulation) per million spleen cells remains constant in both groups during the depletion and recovery process.

TABLE II

Recovery of Hemolytic Plaques after Sublethal Irradiation, and after Lethal Irradiation and Bone Marrow in Aged Mice

Exp. No.	Radiation dose	Age when irradiated	Time after irradiation	Normal* controls	Irradiated* normals	Irradiated* thymectomized
	r	*wk*	*wk*			
1	525	9	2½	41,000	1,520	640
			5	115,700	11,100	410
			10		15,900	1,580
			20		37,900	7,600
			30	124,000	51,000	7,900
2	875‡	25	10		17,300	1,620
3	875‡	30	10		67,500	4,200

* Expressed in plaques per spleen, arithmetic mean of four to six separately plaqued spleens.

‡ 4×10^6 isogeneic bone marrow cells.

The second point of interest is that the proliferative response to antigen (the increase in total spleen cells) is lost in the unresponsive thymectomized, irradiated animal.

Recovery after Sublethal Irradiation and after Lethal Irradiation in Older Mice.—Two additional points are considered in Table II. First, the immune recovery of animals with intact thymus who have been sublethally irradiated and have not received bone marrow appears to be a slower process than the recovery of lethally irradiated mice given bone marrow. However, recovery of the sheep cell response in sublethally irradiated mice is also thymus-dependent, although sublethally irradiated thymectomized mice, at any time point, are more responsive than their lethally irradiated operated counterparts. Secondly, recovery of the hemolysin response remains thymus-dependent even in animals irradiated at 25 and 30 wk of age (Table II, Experiments 2 and 3), but the ratio of plaque-forming cells of lethally irradiated intact animals to lethally irradiated thymectomized animals appears to be somewhat less in the older mice.

Manipulations of Transfused Marrow.—Table III contains three experiments in which the bone marrow transfused to the lethally irradiated nonthymectomized animal was altered in an attempt to achieve hematopoietic restoration without immunologic restoration. In no instance was this achieved. Thus in Experiment 1 all doses of marrow which allowed the survival of irradiated animals produced equal restoration of plaque-forming spleen cells. In a more

TABLE III

Bone Marrow Manipulations

Exp. No.	Radiation dose r	No. of animals	Survivors	Bone marrow cells*	Source of bone marrow	Plaques‡
1	0	4	4	0		151,000
	875	5	2	2.0	Normal	7,300
	875	5	3	0.5	Normal	4,800
	875	5	3	0.15	Normal	6,900
	875	5	3	0.05	Normal	21,000
	875	5	0	0.02	Normal	
2	0	8	8	0		62,800 ± 23,000
	875	8	8	4.0	Normal donor irrad. 15 wk before§	18,500 ± 12,000
	875	8	8	4.0	Donor thymectomized, irrad. 15 wk before§	18,900 ± 8,600
3	875	6	6	4.0	Normal	20,700
	875	6	0	5.2	Irrad.¶-525 r	
	875	6	3	5.7	Irrad.¶-262 r	12,100

* $\times 10^6$, recipients nonthymectomized.

‡ Arithmetic mean expressed in plaques per spleen, ± standard deviation.

§ Donors received 875 r followed immediately by normal bone marrow 15 wk earlier.

¶ Donor irradiated on the day of marrow grafting.

refined experiment (Experiment 2 of Table III) animals were restored with marrow from animals which had themselves been irradiated and transfused with marrow 15 wk before. Such marrow derived from thymectomized, irradiated animals (unable to respond to this antigenic stimulus) was as effective in restoring the sheep cell response of the secondary recipient as marrow from nonthymectomized, irradiated donors.

Finally, in Experiment 3 the marrow donor was irradiated immediately before the marrow was transfused to lethally irradiated animals. The finding in this experiment was unexpected: while control animals that received 525 r survived, recipients of marrow from these animals did not. Indeed, there were only three survivors out of the six recipients of marrow from animals that

received 262 r. The plaque response of the survivors was comparable to that of animals that received unirradiated marrow.

Delayed Thymectomy and Thymus Grafting.—Tables IV and V represent an effort to establish the time within the period of radiation recovery that the

TABLE IV

Effect of Delayed Thymectomy on the Recovery of the Hemolytic Plaque Response after Lethal Irradiation and Bone Marrow

Experimental group	No. of animals	Average*	Standard deviation
Intact, irradiated	14	29,400	18,400
Thymectomy before irradiation	14	1,040	960
Thymectomy 18 days after irradiation	14	2,150	2,060

* Plaques per spleen.

TABLE V

Reconstitution of Immune Function with Thymus Grafts

Thymectomy	Period graft in place*	Strain of thymus donor	No. of animals	Average plaques per spleen	Standard deviation	Ratio‡
−	Not grafted		18	41,400	±38,000	1.0
+	Not grafted		12	930	±700	0.02
+	1–70	CBA	12	57,900	±18,200	1.4
+	1–70	C57BL	14	1,020	±930	0.02
+	7–70	CBA	5	33,200	±11,500	0.8
+	14–70	CBA	6	53,000	±33,000	1.3
+	35–70	CBA	5	14,800	±9,400	0.4
+	50–70	CBA	5	3,100	±3,100	0.1
+	1–8	CBA	4	10,020	±8,000	0.2
+	1–15	CBA	5	7,040	±3,400	0.2
+	1–35	CBA	7	38,800	±12,600	0.9

* A single whole newborn thymus was placed under the kidney capsule of irradiated thymectomized CBA mice on the day indicated (day 1 is the day following radiation). Grafts were removed, if so indicated, either by nephrectomy or simple excision of the graft.

‡ Ratio of plaques in experimental group to plaques in nonthymectomized, nongrafted, irradiated controls.

thymus is operative. The findings again were not anticipated. Although considerable cell repopulation after radiation injury is evident by 10 days (Table I), mice thymectomized 18 days after irradiation were as deficient as those thymectomized before irradiation. This finding is confirmed by the grafting work.

CBA thymus grafts placed under the kidney capsule immediately after irradiation are very effective in restoring thymus function; indeed such grafted animals are more responsive than nonthymectomized, irradiated mice. Furthermore, grafts placed as late as 14 days after radiation result in complete restoration. Immediate grafts left in place for 1 or 2 wk provide only partial restoration, and grafts placed under the kidney on day 50 restore only slightly, though such grafts grow to large size by day 70. Finally, C57BL thymus grafts do not restore the hemolysin response of irradiated, thymectomized CBA mice.

TABLE VI

Immunological Restoration with Irradiated CBA Thymus Grafts

Experimental group	No. of animals	Weight of thymus graft	Plaques per spleen
		mg	
Intact, irradiated	6		30,300*
Thymectomy before irradiation	3		300*
Thymectomy before irradiation; normal thymus graft day after	6	28–51	45,600*
Thymectomy before irradiation; irradiated‡ thymus graft day after	1	3	7,400
	1	16	65,600
	1	21	25,000
	1	24	30,000
	1	12	11,300
	1	22	23,600
	6*		27,100*

* Arithmetic mean.

‡ Newborn donor received 875 r the day of grafting.

Immunological Restoration with Irradiation Grafts.—We have explored the reason thymectomized animals with thymus grafts are more responsive than irradiated, intact animals by means of an experiment with grafts from irradiated neonates (Table VI). Thymectomized animals, lethally irradiated and grafted with irradiated thymuses, recover a plaque-forming capacity that is closely similar to that of intact irradiated animals but is below the level of animals receiving unirradiated thymuses. In such animals the size of the irradiated thymus correlates with the responsiveness of the spleen of the animal that contains it (Table VI) These data suggest that irradiation of the thymus of nonoperated animals prevents complete recovery of responsiveness in these animals.

DISCUSSION

There appears little room to doubt that the hemolysin response to sheep erythrocytes of adult CBA mice is largely thymus-dependent. This dependence can be demonstrated after lethal and sublethal irradiation as reported both here and by others (8), after neonatal thymectomy (9), and in the recovery from drug-induced tolerance (2). We assume that the same cellular mechanism mediates these various thymus-dependent parameters. Recovery from nonspecific lymphoid damage induced with the drug cyclophosphamide (in contrast to drug-induced immunological tolerance [2]) differs from radiation recovery and is not thymus-dependent. Again, it seems logical that a different cell (or mechanism) is the target of this simple drug injury than is the cell target of the aforementioned thymus-dependent activities.

Our failure to obtain myeloid restoration without lymphoid restoration (in nonthymectomized animals) corroborates existing evidence that the same stem cell subserves the two processes (10). However, our own work is certainly not conclusive on this point. The finding that bone marrow from immunologically incompetent (irradiated, thymectomized) donors provides lymphoid stem cells as effectively as marrow from unoperated animals is of interest, but again could be anticipated (10). On the other hand, it is not clear why sublethally irradiated marrow fails to sustain lethally irradiated animals over the period of radiation injury. Why should marrow which has received 525 r function adequately *in situ*, but fail to function when transferred to animals that have received 875 r? Two explanations can be put forward, neither of which is at present backed by experimental evidence. Perhaps restoration is mediated by several cell populations of varying radiation susceptibility and only one is obtained (a cell that is radiation sensitive) in the marrow populations we employ. Alternately, a simpler explanation is that mechanical injury attendant upon making the marrow preparation adds to radiation injury and reparation is no longer adequate.

Since the CBA mouse does not reach full immunological competence until 15 to 20 wk of age (2), it was important to establish that the thymus continues to function beyond this age. Reported studies of adult thymus function (8, 11, 12) have not employed mice more than 12 wk old, apparently because of the difficulty in older animals in freeing the organ satisfactorily from adjacent structures. We have experienced no difficulty in removing the thymus from 25 and 30 wk old CBA mice, and find that the thymus continues to function in these older animals.

The relationship of the base line plaque (the plaque-forming cell of the unstimulated animal) to the specific cell or "clone" which responds to antigen remains unsettled. In earlier work we observed the disappearance of the base-line plaque with the induction of tolerance with the drug cyclophosphamide and

return of the plaque with recovery (2), and concluded from this that the base line plaque was related to the responding "clone." The present findings that the baseline plaque is preserved in the unreactive, irradiated thymectomized animals allows an interesting, if tentative, deduction: i.e., thymus-dependent immunological unreactivity occurs in the presence of this specific "clone."

This raises the important question of how the thymus is related to immunological tolerance (13). Isković et al. (14) feel, on the basis of experiments with thymus grafts from tolerant adult animals, that specific tolerance is a thymus function. At present we prefer the divergent view that specific immunological reactivity to sheep erythrocytes is not thymus mediated, but that the thymus amplifies the specific signal to a magnitude that is immunologically audible. (The degree of amplification may vary for different antigens and types of immune response). Clearly, the presence of base line plaques in the incompetent, thymectomized irradiated mouse is very indirect support for the preservation of the specific antigen recognition mechanism in the thymus-free animal. Perhaps an additional point in favor of this thesis is the observation that the thymectomized animal, in our experience, is never completely devoid of responsiveness; a residue of reactivity always remains. Perhaps such a formulation would also help explain the puzzling sporadic nature of the defect in thymectomized mice observed by Humphrey et al. (15). Finally, such a formulation would be consistent with the observations of Mitchell and Miller (16) and of Davies et al. (17) that thymus-derived cells fail to produce antibody.

A second and equally important question is whether thymus function is mediated by cells or a soluble factor, a question which has not been satisfactorily answered despite its investigation in a number of laboratories (18–24). While our studies do not settle this point, the experiments with late thymectomy and with thymus grafts help to delineate the question in several ways. First, it is clear that the thymus is not essential (for recovery of the hemolysin response) during the first two and one-half wk after radiation, during which time much of the cellular repopulation of spleen and lymph nodes takes place. Thus, animals thymectomized 18 days after irradiation are as deficient in hemolysin response as animals thymectomized before, and thymus grafts placed 2 wk after irradiation are as effective as immediate thymus grafts in restoring the thymectomized animal. Similar results were obtained with drug-induced tolerance in the same mouse strain, a parallel thymus-dependent function in which delayed thymectomy is as effective as immediate ablation (2).

It is also evident that the thymus graft must be in residence for a considerable period of time in order to achieve complete restoration of the hemolysin response. In our 10 wk experiments, a graft for the initial 2 wk produces only slight restoration, although by 2 wk a thymus graft under the kidney capsule is

well developed. Similarly a graft in place for the final 3 wk of the 10 wk period produces only slight restoration. Finally, our experiments suggest that the thymus itself is sensitive to radiation. It appears that the CBA mouse thymus undergoes sufficient radiation injury after 875 r so that it functions irregularly in restoring the hemolysin response.

The results with allogeneic thymus grafts deserve special comment. In contrast to our failure to observe restoration of the hemolysin response with allogeneic thymus grafts, Miller et al. have noted that rejection of skin homografts derived both from the thymus donor and a third party is restored by this manipulation. The strains and other experimental details employed by Miller et al. were similar to our own, and we have preliminary skin graft data which appear to confirm their findings. However, we feel that much more data with additional strain combinations are needed before we can comment on the extent to which the homograft reaction is restored by allogeneic thymus grafts.

This last point is an important one. If it proves possible to restore the homograft reaction without restoring the hemolysin response, then it appears likely that the allogeneic thymus graft has dissociated two separate functions of the mammalian thymus. In view of the known separation in birds of immunological function into humoral immunity subserved by the bursa of Fabricius and cellular immunity subserved by the avian thymus (25–27), this dissociation would suggest that the mammalian thymus combines the function of the two avian organs. However, at the present time the only point that can be made with assurance is that conclusions based on the role of the thymus in restoring the hemolysin response should not be applied to cellular immunity.

SUMMARY

The role of the thymus in the recovery of the sheep erythrocyte response after lethal irradiation has been studied in adult CBA mice with the hemolytic plaque technique of Jerne. This immunological parameter is markedly thymus-dependent. 10 wk after irradiation and after antigenic challenge the thymectomized animal has only one-twentieth to one-fortieth the number of plaque-forming cells as does the irradiated animal with intact thymus. The thymus continues to function into the 7th and 8th month of life in this strain. Unlike the drug-tolerant animal, the incompetent irradiated thymectomized mouse retains base line plaques (plaques without antigenic stimulation).

Thymectomy 18 days after irradiation is as effective as prior thymectomy in preventing recovery of the sheep cell response. Thymectomized animals receiving grafts of isogenic neonatal thymus (placed beneath the kidney capsule) 1 day, 1 wk, or 2 wk after irradiation are somewhat more responsive at 10 wk than intact animals. Grafts in place for 1 or 2 wk after irradiation and then removed result in one-fifth the recovery of grafts in place the entire time, while only slight restoration is obtained from grafts in place for the final

3 wk of the experiment. The results indicate that the thymus is not required for the 18 days after irradiation, that a period of at least 3 wk residence is required for complete restoration, and that the thymus itself is somewhat radiation-sensitive.

Allogeneic thymus grafts failed to restore the hemolysin response of irradiated thymectomized animals.

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