STUDIES ON THE RECOVERY OF THE IMMUNE RESPONSE IN IRRADIATED MICE THYMECTOMIZED IN ADULT LIFE*

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It has been shown that mice thymectomized at 2 months of age have diminished numbers of lymphocytes in their peripheral blood and lymphoid tissues (1) but no defects in immune responses (2). When, however, thymectomy was followed by a potentially lethal dose of ionizing irradiation and bone marrow therapy, the mice failed to respond, at 2 months postirradiation, to skin homografts and sheep erythrocytes. Sham-thymectomized control mice, on the other hand, had perfectly normal immune responses when tested 4 to 10 weeks after irradiation and marrow therapy. It was concluded that the recovery of the immune mechanism after total body irradiation is thymus-dependent (2).

The present experiments were undertaken to study the effects of varying different parts of this system such as the number, source, and type of cells used for therapy and the age of the host at thymectomy. In addition, the growth of the mice and their immune response at later times after irradiation were studied.

Materials and Methods

Animals.—Male mice of the CBA strain were used throughout as hosts and donors of bone marrow, spleen, and fetal liver cells. Male mice of the strains Ak, C3H, C57BL, and BALB/c were used as donors of skin. The strains C57BL and BALB/c differ from CBA at the strong histocompatibility locus H-2. The strains Ak, C3H, and CBA differ from one another at histocompatibility loci other than H-2 and thus have only weak immunogenetic differences. All the mice used have been highly inbred at the Chester Beatty Research Institute.

Thymectomy and Irradiation.—Thymectomy was performed as described previously (3). Mice were irradiated in groups of 5 in a perspex box, by means of a 220 kv Westinghouse

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x-ray machine, half value layer 0.4 mm Cu, focal distance 100 cm, dose rate 60 roentgens/minute. The dose of radiation used throughout was 850 r which is an LD_{99}/30 days for Chester Beatty CBA mice. Bone marrow and spleen cells were taken from 8-week-old donors, counted, suspended in 0.4 ml of 199 culture medium, and injected 4 to 6 hours after irradiation. Foetal liver cells were taken from foetuses in the 3rd week of gestation. No effort was made to assess the exact age of the fetuses. A cell suspension was prepared by means of a ground glass homogenizer, washed, and injected as above.

Tests for Immune Responses.—Two methods of testing the immune response were used: (*a*) skin grafting according to the method of Billingham and Medawar, (4), and (*b*) the production of haemagglutinins after intraperitoneal injection of sheep erythrocytes.[1] The CBA mice used had no naturally occurring agglutinins to sheep erythrocytes.

The basic experimental design used throughout is shown in Table I.

TABLE I

Experimental Schedule

Age, *wks*.....	8	10	14	18	19	20
Postirradiation, *days*.....	—	0	28	60	67	72
Control group I	Sham thymectomy	850 r and therapy				
Control group II	Thymectomy	Sham irradiation and therapy				
Experimental group I	Thymectomy	850 r and therapy (cells from normal donors)	Skin grafted	Sheep erythrocytes injected (first injection)	Haemagglutinins titrated and sheep erythrocytes injected (second injection)	Haemagglutinins titrated
Experimental group II	Thymectomy	850 r and therapy (cells from neonatally thymectomized donors)				

RESULTS

Long-Term Effects.—In previous experiments many thymectomized irradiated mice had lost weight, and about 25 per cent died between 30 and 80 days' postirradiation. In order to study changes in weight and mortality, mice were thymectomized and irradiated, given 5 million bone marrow cells, and left without further treatment. Controls received irradiation and bone marrow only. The results are shown in Fig. 1. After an initial loss of weight during the 1st week postirradiation, the mice in both groups gained weight at approximately the same rate during the next 2 weeks. Thereafter, only the mice in the control group continued to gain weight. The weights of the mice in the experimental group remained stationary from 30 to 70 days' postirradiation and began to rise only after 70 days. In this experiment, although the condition of the experimental mice was poor during the period up to 70 days, there was no mortality.

[1] Sheep erythrocytes were obtained from Wellcome Research Laboratories, Beckenham, England.

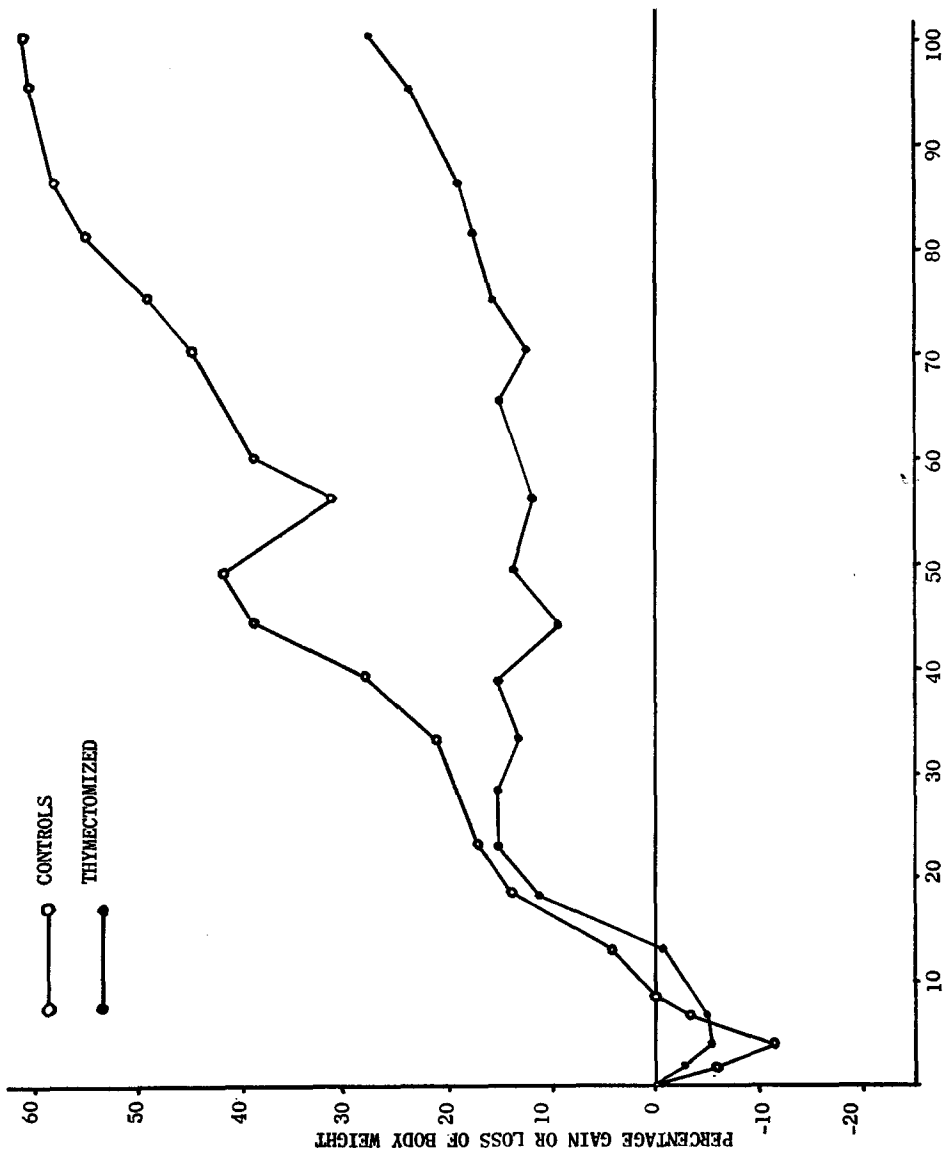

FIG. 1. Percentage increase or decrease in body weight of thymectomized irradiated CBA mice and sham-thymectomized irradiated controls.

The deaths recorded in previous studies may have been due to the fact that other manipulations such as skin grafting and bleeding had been performed.

In view of the fact that both the appearance and weight of the experimental mice improved after 70 days, the immune responses of groups of mice similarly treated were tested at times later than 70 days' postirradiation. Mice which had first been skin grafted at 28 days' postirradiation were regrafted at 128 days (Table II): 23 out of 24 mice still carried the Ak skin grafts and 17 out of 24 carried the C57BL grafts. On regrafting, some of the controls showed a rather

TABLE II

Skin Graft Survival Times in CBA Mice Thymectomized and Irradiated at 2 Months of Age

Treatment	No. of mice in group	Skin grafted at 30 days	No. of mice with skin surviving 100 days	No. of mice grafted again at 100 days	Skin grafted at 100 days	No. of mice showing survival of second skin graft for:		
						<20 days	20–50 days	>50 days
Control group I								
Sham-thymectomized and irradiated; syngeneic bone marrow	18	Ak	0	4	C3H	2	2	0
		C57BL	0		BALB/c	4	0	0
					C57BL	4	0	0
Control group II								
Thymectomized and sham-irradiated; syngeneic bone marrow	25	Ak	0	24	C3H	14	10	0
		C57BL	0		BALB/c	24	0	0
					C57BL	24	0	0
Experimental group I								
Thymectomized and irradiated; syngeneic bone marrow	24	Ak	23	24	C3H	5	4	15
		C57BL	17		BALB/c	7	5	12
					C57BL	7	9	8

slow rejection of the C3H graft. However, only in the experimental group were grafts retained for periods longer than 50 days. Previous challenge with C57BL skin prejudiced, in some mice, the survival of a second C57BL graft to a slight extent, with only 8 of these remaining intact for longer than 50 days as compared to 12 BALB/c grafts. Seven mice rejected the second C57BL graft while the first remained intact.

Mice previously challenged with sheep erythrocytes at 60 and 67 days after irradiation, were again challenged at 150 days (Table III). Most of the mice in the experimental group still produced no detectable titres of antibodies but the mean titre was significantly higher than at 60 days' postirradiation ($P < 0.01$). A group of mice, which had not previously been challenged in any way,

TABLE III

Immune Response of Thymectomized Irradiated CBA Mice to Sheep Erythrocytes

Treatment	Antigen challenge post-irradiation	No. of mice in group	No. of mice showing following $\log_4$ haemmagglutinin titers:										Mean $\log_2$ titer and standard deviation
			0	1	2	3	4	5	6	7	8	9	
	days												
Control group I													
Sham-thymectomized and irradiated; syngeneic bone marrow	60	14					14						8.86 ± 0.2673
	150	12					1	6		4	1		11.67 ± 2.416
Control group II													
Thymectomized and sham-irradiated; syngeneic bone marrow	60	12					12						8.75 ± 0.4085
	150	13					1		4	2	1	5	14.62 ± 3.162
Experimental group I													
Thymectomized and irradiated; syngeneic bone marrow	60	28	25	2		1							0.464 ± 1.210
	150	24	14	2	5	3							2.208 ± 2.973
Experimental group I*													
Thymectomized and irradiated; syngeneic bone marrow	150*	12			5	6	1						3.83 ± 1.354

* No previous challenge with antigen.

TABLE IV

Response of CBA Mice, Thymectomized at 5 Months of Age, to Allogeneic Skin Grafts

Treatment	No. of mice in group	Donor skin	No. of mice showing skin graft survival for:		
			<20 days	20–50 days	>50 days
Thymectomized, irradiated; syngeneic bone marrow	5	C3H	0	0	5
		C57BL	0	1	4
Thymectomized, not irradiated	6	C3H	6	0	0
		C57BL	6	0	0

was given sheep red cells at 150 days. The mean titre in this group was rather higher than that in the previous group but the differences were not significant.

Effect of Thymectomy in Older Mice.—In a small group of mice, thymectomy was performed at 5 months instead of 8 weeks of age (Table IV). These mice

TABLE V

Effect of Various Doses of Bone Marrow Cells on the Response of Thymectomized Irradiated CBA Mice to Allogeneic Skin Grafts

No. of marrow cells injected	No. of mice in group	Donor skin	No. of mice showing skin graft survival for:		
			<20 days	20–70 days	>70 days
1×10^6	3	Ak	0	0	3
	4	C57BL	0	0	4
5×10^6	6	Ak	0	0	6
	6	C57BL	0	0	6
10×10^6	6	Ak	0	0	6
	6	C57BL	0	0	6
20×10^6	4	Ak	0	1	3
	4	C57BL	0	3	1
40×10^6	8	Ak	0	0	8
	11	C57BL	0	6	5

TABLE VI

Effect of Various Doses of Bone Marrow Cells on the Response of Thymectomized Irradiated CBA Mice to Sheep Erythrocytes

No. of marrow cells injected	Antigen challenge	No. of mice in group	No. of mice showing following $\log_2$ haemagglutinin titer:										Mean $\log_2$ titer and standard deviation
			0	1	2	3	4	5	6	7	8	9	
1×10^6	First	4	3	1									0.75 ± 1.300
	Second	4	2	1		1							2.05 ± 1.581
5×10^6	First	6	5	1									0.1667 ± 0.373
	Second	6	3	1	1		1						1.1667 ± 1.472
10×10^6	First	6	5				1						0.6667 ± 1.472
	Second	6	4	1		1							1.1667 ± 1.472
20×10^6	First	4	3	1									0.25 ± 0.433
	Second	4	2	1		1							1.05 ± 1.225
40×10^6	First	13	2	3	1	5	1	1					2.231 ± 1.467
	Second	13	2	2	3	1	1	1	2	1			3.231 ± 2.632

showed the same inability to reject allogeneic skin grafts as those thymectomized at an earlier age.

Variation of Cell Number.—In the experiments described above, the mice were given a dose of 5 million bone marrow cells after irradiation. The effect

of varying this dose from 1 to 40 million cells was studied. The injection of as many as 40 million cells failed to restore more than a weak immune response and in many mice there was no effect. Only a few mice gave evidence of any ability to reject skin homografts (Table V). A similar picture was seen when the mice were challenged with sheep erythrocytes (Table VI). In this case, the titres were not increased except in mice given 40 million cells, and even this increase was not statistically significant.

TABLE VII

Effect of Pretreatment of Bone Marrow Donor on Recovery of Immune Response in Syngeneic Radiation Chimeras

Marrow donor		No. of irradiated recipients	Donor skin	No. of mice showing skin graft survival for:		
Age	Pretreatment			<20 days	20–50 days	>50 days
mos.						
2	Nil	18	Ak	18	0	0
			C57BL	18	0	0
	Neonatal thymectomy	10	Ak	6	4	0
			C57BL	10	0	0
9	Sham thymectomy, 850 r, syngeneic bone marrow	8	Ak	0	8	0
			C57BL	1	7	0
	Thymectomized, sham-irradiated, syngeneic bone marrow	11	Ak	0	11	0
			C57BL	0	11	0
	Thymectomized, 850 r, syngeneic bone marrow	11	Ak	0	11	0
			C57BL	1	10	0

Use of Marrow from Thymectomized Mice.—An experiment was performed to determine whether bone marrow cells from mice, which were themselves unable to reject skin grafts or produce sheep haemagglutinins, could restore immunological capacity in irradiated non-thymectomized mice (Table VII). Animals which had received bone marrow cells from 2-month-old neonatally thymectomized mice showed almost normal immune responses. Only in a few cases was there a slight prolongation of the survival time of allogeneic grafts from donors with the same H-2 locus as the host. All mice given bone marrow cells from 9-month-old donors rejected skin grafts more slowly than normally, but experimental mice, given marrow from thymectomized irradiated donors, gave the same rejection times as the controls.

Variation of Cell Type.—It is clear, from the above results, that bone marrow cells will not restore immunological capacity in the absence of the thymus.

It was, therefore, decided to see whether a different type of tissue would be effective.

Mice were given 10 million spleen cells after irradiation and the experimental schedule from Table I was used. The skin grafting results are given in Table VIII. It can be seen that, although the mice in the first experimental group showed rather long rejection times, there were no mice which showed survival of grafts for longer than 40 days. Similarly, the haemagglutinin titres of mice

TABLE VIII

Effect of Syngeneic Spleen Cells on the Response of Thymectomized Irradiated CBA Mice to Allogeneic Skin Grafts

Treatment	No. of mice in group	Donor skin	No. of mice showing skin graft survival for:			
			<20 days	20–40 days	40–70 days	>70 days
Control group I						
Sham thymectomized and irradiated;	16	Ak	13	3	0	0
syngeneic spleen cells	16	C57BL	16	0	0	0
Control group II						
Thymectomized, and sham-irradiated;	28	Ak	28	0	0	0
syngeneic spleen cells	28	C57BL	28	0	0	0
Experimental group I						
Thymectomized and irradiated; syngeneic spleen cells from normal donors	40	Ak	12	28	0	0
	40	C57BL	30	10	0	0
Experimental group II						
Thymectomized and irradiated; syngeneic spleen cells from neonatally thymectomized donors	6	Ak	0	1	1	4
	9	C57BL	1	1	0	7

in the first experimental group (Table IX) were significantly lower than those of control mice (first challenge, $P < 0.01$; second challenge $P < 0.05$), but were much higher than those seen in mice given bone marrow. A different picture is seen when spleen cells were taken from neonatally thymectomized donors (experimental group II). In most of these animals, skin grafts were retained for long periods (Table VIII) and haemagglutinins were produced in low titres or were not detectable (Table IX).

In a subsequent experiment, mice were given 5 million foetal liver cells after irradiation. They reacted in a similar way to those given bone marrow, being unable to reject allogeneic skin grafts (Table X) and to produce normal titres of sheep haemagglutinins (Table XI).

TABLE IX

Effect of Syngeneic Spleen Cells on the Response of Thymectomized Irradiated CBA Mice to Sheep Erythrocytes

Treatment	Antigen challenge	No. of mice in group	No. of mice showing following $\log_4$ haemagglutinin titers:									Mean $\log_2$ titer and standard deviation
			0	1	2	3	4	5	6	7	8	9	
Control group I													
Sham-thymectomized and irradiated; syngeneic geneic spleen cells	First	11				3	8						7.45 ± 0.9045
	Second	15					9	6					8.80 ± 0.9660
Control group II													
Thymectomized and sham-irradiated; syngeneic spleen cells	First	15					14	1					8.133 ± 0.5164
	Second	14						13	1				10.140 ± 0.5436
Experimental group I													
Thymectomized and irradiated; syngeneic spleen cells from normal donors	First	30				22	8						6.533 ± 0.8944
	Second	25				1	21	3					8.160 ± 0.7746
Experimental group II													
Thymectomized and irradiated; syngeneic spleen cells from neonatally thymectomized donors	First	9	6	3									1.330 ± 1.300
	Second	9	4	2	2	1							2.500 ± 1.699

TABLE X

Effect of Syngeneic Foetal Liver Cells on the Response of Thymectomized Irradiated CBA Mice to Allogeneic Skin Grafts

Treatment	No. of mice in group	Donor skin	No. of mice showing skin graft survival for:		
			<20 days	20–70 days	>70 days
Control group I					
Sham-thymectomized and irradiated; syngeneic foetal liver cells	18	C3H	11	7	0
	19	C57BL	15	4	0
Control group II					
Thymectomized and sham-irradiated; syngeneic foetal liver cells	21	C3H	18	3	0
	23	C57BL	22	1	0
Experimental group I					
Thymectomized and irradiated; syngeneic foetal liver cells	21	C3H	0	3	18
	21	C57BL	0	3	18

DISCUSSION

The results presented here confirm and extend previous observations (2, 5) indicating that there is a failure of the recovery of the immune apparatus in adult mice thymectomized and irradiated. Recovery of immunological function in the irradiated CBA mice, used in the present experiments, could take place only if either the thymus was present or if spleen cells from normal mice were provided. Adult marrow and foetal liver cells did not promote such a recovery in the absence of the thymus.

TABLE XI

Effect of Syngeneic Foetal LiverCells on the Response of Thymectomized Irradiated CBA Mice to Sheep Erythrocytes

Treatment	Antigen challenge	No. of mice in group	No. of mice showing following $\log_4$ haemagglutinin titers:									Mean $\log_2$ titer and standard deviation
			0	1	2	3	4	5	6	7	8	9	
Control group I													
Sham-thymectomized and	First	16					9	7					9.625 ± 0.7906
irradiated; syngeneic	Second	14					1	8	5				10.93 ± 0.9636
foetal liver cells													
Control group II													
Thymectomized and sham-	First	15				5	9	1					7.867 ± 0.9661
irradiated; syngeneic	Second	15					1	4	8	2			11.93 ± 1.4600
foetal liver cells													
Experimental group I													
Thymectomized and ir-	First	15	5	9	1								1.667 ± 1.1830
radiated; syngeneic foe-	Second	13	5	5	3								2.154 ± 1.7100
tal liver cells													

Strong evidence has recently been obtained to indicate that primary immune responses, in general, may be initiated by small lymphocytes (6). Depletion of small lymphocytes by prolonged lymph drainage from the thoracic duct suppresses primary antibody responses to tetanus toxoid and sheep erythrocytes (7). Neonatal thymectomy, which eventually leads to marked lymphoid atrophy (8), is associated with an inability to produce primary immune responses to standard antigens and to skin homografts (9). The failure of thymectomized irradiated mice to recover normal levels of small lymphocytes (2) may thus explain their immunological inadequacies. The unresponsive state in lymphocyte-depleted animals can be corrected by an injection of small lymphocytes or of lymph node and spleen cells from normal animals of the same inbred strain (7, 10). Likewise, in the present experiments,

injection of spleen cells from normal, but not from neonatally thymectomized animals, restored immunological capacity. These results indicate (*a*) that immunologically competent cells are present in the circulation, spleen, and lymph nodes of normal animals and can function in the absence of the thymus, and (*b*) that only the development of an adequate population of such cells is thymus-dependent.

Large numbers of cells from bone marrow of adult donors failed to restore immunological capacity in thymectomized irradiated mice. Bone marrow suspensions contain about 20 per cent of cells classifiable morphologically as small lymphocytes. It is thus apparent that there is not in marrow an adequate population of immunologically competent cells, and that the majority of marrow small lymphocytes must be functionally different from the majority of the small lymphocytes circulating in blood and lymph and present in spleen and lymph nodes of normal adult animals. The marrow small lymphocytes may thus belong to a population of cells that has, in its present state, no immunological function whatsoever. Whether this population ever gives rise to immunologically competent cells cannot be decided on the basis of present evidence.

It has been clearly established that after doses of irradiation such as those used in the present experiments, both the haematopoietic and lymphoid tissues of the host are repopulated by donor cells (11, 12). There must thus exist, in the marrow, a lymphoid precursor cell the descendants of which can become immunologically competent in the presence of thymus tissue. Such a precursor cell must also exist in neonatally thymectomized mice since marrow from such animals effectively restored immune competence in irradiated mice. Neonatal thymectomy is not, therefore, associated with the absence of lymphoid precursor cells or cells capable of acquiring competence. The identity of such cells in marrow tissue is not established. There is no evidence at the present time that the marrow small lymphocytes may be such cells.

Recent experiments indicate that the immunological inadequacies of the thymectomized irradiated mouse protected with marrow can be corrected by grafting syngeneic or allogeneic thymus tissue. Furthermore, it can be shown that in this system, the immunologically competent cells have been derived from cells of the marrow donor. The mechanism by which the thymus enables such cells to become immunologically competent has to be determined. The thymus could conceivably exert an influence through lymphoid precursor cells which migrate into the thymus first to reconstitute it and then recolonize the depleted lymphoid tissues. Alternatively, it could, by means of a humoral factor, induce the maturation of such cells in lymphoid tissues. Evidence has been given to show that a humoral thymus mechanism is involved in the maturation of immunological faculties in the neonatal mouse (13, 14). It now seems probable that a similar mechanism is also operative in the adult irradiated mouse (15).

Animals with depleted lymphoid tissues in general develop marasmus. This is seen in infant mice following the neonatal injection of allogeneic lymphoid cells (16), in lethally irradiated animals injected with allogeneic bone marrow (17) or with low numbers of syngeneic marrow or foetal liver cells (18), in mice thymectomized at birth (9, 19), and in human infants with essential lymphocytophthisis (20), a disease characterized by thymus atrophy. Since all these conditions have both lymphoid atrophy and trophic disturbances, it has been suggested that the lymphocyte may be a "feeder" cell or trephocyte (21, 22). The thymectomized irradiated mice in the present experiments exhibited some degree of trophic disturbances during the period 30 to 70 days postirradiation. Thereafter, however, their appearance improved and they began to increase in weight. It may be argued that the trophic disturbances observed between 30 and 70 days were dependent on lymphoid dysplasia. On this theory, however, it is difficult to explain the resumption of the increase of body weight which took place after 70 days at a time when the mice showed neither a significant increase in their peripheral blood mononuclear leucocyte counts (2) nor any apparent improvement in their immunological capabilities.

SUMMARY

Experiments performed on CBA mice thymectomized in adult life, exposed to lethal doses of irradiation and given tissue therapy are described. Marrow, foetal liver, or spleen cells from syngeneic donors could protect the mice against the lethal effects of irradiation. Between 30 and 70 days' postirradiation, however, marrow-treated, thymectomized irradiated mice showed evidence of trophic disturbances, such as failure to gain weight, in contrast to sham-operated, irradiated, marrow-treated controls. The immune responses of experimental and control mice were tested up to 150 days' postirradiation by challenging with sheep erythrocytes and allogeneic skin grafts. Sham-operated irradiated controls, whether protected with marrow, foetal liver, or spleen cells, produced normal immune responses when challenged at 28, 60, or 150 days after irradiation. Neither foetal liver cells nor marrow cells, in doses of up to 40 million cells per mouse, enabled thymectomized irradiated mice to recover normal immune functions. Spleen cells, from normal donors but not from neonatally thymectomized donors, restored immunological capacity in such mice. It is concluded that immunologically competent cells are present in the spleen of normal adult donors and can function in the absence of the thymus. Bone marrow, on the other hand, does not contain an adequate population of such cells but has lymphoid precursor cells, the descendants of which can become immunologically competent only in the presence of a functioning thymus mechanism.

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