CELLULAR RECOGNITION IN VITRO BY MOUSE LYMPHOCYTES

Effects of Neonatal Thymectomy and Thymus Graft Restoration on Alloantigen and PHA Stimulation of Whole and Gradient-Separated Subpopulations of Spleen Cells

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The thymus is influential in establishing and maintaining elements of immune function throughout life (1, 2) and appears to be required for elicitation of delayed hypersensitivity reactions, for effective rejection of allografts (3, 4), for resistance to tumor induction (5, 6), and for response to certain classes of antigens (7, 8). The objective of the studies to be reported here was to examine further the role of the thymus in cellular recognition. The susceptibility of whole and albumin gradient-separated subpopulations of cells taken from mice thymectomized at birth to stimulation in vitro by alloantigens and PHA was assayed. The capacity of thymus grafts of differing origins to restore cellular reactivity was also explored. These studies confirm that the thymus is required for normal levels of responsiveness of lymphoid cells to primary stimulation in vitro by alloantigens and PHA.

Materials and Methods

Experimental Animals.—Mice of the inbred strains, C57BL/6J, A/J, BALB/c, DBA/2, and CBA/J, and the F_1 hybrids of these strains, were derived from inbred stocks maintained in this laboratory or purchased directly from the Jackson Laboratories, Bar Harbor, Maine.

Neonatal Thymectomy and Thymus Grafting Procedure.—Thymectomy was performed within 24 hr of birth by the method described by Sjodin et al. (9). Macroscopic examination when the animals were sacrificed for an experiment verified the success of thymectomy.

The thymus graft procedure was a modification of that of Metcalf et al. (10) in which a polyethylene trocar containing one lobe of newborn thymus tissue was inserted under the left kidney capsule under direct vision. The trocar was approximately the size of an 18 gauge needle and was mounted on a syringe containing 0.5 ml of air. The cannula was inserted under the

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kidney capsule from the lower pole of the kidney, and pushed up to the upper pole. The graft was then deposited in this site by gentle pressure on the syringe plunger. Histologic examination of the thymus at the time of sacrifice of the animals showed normal thymus morphology with cortical-medullary differentiation and Hassall's corpuscles.

Cell Preparations.—Thymic and splenic lymphocytes were prepared for culture in the same manner as previously described (11).

Cell-Separation Method.—Spleen and thymus cells were separated by albumin density gradient centrifugation by a modification of the method of Raidt et al. (12), as described previously in detail (13). Briefly, dispersed spleen and thymus cells were centrifuged in Falcon culture tubes at 1000 rpm for 10 min in the cold. The cells were resuspended in 3 ml of 35% BSA (Pentex Co., Kankakee, Ill., Lot No. 50). 1 ml of the mixture was loaded in each of three 5 ml cellulose nitrate tubes for use in a Beckman SW 39 rotor. The cell mixture was then carefully overlayered with 1.0 ml quantities of 29, 26, 23, and 10% albumin, diluted from 35% BSA with RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.). Centrifugation was then carried out for 30 min at 13,500 rpm at 4°C.

Discrete bands of cells which formed at the interfaces between albumin solutions of differing density were harvested with sterile Pasteur pipettes. The A band was defined as that population of cells harvested from the interface above the 23% albumin layer; B band cells were from the interface above 26% albumin; C band cells were from the interface above 29% albumin; and D band cells were from the interface above 35% albumin. Cells were then washed twice in an excess of RPMI 1640 before counting and distribution to the culture tubes.

Tissue Culture Method.—The method of culture used in these studies has been described in detail in previous papers from this laboratory (11, 13, 14). The medium employed was RPMI 1640, containing 100 units of penicillin and 100 μg of streptomycin per ml, with 5% fresh, heated (56° for 30 min) human serum. 10×10^6 (PHA stimulation) or 15×10^6 (alloantigen stimulation) thymus or spleen cells taken from the various density gradient fractions or from whole thymus or spleen cell populations were distributed in 3 ml of medium in Falcon plastic, disposable, screw-capped tubes (Falcon Plastics, Los Angeles, Calif. No. 3033); then they were incubated at 37°C, loosely capped for 2 (PHA stimulation) or 3 days (alloantigen stimulation) in 5% CO_2 in air and 80% humidity, and were inclined at a 5° angle from the horizontal.

PHA-P (Difco Laboratories, Inc., Detroit, Mich.) was employed as supplied suspended in sterile water at a dose of 10 μl per culture.

Mitomycin-C (Nutritional Biochemicals Corp., Cleveland, Ohio) was used to block target cells in mixed-cell cultures for detecting alloantigen stimulation, in a modification of the method of Bach and Voynow (15), as described previously (14). 15×10^6 reacting cells were added to the 15×10^6 mitomycin-treated target cells and cultured in 3 ml of medium. Control mixed-cell cultures containing 15×10^6 mitomycin-blocked syngeneic target cells were added to the same number of syngeneic reacting cells.

DNA synthesis in the cultures was estimated by adding 1.0 μCi of tritiated thymidine[1] (^{3}H-Tdr, Schwarz BioResearch, Orangeburg, N. Y.; specific activity 1.9 Ci/mmole, 0.25 μCi in 0.5 ml) to each tube for the final 24 hr of culture. The method for scintillation counting has been described previously (11).

The data obtained are presented as mean values of replicate tubes, and are indicated in the tables as the averages of means obtained in different experiments done under identical conditions.

RESULTS

Effect of Thymectomy on In Vitro Stimulation of Spleen Cells

Effect of thymectomy.—The number of nucleated cells per spleen was enumerated on a total of 30 pairs of neonatally thymectomized and control C57BL/6

[1] Abbreviation used in this paper: ^{3}H-Tdr, tritiated thymidine.

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mice, ranging from 3 to 18 wk of age. As will be shown below (see Fig. 1), the total number of spleen cells during the first few weeks of life were lower in thymectomized than in control mice. However, by 13–16 wk of age, the initial deficit in numbers produced by thymectomy was no longer evident and cell numbers were equal in the two groups.

In general, peripheral lymphocyte levels in neonatally thymectomized mice were between 3600 and 8000 per mm^3, but no correlation was found between the number of spleen cells and the number of peripheral lymphocytes in individual thymectomized mice. In addition, thymectomy was associated with

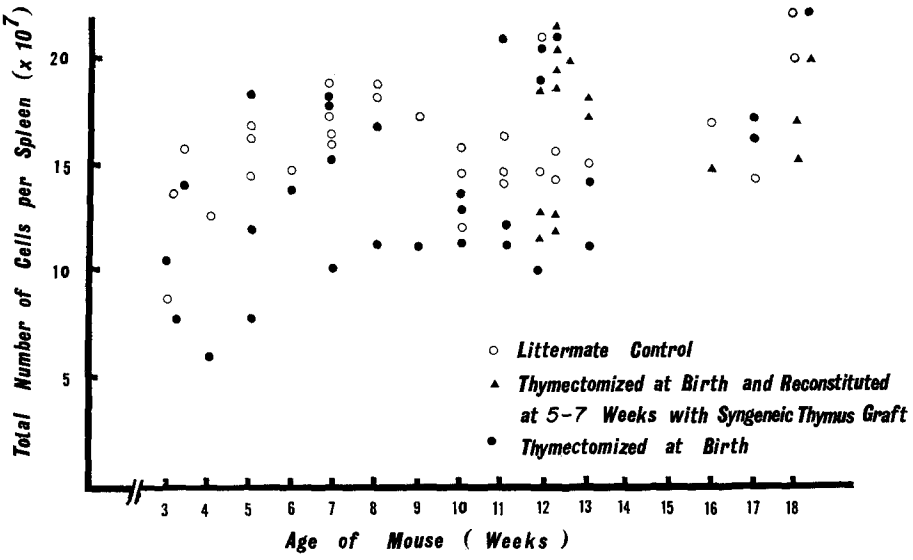

FIG. 1. Correlation of total numbers of spleen cells with age, in thymectomized, thymectomized and thymus graft-reconstituted, and control C57BL/6 mice.

no reduction in spleen weights, nor was evidence of wasting disease observed in the C57BL/6 strain.

PHA Stimulation of Spleen Cells from Neonatally Thymectomized C57BL/6 Mice.—Spleen cells from various strains of mice which had been thymectomized at birth or kept as paired littermate controls, and ranging from 3 to 19 wk of age, were divided into age groups, and the in vitro responses to PHA were assessed. Table I shows the results of experiments which compared PHA stimulation of spleen cells taken from these pairs. As no differences were found in sham operated and nonoperated littermates, the control population employed was the littermates.

As previously described, the titer of a naturally occurring antilymphocyte

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globulin present in human serum determines, to a large extent, the background level of ^{3}H-thymidine incorporation by spleen cells (11). The same human serum was used to supplement the culture medium employed to test the response of each pair of animals. The donors of serum were not always the same, however, for the various experiments. This probably explains the observation that tritiated-thymidine uptake was not equivalent in the controls and stimulated groups between the individual experiments. However, the ratios of incorporation by PHA-stimulated cultures to that of control cultures was com-

TABLE I

*Comparison of PHA Stimulation of Spleen Cells Taken from Various Strains of Neonatally Thymectomized and Paired Controls**

Strain tested	Age tested	No. of pairs	^{3}H-thymidine incorporation (mean/culture)					
			Littermate controls			Thymectomy at birth		
			Unstimulated	10 μl PHA added	Ratio of incorporation	Unstimulated	10 μl PHA added	Ratio of incorporation
	(*wk*)							
C57BL/6	3–4	6	8,383	54,793	6.54	9,573	9,760	1.0
	5–7	6	8,851	116,517	13.2	9,791	18,071	1.84
	8–11	6	4,120	77,418	18.8	7,295	25,796	3.5
	12–13	6	2,965	83,385	28.1	14,764	37,707	2.6
	17–18	3	1,587	71,345	45.0	1,834	17,003	9.2
BALB/c	7	1	3,979	183,691	46.2	6,435	38,502	6.0
DBA/2	7	6	21,685	69,835	3.2	28,705	47,509	1.7
CBA	8	3	12,045	111,547	9.3	17,702	15,941	<1
	16	2	13,248	104,459	7.9	23,164	73,747	3.2
A/J	7–8	2	11,023	89,010	8.1	8,994	28,558	3.2
(CBA × C57BL/6)F_1	16	2	8,120	98,561	12.1	12,131	73,570	6.1
(C57BL/6 × A/J)F_1	13–14	4	7,286	86,057	11.8	9,245	60,189	6.5
(DBA × C57BL/6)F_1	15–19	2	4,253	101,529	23.8	5,960	35,389	5.9
(CBA × DBA/2)F_1	6	1	1,926	82,204	42.6	3,971	7,015	1.76

* In these experiments, 10×10^6 spleen cells taken from thymectomized or paired control littermates of the mouse strain indicated were incubated with 10 μl of PHA in a 3 ml volume of RPMI 1640 containing 5% heated human serum. ^{3}H-thymidine incorporation was assayed during the final 24 hr of a 2-day culture period on triplicate tubes and expressed as mean cpm per tube.

parable between experiments. As shown in Table I, the background level of incorporation was always higher and the response to PHA was much lower in spleen cells from thymectomized animals, as compared to that of controls. The differences in the degree of stimulation between the two groups as reflected in the ratio of incorporation was highly significant.

Stimulation by Alloantigens in Mixed-Cell Cultures of Spleen Cells Taken from Neonatally Thymectomized Mice.—Table II compares the degree of stimulation in one-way mixed-cell cultures consisting of reacting cells taken from neonatally thymectomized or control animals of several strains and ages, with mitomycin-blocked normal allogeneic target cells. Responsiveness to alloantigenic stimulation in culture was much less in the thymectomized group than in the littermate

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controls. Control syngeneic cell cultures from C57BL/6 and BALB/c thymectomized mice gave background counts which were higher than those of controls, as seen in the PHA experiments cited above, but DBA-2 did not show this effect.

Effect of Thymectomy on In Vitro Stimulation of Subpopulations of Spleen and Thymus Cells Obtained by Density-Gradient Fractionation

Cells accumulated in the spleen are heterogeneous in morphology, life span, migratory behavior, origin, immunologic competence, and immunologic destiny.

TABLE II

*Comparison of Alloantigen Stimulation of Spleen Cells Taken from Various Strains of Neonatally Thymectomized and Control Mice in Mixed-Cell Culture**

Reacting cell strain	Age	No. of pairs	Allogeneic target cell strain	^{3}H-thymidine incorporation (mean/tube)					
				Littermate controls			Thymectomy at birth		
				Syngeneic target cell	Allogeneic target cell	Ratio of incorporation	Syngeneic target cell	Allogeneic target cell	Ratio of incorporation
	(*wk*)								
C57BL/6	3.5–6	3	A/J	634	5407	8.5	1187	2613	2.2
	7–8	3		664	2701	4.1	750	1937	2.6
	12–13	3		1007	8450	8.4	1814	4434	2.4
	17–18	3		645	5172	8.0	930	2846	3.1
DBA-2	7	1	C57BL/6	1087	4914	4.5	863	2298	2.7
	7	1	BALB/c	1087	2188	2.0	863	1373	1.6
BALB/c	7	1	DBA-2	787	3850	4.9	1275	4029	3.2
	7	1	A/J	787	3612	4.6	1275	4665	3.7

* In these experiments, 15×10^6 spleen cells taken from thymectomized or control paired littermates of the strain indicated in the first column, were mixed with an equal number of mitomycin-treated target cells of the strain indicated in the fourth column, in a 3 ml volume of RPMI 1640 containing 5% heated human serum from a single donor. The incorporation of ^{3}H-thymidine was assayed in triplicate tubes during the final 24 hr of a 3-day culture period, the values averaged and expressed as mean cpm per tube.

Consequently, interpretation of in vitro responses of whole spleen cell populations to antigens and other mitogenic stimuli can only be inferential and gives limited insight into the reactivities of the multiple subpopulations which comprise this organ. In order to examine further the influence of the thymus on spleen cell reactivity, subpopulations of spleen cells which varied in density were prepared by albumin-gradient fractionation. The resultant subpopulations of spleen cells from littermate normal and thymectomized animals were tested for reactivity to alloantigens and PHA. In addition, the reactivities of subpopulations of thymus cells were tested.

In the experiment shown in Table III, the cell numbers in each fraction and

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their reactivity to PHA is compared in the two groups in a typical experiment. Approximately 80% of the total number of spleen cells loaded onto the gradients were recovered at the four interfaces. The homogeneity of each fraction sepa-

TABLE III

*Comparison of Distribution in Density Gradients of PHA-Reactivity of Spleen and Thymus Cells from Neonatally Thymectomized and Control C57BL/6 Mice**

Cell source and treatment of mice	Fraction of gradient	Cell Distributions		^{3}H-thymidine incorporation (mean/tube)		Ratio of incorporation
		Number of cells	Per cent in group	Unstimulated	10 μl PHA added	
Spleen cells from neonatally thymectomized mice	A‡	10×10^6	1.9%	—	—	—
	B	130×10^6	23.61%	80,172	32,246	<1
	C	315×10^6	57.8%	18,406	27,678	1.5
	D	90×10^6	16.5%	12,136	22,191	1.83
		545×10^6§		19,500‖	29,422	1.5
Spleen cells from littermate controls	A	15×10^6	5.1%	—	—	—
	B	100×10^6	30.0%	23,594	45,303	1.92
	C	165×10^6	48.8%	10,697	65,299	6.1
	D	54×10^6	16.1%	1,773	56,259	32.0
		334×10^6		2,976	73,250	24.7
Thymus cells from controls	A	5×10^6	0.8%	—	—	—
	B	90×10^6	14.4%	5,108	38,856	7.6
	C	345×10^6	55.2%	716	2,430	3.4
	D	185×10^6	29.6%	311	411	1.4
		625×10^6		675	9,685	14.3

* In this experiment, $500–800 \times 10^6$ cells from neonatally thymectomized or paired control littermate C57BL/6 mice were suspended in 35% BSA and introduced into the lowest portion of a discontinuous albumin density gradient. The tubes were then centrifuged at 13,500 rpm for 30 min in a SW-39 swinging bucket rotor. The cells were carefully harvested by aspiration, then enumerated as indicated. 10 μl PHA was added to duplicate tubes, each containing 10×10^6 cells from the individual fractions, and ^{3}H-thymidine incorporation was assayed during the last day of a 2-day culture period. Results are expressed as mean cpm per tube containing 10×10^6 cells from each fraction.

‡ Fraction A consisted of cells above the 23% albumin layers, layer B above the 26%, layer C above the 29%, and layer D above the 35%.

§ Total cells counted in all fractions.

‖ Mean cpm in unfractionated cells.

rated according to density has been previously described (13). In both groups the highest proportion of all spleen cells was recovered in the C layer, and the representation in each of the other three subpopulations was nearly identical. Thymectomy, therefore, did not affect the distribution of spleen cells of differing

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density. Simultaneous gradient separation of normal thymus cells, also shown in Table III, gave more than 80% recovery of cells in the dense C and D layers.

PHA Response of Fractionated Spleen and Thymus Cells.—Susceptibility of the various subpopulations of spleen cells from thymectomized and control mice to PHA stimulation is also shown in Table III and is illustrated in Fig. 2. Striking differences were found between thymectomized and control groups in terms of PHA-induced incorporation of thymidine. Spleen cells taken from B subpopulations showed high background levels of incorporation and a low degree of PHA stimulation. Control groups C and D subpopulations of spleen

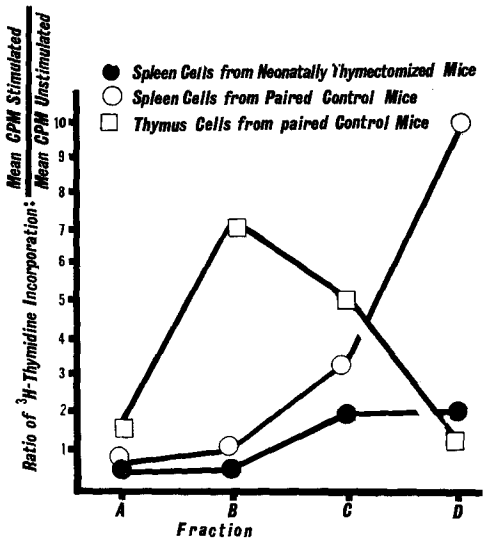

FIG. 2. PHA response of albumin gradient-fractionated thymus or spleen cells from normal or thymectomized C57BL/6 mice.

cells showed a lower background incorporation and a higher degree of PHA stimulation as described previously (13). All subpopulations of spleen cells from thymectomized mice were relatively unresponsive to PHA stimulation.

Fractionation of thymus cells as exemplified by the experiment shown in Table III yielded PHA-responsive B and C subpopulations. The B layer thymus cells gave incorporation values on the order of 7 to 10-fold over background. C and D layer cells had low degrees of background thymidine incorporation and D layer cells gave minimal response to PHA. In effect a small, but highly PHA-responsive subpopulation of cells exists in the thymus, the influence of which is diluted by the much greater population of nonsusceptible cells.

Alloantigen Stimulation of Fractionated Spleen Cells from Thymectomized and Control C57BL/6 Mice.—Spleen cell subpopulations from thymectomized and

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control C57BL/6 mice fractionated as described above were then reacted in mixed-cell culture with mitomycin-treated A/J target spleen cells. Table IV shows the results of a typical experiment involving the mixed-cell reaction. As was the case in responses to PHA, cells reacting most vigorously to alloantigens were from the higher density fractions of the gradient, the C and D layers. As was generally the case in other experiments, subfractions of spleen cells taken from thymectomized mice showed a higher background and were less responsive to alloantigen stimulation than cells from normal mice.

TABLE IV

*Effect of Neonatal Thymectomy on Allogeneic Stimulation of Gradient-Fractionated Spleen Cells Taken from C57BL/6 Mice**

Source of reacting cells	Gradient fraction of reacting cells	^{3}H-thymidine incorporation (mean/tube)		Ratio of incorporation
		Syngeneic target cells	Allogeneic target cells	Allogeneic/ Syngeneic
Thymectomy at birth:	B	6319	8278	1.3
	C	1502	1662	1.1
	D	1363	1477	1.1
	Unfractionated cells	2401	2624	1.1
Littermate controls:	B	3493	7836	2.2
	C	1075	3463	3.2
	D	448	1573	3.5
	Unfractionated cells	998	2807	2.8

* Spleen cells from the various fractions resulting from gradient separation of the indicated neonatally thymectomized or control mice, 15×10^6 cells per tube, were incubated with an equal number of C57BL/6 (syngeneic) or A/J (allogeneic) mitomycin-treated unfractionated target cells. Insufficient numbers of A layer cells were obtained for assay in this experiment. ^{3}H-thymidine incorporation was measured during the final 24 hr of a 3-day culture period, and the results expressed as the mean/tube (15×10^6 reacting cells) for two or three replicate cultures.

Reconstitution of In Vitro Responsiveness by Thymus Grafts

The immunologic deficiency produced by neonatal thymectomy, including prolonged allograft rejection time and lessened antibody responses are restored in part by thymus grafts (16, 17), by thymic cells, (18, 19), perhaps by thymic extracts (20–22), and by thymic tumors (23). It was, therefore, of interest to examine the effects of thymus grafts on the in vitro reactions of spleen cells of thymectomized-reconstituted mice. Accordingly, experiments were designed to test the effects of restoration by transplanting either syngeneic, allogeneic, or semisyngeneic thymus beneath the renal capsule by a modification of the technique of Metcalf et al. (10). As shown in Fig. 1, mice which had received a thymus graft had total spleen cell numbers which were about equal in thymecto-

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mized and control groups. PHA responsiveness of spleen cells taken from thymectomized mice which had received syngeneic thymus grafts was nearly completely restored, as shown by the results of experiments summarized in Table V. The results indicate that earlier grafts, given more time to become operational, are most effective in restoring in vitro PHA responsiveness. It was noteworthy that background thymidine incorporation of spleen cells from reconstituted mice, although variable, generally gave intermediate values between those of spleen cells from the thymectomized and from the control animals.

Restoration of in vitro PHA responsiveness in F_1 hybrids with parental 1-day old thymus grafts was very irregular, being detectable in only 5 out of 18 animals, as illustrated by experiments shown in Table V. (C57BL/6 × A/J) F_1 hybrid animals, thymectomized at birth and given parental C57BL/6 thymus implants, showed splenic enlargement, and the spleen cell cultures from such animals gave high background values and minimal evidence of a response to PHA. A graft-*versus*-host reaction might explain failure of the graft to restore PHA responsiveness in this combination. Allogeneic thymus grafts never restored responsiveness in any combination tested.

Spleen cells taken from thymectomized and thymus-grafted animals were assayed also in mixed-cell culture for stimulation by mitomycin-blocked allogeneic target cells. Table VI gives data indicating that syngeneic thymus grafts were effective in restoring the in vitro responsiveness of C57BL/6 spleen cells to A/J target cells, for example. The degree of restoration achieved was usually less than that seen to PHA stimulation, and it varied considerably in individual animals. Semisyngeneic grafts gave variable results, but never full restoration of all antigenic responsiveness, while allogeneic grafts were never effective in the many combinations tried.

PHA Stimulation of Gradient-Separated Subpopulations of Spleen Cells From Thymectomized Mice Grafted With Syngeneic Thymus.—As reported earlier (13), the D and C layer cells from density gradient fractions of the spleens of normal mice were the most reactive to PHA. The gradient fractions most responsive to PHA in normal thymus cell populations were however, from the B layer. Since thymectomized mice responded poorly to PHA stimulation, but showed no deficit in numerical representation of any of the subpopulations, and since thymus grafts did restore the PHA reactivity, it was of interest to determine which cells were responsible for PHA reactivity in the reconstituted mice.

Spleen cells from neonatally thymectomized C57BL/6 mice which had received a syngeneic thymus graft at 7.5 wk of age, were separated in albumin density gradients and the resultant subpopulations were tested for response to PHA. Table VII illustrates the results of such experiments. The subpopulations reactive to PHA were the same C and D layer cells found in spleen cells taken from normal controls. Since the thymus cell population responsive to PHA was

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TABLE V

*Comparison of PHA Stimulation of Spleen Cells from Normal Thymectomized and Thymectomized-Reconstituted mice which had been Grafted with either Syngeneic, Semisyngeneic, or Allogeneic Thymus**

Strain and age of mice	Number of mice	Thymectomy performed	Thymus grafted	Strain of origin of thymus graft and age at grafting	^{3}H-Tdr incorporation (mean and range)‡		Ratio of incorporation PHA/control (mean and range‡)
					Control	PHA-stimulated	
(*wk*)				(*wk*)			
C57BL/6	6	no	no	—	2,965	83,385	28.1
(12–13)					(634–4,503)	(64,856–111,632)	(20.5–133.0)
	6	yes	no	—	14,764	37,707	2.6
					(2,465–32,337)	(10,984–82,722)	(1.5–9.2)
	12	yes	yes	C57BL/6	5,609	64,633	11.5
				(7–9)	(2,226–10,720)	(21,038–307,480)	(2.7–24.8)
(15)	1	no	no	—	3,404	51,701	15.2
	2	yes	no	—	4,960	16,098	3.1
					(3,666–6,560)	(8,021–24,175)	(2.2–3.8)
	4	yes	yes	C57BL/6	4,617	56,569	12.3
				(8)	(3,724–5,056)	(49,657–72,238)	(10.0–15.2)
	1	yes	yes	CBA	7,466	37,791	5.0
				(8)			
	3	yes	yes	(CBA × BL)F_1	11,687	13,802	1.2
				(4)	(10,051–13,045)	(10,515–17,647)	
	1	yes	yes	A/J	14,430	15,083	1.1
				(6)			
(17–18)	3	no	no	—	1,587	71,345	45.0
					(1,044–1,991)	(61,267–87,764)	(32.6–58.7)
	3	yes	no	—	1,834	17,003	9.2
					(1,688–2,076)	(9,700–25,455)	(5.7–14.6)
	4	yes	yes	C57BL/6	1,637	58,294	35.6
				(5–6)	(1,172–2,517)	(54,340–63,737)	(23.2–48.5)
C57BL/6	2	no	no	—	3,573	71,061	19.9
(13–14)					(3,265–3,881)	(60,018–82,104)	(15.5–25.2)
	2	yes	no	—	25,049	62,371	2.5
					(17,760–32,337)	(42,019–82,722)	(2.4–2.6)
	3	yes	yes	(DBA-2 × C57BL/6)F_1	8,116	18,333	2.2
				(6)	(4,747–13,804)	(11,262–22,347)	(1.6–3.7)

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(A/J × C57BL/6)F_1 (13–14)	2	no	no	—	6,253 (2,133–10,373)	58,688 (36,697–80,678)	9.3 (7.8–17.2)
	2	yes	no	—	8,204 (5,050–11,358)	42,212 (39,392–45,031)	5.1 (4.0–7.8)
	2	yes	yes	C57BL/6 (6)	11,326 (10,370–12,282)	38,759 (26,309–51,209)	3.4 (2.5–4.2)
(14)	2	no	no	—	8,318 (7,561–9,075)	116,426 (115,465–117,487)	13.9 (12.9–15.2)
	1	yes	no	—	10,285	78,166	7.6
	4	yes	yes	C57BL/6 (4.5)	11,129 (7,274–18,576)	59,771 (14,850–88,263)	5.4 (1.0–12.1)
	1	yes	yes	A/J (4.5)	12,021	76,232	6.3
(CBA × C57BL/6)F_1 (16)	1	no	no	—	8,120	98,561	12.1
	2	yes	no	—	12,131 (10,201–14,060)	73,620 (70,990–76,250)	6.1 (5.4–7.5)
	2	yes	yes	C57BL/6 (8)	9,763 (8,397–11,127)	75,293 (73,310–77,275)	7.7 (6.7–8.7)
(DBA-2 × C57BL/6)F_1 (15)	1	no	no	—	5,973	92,686	15.5
(15)	1	yes	no	—	5,174	30,784	5.9
(15)	3	yes	yes	DBA-2 (7)	8,473 (4,434–15,271)	40,913 (33,684–54,897)	4.8 (2.2–12.3)
(15)	1	yes	yes	C57BL/6 (7)	6,322	43,789	6.9
(19)	2	no	no	—	5,372 (3,432–7,312)	114,169 (110,371–117,966)	21.8 (24.3–32.0)
(19)	1	yes	no	—	6,746	39,994	5.9
(19)	4	yes	yes	C57BL/6 (6)	4,682 (2,539–9,826)	48,250 (21,754–63,454)	10.3 (6.1–25.1)

* In these experiments, 10×10^6 spleen cells from the various mice were cultured with or without PHA for 48 hr. Thymidine incorporation was assayed during the final 24 hr of culture. Thymectomy was performed at birth and thymus grafts were implanted when the thymectomized mice were of the indicated age.

‡ Values for ^{3}H-Thymidine incorporation and ratio of incorporation for cultures of spleen cells from single animals represent means of duplicate or triplicate cultures. In those instances where more than one pair was examined in a single experiment, the mean values for all animals were averaged, and the range of these means are given in parentheses in the three columns on the right.

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in the less dense B population, it appears that the mechanism of reconstitution of a thymectomized animal by a thymus graft is not simply the grafting of an unchanging subpopulation of PHA-responsive cells.

TABLE VI

Comparison of One-Way Allogeneic Stimulation in Mixed-Cell Culture Between Normal or Thymectomized C57BL/6 Mice and those Thymectomized and Reconstituted with Syngeneic, Allogeneic, or Semisyngeneic Thymus Grafts

Age of mice	Thymectomy	Thymus graft	Strain of origin of thymus graft	^{3}H-thymidine incorporation		Ratio of incorporation
				Syngeneic target cells (mean/culture)	Allogeneic target cells (mean/culture)	Allogeneic/Syngeneic
(*wk*)						
13	no	no	—	1532	14,459	9.0
14	no	no	—	2511	16,540	6.6
15	no	no	—	417	4,034	9.6
18	no	no	—	366	3,523	9.4
18	no	no	—	629	5,826	9.3
13	yes*	no	—	1778	4,231	2.4
13	yes	no	—	2365	6,247	2.6
15	yes	no	—	961	2,307	2.4
15	yes	no	—	1499	2,979	2.0
18	yes	no	—	563	1,693	3.0
15	yes	yes	C57BL/6‡	566	3,968	7.0
15	yes	yes	C57BL/6	724	4,977	6.9
18	yes	yes	C57BL/6	760	3,972	5.2
18	yes	yes	C57BL/6	799	3,675	4.6
18	yes	yes	C57BL/6	433	3,367	7.8
13	yes	yes	(DBA × C57BL/6)F_1§	1865	3,856	2.1
13	yes	yes	(DBA × C57BL/6)F_1	948	4,792	5.1
14	yes	yes	(DBA × C57BL/6)F_1	2101	5,436	2.6
15	yes	yes	(CBA × C57BL/6)F_1	1268	7,751	6.1
15	yes	yes	(CBA × C57BL/6)F_1	1038	6,017	5.8
15	yes	yes	(CBA × C57BL/6)F_1	2385	7,214	3.0

In this experiment, 15×10^6 C57BL/6 mouse spleen cells were mixed with 15×10^6 mitomycin-treated syngeneic or 15×10^6 mitomycin-treated A/J spleen cells and the amounts of ^{3}H-thymidine incorporated was compared.

* Mice were thymectomized at birth.

‡ Thymus graft implanted at 5 wk of age.

§ Thymus graft implanted at 6 wk of age.

DISCUSSION

The central role of the thymus in immunity has been defined in many different species, using a variety of experimental methods. In the mouse, elimination of thymic function by neonatal thymectomy, or by adult thymectomy followed by

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irradiation, have proven to be especially useful models in understanding the functions of the thymus. More recently, grafts of thymus tissue have been used successfully to reconstitute both neonatally thymectomized and adult thymectomized irradiated mice in terms of their capacity for antibody production at the cellular level (24–26), graft-*versus*-host reactivity (footnote 2 and reference 27), and restoration of thymus-influenced membrane antigens on peripheral lymphoid cells (28, 29). The series of experiments reported here were directed toward assaying the effects of thymectomy and restoration on in vitro responses of spleen cell populations to a general mitogen, PHA, and to specific membrane alloantigens. Such an approach has the advantage of assaying reactions which appear to involve direct cellular recognition. It possibly avoids the complication of helper functions supplied by other cells.

TABLE VII

PHA Stimulation of Density Gradient-Separated Spleen Cell Subpopulations from Neonatally Thymectomized-Reconstituted C57BL/6 Mice

Gradient fraction	Cell distribution cells in each fraction		^{3}H-thymidine incorporation (mean/tube)		Ratio of incorporation
	No. of cells	Per cent	Unstimulated	10 μl PHA added	
A	35×10^6	4.5	88,243	23,210	0.27
B	150×10^6	19.1	39,607	15,526	0.39
C	415×10^6	52.8	10,650	35,655	3.3
D	185×10^6	23.6	4,136	27,071	6.5
Unfractionated	785×10^6		3,393	43,222	12.6

C57BL/6 mice were thymectomized at birth and received a 1 day old C57BL/6 thymus graft under the kidney capsule at 7.5 wk of age. Spleen cells from these mice at 16 wk of age were separated on albumin density gradients and the cells were cultured with and without PHA.

In general, the data show that the total cell numbers in the spleen and the proportionate distributions of cells in various albumin density gradient subpopulations are the same in neonatally thymectomized and normal animals, despite demonstrable functional deficiency. This suggests that the density characteristics of cells in the spleen are not necessarily related to the presence or absence of the thymus. Earlier studies in this series (14), and some more recent unpublished ones, suggest that the density distribution of cellular subpopulations in the spleen may be directly related to the level of immunologic activity of the host. For example, it was found that animals bearing syngeneic tumors, or who have received alloantigen or other membrane antigen immunization, show shifts in subpopulations of spleen cells in favor of the less dense, larger cell types. Moreover, residual thymus-dependent reactivity to PHA or

[2] Stutman, O. Personal communication.

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alloantigens in thymectomized animals, while markedly reduced, also shifts from the more dense to the less dense layers of cells.

Decreased PHA and alloantigen reactivity of cell populations of the spleens of thymectomized animals probably does not reflect the absence of a particular cell type. Rather, it indicates that the small lymphocyte population in the spleen does not function normally without thymic influence. Syngeneic thymic grafts restored both alloantigen and PHA in vitro reactivity to these cells. The degree of restoration was dependent both upon the age of the recipient at the time when the graft was implanted, and upon the length of time the graft had been in place before in vitro reactivity was assayed. This suggests that either the recirculating small lymphocyte population which passes through the thymus, or the stem cells which differentiate there, require time to accumulate in the periphery and restore susceptibility to in vitro stimulation. On the other hand, when thymus cell populations were examined, it was not the population of small, dense thymocytes, representing a great majority of all cell density groups present, which displayed PHA reactivity. This property was limited to a minor population having B layer density characteristics. The B layer cell population of spleen cells was not the reactive one in the restored animals, and rather the small lymphocytes of the C and D layers were reactive, as was the case in the normal animal population. Experiments in which restoration with thymus cells was attempted wholly in vitro, have not been successful, probably due to the length of time necessary for restoration to be accomplished.

The experiments do not discriminate directly between two possible interpretations of these data. In one, it could be considered that the B layer cells represent a small intrathymic immunologically competent cell population which is differentiated under thymic influence and was poised for peripheralization at the time of grafting. Such cells would then be conceived as establishing in the thymectomized animal an immunocompetent population which then develops new density characteristics as it takes up residence in the spleen or lymph nodes. The second explanation would assume that these minor subpopulations of the grafted thymus are transients, and that the time required for establishment of immunocompetence in the restored animal represents the need for thymic passage and thymic influence of a stem or recirculating population, which eventually restores the animal's in vitro competence. Some recent evidence in this laboratory[3] favors the former interpretation, in that B layer cells have been found to have the capacity to restore antibody production at the cellular level in the Shearer-Cudkowitz model (30). Whether this layer is also responsible for restoration of direct cellular recognition, as we have assayed this property here, is unknown at present.

Restoration experiments establish that complete or nearly complete restora-

[3] Takiguchi, T., W. H. Adler, and R. T. Smith. Restoration of immune competence in irradiated mice by gradient-fractionated subpopulations of thymus cells. Submitted for publication.

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tion of thymic function occurs when syngeneic grafts are put into the renal capsule. This contrasts markedly with the results of the experiments in which reconstitution was performed with parental, F_1 hybrid or allogeneic thymus. In the latter case, no functionally effective restoration was ever detected. In a few animals partial restitution of PHA responsiveness was found when parent thymus grafts were made in F_1 thymectomized mice. In limited numbers of animals F_1 grafts appeared to restore alloantigen responses to parental recipients. This result is consistent with that of Stutman, who has found[2] that F_1 hybrid thymus grafts partially restored graft-*versus*-host reactivity of spleen cells assayed by the Simonsen technique.

Another interpretation of apparent restoration of alloantigen responsiveness by F_1 thymus cells in mice is plausible. Since neonatally thymectomized mice do not lose immunocompetence completely, thymus grafts containing foreign alloantigens may actually immunize the recipient. This might result in apparent in vitro stimulation by alloantigens in those instances in which the grafted thymus shares H-2 isoantigenic specificities with the mitomycin-treated allogeneic target cells employed. As shown previously (14), spleen cells from immunized animals increase in responsiveness to alloantigens in mixed-cell culture when tested against any strain carrying antigenic determinants shared with the strain used for immunization. In the experiments reported here it is conceivable, for example, that the apparent partial restoration of responsiveness to A/J alloantigens in C57BL/6 mice by a (DBA × C57BL/6) F_1 thymus graft may have been due to immunization of the partially competent thymectomized C57BL/6 host by the DBA component of the F_1 graft which shares at least eight major alloantigenic determinents with A/J.

The restoration, or failure of restoration by F_1 and allogeneic thymus grafts, is of considerable theoretical interest in terms of the recent hypothesis of Jerne (31) concerning the generation of antibody diversity. Jerne has attempted to link histoincompatibility between members of the same species and the generation of immunologic diversity by postulating a selection mechanism, perhaps the thymus, in which genetically instructed cells, having recognition sites for self-alloantigens, are thereby either eliminated or permitted to express new antigenic recognition sites which arise by mutation (31). Jerne's hypothesis would predict that an F_1 thymus should eliminate cells carrying alloantigen recognizing sites for both parental strains, thus the F_1 reconsituted animal should remain tolerant of the other parental antigens, and not give appropriate restored recognition responses in vitro. The type of experiments reported here offer opportunities to test the hypothesis, but experiments designed specifically to attack this question are still in progress.

SUMMARY

The effects of thymectomy and thymus graft restoration upon the in vitro primary responses to alloantigens and PHA have been studied. It has been

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found that neonatal thymectomy substantially eliminates both PHA reactivity and responsiveness to alloantigens assayed in vitro in host spleen cell populations. Analysis of albumin density gradient-separated subpopulations of the spleen and thymus in such animals was also performed. It was found that the total and proportional representation of the individual density subpopulations was identical in neonatally thymectomized, in normal, and in thymectomized and thymus graft-restored animals. Therefore, thymectomized mice appear to retain a nonfunctioning, small, dense, lymphocyte population. Reconstitution of thymic-dependent in vitro reactivity was nearly complete when syngeneic, but not allogeneic or semisyngeneic thymus was employed. Occasional partial restoration did occur when F_1 thymus was employed, but never when allogeneic thymus was grafted. The grafted thymus contained PHA and alloantigen-reactive cells in a large, less dense B layer subpopulation, whereas the restored animals, as in the case of normals, showed these reactivities to be a property of a small, more dense cell population.

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