CELL TO CELL INTERACTION IN THE IMMUNE RESPONSE

V. Target Cells for Tolerance Induction*,‡

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Collaboration between thymus or thymus-derived lymphocytes and nonthymus-derived precursors of antibody-forming cells has been implicated in the immune response of mice to sheep erythrocytes (SRBC)[1] (1–3). Neonatal thymectomy impairs the response of mice to SRBC, and this can be reversed by inoculating thymus or thoracic duct lymphocytes simultaneously with SRBC (4, 5). In this system, thymus cells were as effective as thoracic duct cells and semiallogeneic cells were also effective. The identity of the antibody-forming cells produced was determined by using anti-H2 sera in allogeneically reconstituted hosts and chromosome marker analysis in a syngeneic system. These techniques demonstrated that the antibody-forming cells were, in general, derived not from the inoculated lymphocytes, but from cells already present in thymectomized hosts. Irradiated recipients of lymphoid cells from various sources were used to identify the precursors of antibody-forming cells. Thymus cells given together with SRBC failed to produce antibody-forming cells in irradiated mice, even when injected in very large numbers. A synergistic effect between thymus and marrow cells has, however, been described in such mice (6). By means of a chromosome marker method it was shown that all the antibody-forming cells produced were derived not from the thymus donor, but from the marrow donor (7). The capacity of adult thymectomized irradiated and marrow-protected mice to respond to SRBC is depressed but can be restored by the injection of thoracic duct lymphocytes (5). Semiallogeneic lymphocytes were effective in this system, and it was shown, by means of anti-H2 sera, that the antibody-forming cells produced were again derived not from

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[1] *Abbreviations used in this paper*: SRBC, sheep erythrocytes; HRBC, horse erythrocytes; PFC, plaque-forming cells; 19S PFC, direct plaque-forming cells; 7S PFC, developed plaque-forming cells; TDL, thoracic duct lymphocytes; BM, bone marrow; T, thymus lymphocytes; LN, lymph node cells, S, spleen cells; TxBM, adult thymectomized, irradiated, and marrow protected; TxS, spleen from thymectomized, irradiated, marrow protected mice; SE, standard error; NS, not significant.

the inoculated lymphocytes, but from the marrow. It is evident, therefore, that collaboration between thymus-derived and marrow-derived cells is an essential component of the response of mice to SRBC. More recent experiments have shown that a similar type of collaboration takes place with respect to other antigens, such as heterologous serum albumin (8).

The nature of the interaction which takes place between thymus and nonthymus-derived lymphocytes is not understood. Clearly, the antibody-forming cell precursor is not thymus-derived (4, 5, 7), but it is not known whether the thymus-derived cell, the nonthymus-derived cell, or both, can recognize and react specifically with antigen, and thus determine the specificity of the response. In an attempt to study this question, experiments were performed with mice rendered specifically tolerant of SRBC. In this paper, we present results which indicate that target cells for tolerance induction exist in the thymus-derived lymphocyte population. They cannot, however, be demonstrated in the nonthymus-derived population in the system used here. These findings are discussed with reference to the nature of the interaction which takes place between the two classes of lymphoid cells.

Materials and Methods

Animals.—Male and female mice of the highly inbred CBA strain, originally obtained from Harwell, Didcot, Berkshire, England, were used. They were raised and maintained at the Hall Institute and were fed Barastoc cubes with an occasional green feed supplement of cabbage and water *ad libitum*. Neonatally thymectomized mice were reared on foster mothers of a randomly bred Hall Institute strain with a view to minimizing losses from cannibalism after the operation. Penicillin, at a dose level of 600,000 units per liter, was added to the drinking water each day.

Cell Suspensions.—Cell suspensions from thymus, spleen, and mesenteric lymph nodes were prepared by teasing with fine forceps through an 80-mesh stainless steel sieve in cold Eisen's solution.[2] Further disruption was achieved by gentle aspiration with a Pasteur pipette. The suspensions of single cells were washed three times in Eisen's solution in the case of thymus and once or twice in the case of lymph nodes or spleen. The cells were finally resuspended in Eisen's solution and counted in a hemocytometer, the volume being adjusted so that the number of cells required for injection was contained in 0.2–0.6 ml, depending on the experiment.

Marrow cells were expressed from the femurs and tibiae by means of a syringe and a needle using cold Eisen's solution. The marrow plugs were gently disrupted by aspiration through a 25-gauge needle. The suspension of single cells was washed once, resuspended in cold Eisen's solution, counted, and the volume adjusted so that the required dose could be injected.

Thoracic duct lymphocytes were obtained from mice cannulated and restrained in modified Bollman cages. The cells were collected in cold Dulbecco's solution[3] containing 10% fetal calf serum.[4] After gentle centrifugation, the cells were resuspended in a volume suitable for injection.

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[2] Kern, M., and H. N. Eisen. 1959. The effect of antigen stimulation on incorporation of phosphate and methionine into proteins and isolated lymph node cells. *J. Exp. Med.* **110**:207.

[3] Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **99**:167.

[4] Fetal calf serum was obtained from Commonwealth Serum Laboratories, Melbourne, Australia.

SRBC were obtained from a single animal. The jugular vein was punctured at weekly intervals, the blood collected, and stored in Alsever's solution for 1 wk prior to use. When required, the cells were washed three times in saline and finally resuspended in an appropriate volume. The number of cells used for immunization was $2–5 \times 10^8$. Horse erythrocytes were obtained from Commonwealth Serum Laboratories, Melbourne, Australia, and stored in citrate saline. When required, they were washed three times in saline and resuspended to an appropriate volume. The same number of cells was used for immunization as in the case of SRBC.

Injections.—Cell suspensions were injected into the tail vein unless otherwise stated. In the case of thymus cells given in doses exceeding 10 million per mouse, it was absolutely essential to spread the injection over 1–2 min and to give a large volume (up to 0.6 ml) to prevent death from emboli. Mice were not heparinized before injection.

Operative Procedures.—Thymectomy or sham-operation was performed in newborn mice, less than 36-hr old or in 8–10-wk old mice, according to the method of Miller (9). Whenever thymectomized mice were killed, the mediastinum was examined macroscopically and, in some cases microscopically, to check for the presence of thymus remnants. Only very few mice were found with such remnants and were discarded from the experiments.

The technique used to establish a thoracic duct fistula has already been described in the first paper in this series (4).

Irradiation.—Intact or adult thymectomized mice were exposed to total body irradiation in a Perspex box. In the case of thymectomized mice, irradiation was performed generally 1–2 wk after thymectomy. The dose given was 800 r to midpoint with maximum backscatter conditions and the machine operated under conditions of 250 kv, 15 ma, and an HVL (half value layer) of 1 mm Cu. The focal skin distance was 50 cm and the absorbed dose rate was 170 r/min. When thymectomized irradiated mice were to be protected with bone marrow, they received an intravenous injection of 3–5 million cells in 0.1 ml of Eisen's solution 1–3 hr after irradiation. All irradiated mice were given penicillin in the drinking water.

Plaque Forming Cell (PFC) Assays.—Spleen cell suspensions for assays were prepared as mentioned above, washed once, and diluted to an appropriate volume in Eisen's solution so that 0.1 ml contained an estimated number of 100–500 plaque-forming cells. The actual number of plaque-forming cells was determined according to the method of Cunningham and Szenberg (10). In order to estimate the number of developed plaques, a rabbit anti-mouse gammaglobulin serum was added to the reaction mixture in the assay. This number was derived, by difference, from assays done in the presence or absence of antiglobulin antibody.

Induction of Immunological Tolerance to Sheep Erythrocytes.—8–12-wk old CBA mice were made tolerant of sheep erythrocytes according to the method of Dietrich and Dukor (11). They received one intraperitoneal injection of 0.5 ml of packed sheep erythrocytes and 24 hr later a subcutaneous injection of cyclophosphamide[5] in phosphate buffer in a dose of 1 mg per 10 g body weight. Control mice were injected only with cyclophosphamide.

Statistical Analysis.—The geometric means and standard errors of the mean were calculated from the $\log_{10}$ of the plaque-forming cell counts. P values were determined by the Rank test. In the comparison of the means of any two groups of observations, a significance level of 0.05 was chosen.

RESULTS

Induction of Tolerance to Sheep Erythrocytes.—8–12-wk old CBA mice were given packed SRBC intraperitoneally and 24 hr later, cyclophosphamide subcutaneously. Control mice received cyclophosphamide only. Three wk later the mice were challenged with a mixture of SRBC and horse red blood cells (HRBC)

[5] Cyclophosphamide (Endoxan, Asta), Charles McDonald, Caringbah, Australia.

and the number of direct and indirect plaque-forming cells (PFC) per spleen produced against both SRBC and HRBC was determined at intervals from 2 to 10 days after challenge. The results are shown in Figs. 1 and 2. It can be seen that the peak 19S response to SRBC and to HRBC occurred at 4 days after

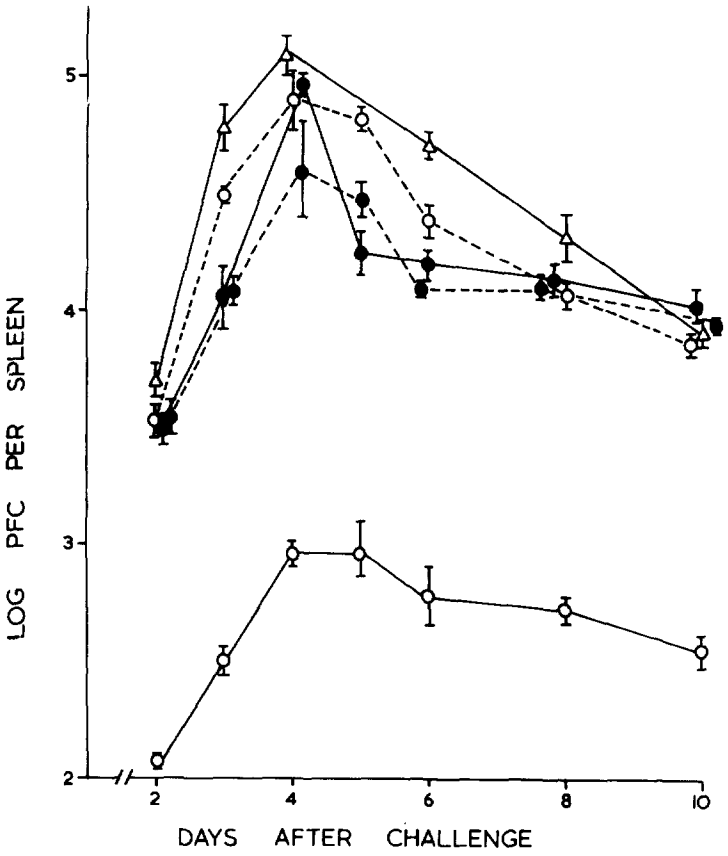

FIG. 1. Direct (19S) PFC produced in the spleens of CBA mice challenged with SRBC (○) and HRBC (●) 3 wk after pretreatment with SRBC and cyclophosphamide (——), or with cyclophosphamide alone (---). △——△ indicates the response of normal mice to SRBC. The number of mice per point was 4–15. Geometric means are shown and the upper and lower limits of the standard error are indicated by the vertical bars.

challenge in mice treated with cyclophosphamide alone and that pretreatment with both cyclophosphamide and SRBC did not significantly alter the response to HRBC, but markedly depressed that to SRBC. In the case of developed PFC the peak response was achieved at 6 days after challenge and, similarly,

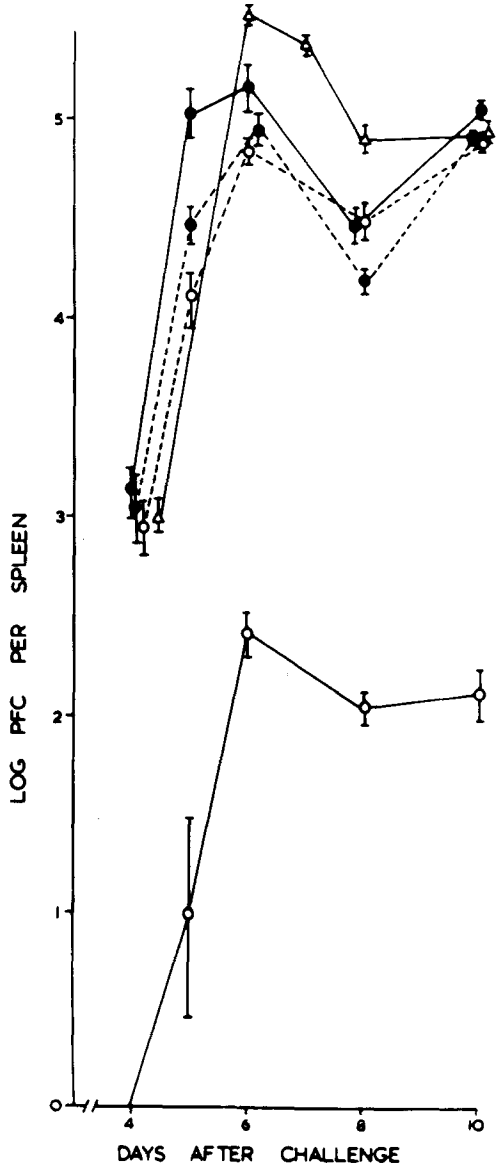

FIG. 2. Indirect (developed) PFC produced in the spleens of CBA mice challenged with SRBC (○) and HRBC (●) 3 wk after pretreatment with SRBC and cyclophosphamide (——) or with cyclophosphamide alone (----). △——△ indicates the response of normal mice to SRBC. The number of mice per point was 4–5. Geometric means are shown and the upper and lower limits of the standard error are indicated by the vertical bars.

only the response to SRBC was markedly depressed by pretreatment with both cyclophosphamide and SRBC.

The results shown in Figs. 1 and 2 indicate that recovery from the immunosuppressive effect of cyclophosphamide was evident 3 wk after pretreatment

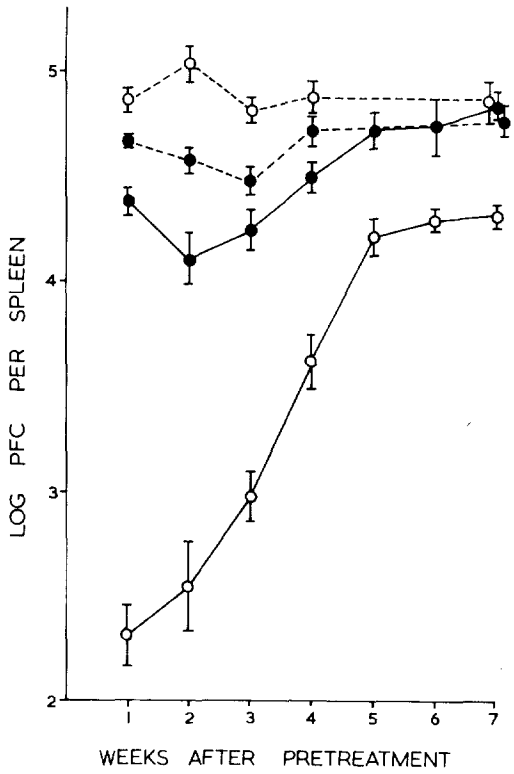

FIG. 3. Direct (19S) 4–5 days PFC response per spleen of mice challenged with SRBC (○) and HRBC (●) at various weekly intervals after pretreatment with SRBC and cyclophosphamide (——) or with cyclophosphamide alone (----). The number of mice per point was from 4 to 12. Geometric means are shown and the upper and lower limits of the standard error are indicated by the vertical bars.

with the drug alone. It was of interest to determine how soon after such pretreatment evidence of recovery could be noted and how long after pretreatment with both SRBC and cyclophosphamide specific tolerance to SRBC could be expected. The results of a time course study on the 4–5 day 19S PFC response of mice pretreated with either cyclophosphamide alone or together with SRBC are shown in Fig. 3. It is evident that a significant response to heterologous erythrocytes can be expected as early as 1 wk after pretreatment with the drug alone, and that a marked specific depression of the response to SRBC is maintained for

up to 4 wk after pretreatment with both drug and SRBC. These rates of recovery from the specific and nonspecific suppressive effects of cyclophosphamide are more rapid than those reported by others using higher doses of the drug and a different injection schedule (12).

In most experiments, therefore, SRBC–cyclophosphamide-pretreated mice were used 2–3 wk after the tolerization regime. At that period, as can be seen from Table I, tolerance was strictly specific: SRBC-tolerant mice could produce a normal response to HRBC, and HRBC-tolerant mice could produce a normal response to SRBC. Furthermore, inducing the response to HRBC did not break tolerance to SRBC, when recipients were challenged simultaneously with both

TABLE I

19S PFC Response of CBA Mice Pretreated with Cyclophosphamide and SRBC and Challenged with SRBC and HRBC

Pretreatment		Erythrocytes used for challenge (days 21–28)	19S PFC count 4–5 days after challenge			
			Anti-SRBC		Anti-HRBC	
Day 1	Day 2		*PFC/spleen*		*PFC/spleen*	
SRBC	Cyclophosphamide	SRBC	1,540(1,740–1,360)*‡	(35)§	100(190–50)‡	(4)
		HRBC	80(110–60)	(6)	18,960(23,030–15,610)	(12)
		SRBC + HRBC	950(1,240–720)‖	(11)	17,620(21,830–14,220)	(11)
HRBC	Cyclophosphamide	SRBC	92,200(104,610–81,270)	(8)	2(3–1)	(8)
		HRBC	14(16–11)	(8)	530(610–460)	(8)
		SRBC + HRBC	101,850(111,400–93,130)	(8)	2,950(3,600–2,420)¶	(8)
—	Cyclophosphamide	SRBC	94,570(110,270–81,110)*	(21)	260(310–210)	(6)
		HRBC	300(440–200)	(6)	22,110(27,300–17,910)	(14)
		SRBC + HRBC	64,750(72,560–57,780)‖	(15)	29,730(34,400–25,690)¶	(15)

*, ‖, ¶ $P < 0.005$.

‡ Geometric mean, upper and lower limits of SE.

§ Single number in parentheses refers to number of mice involved in the assays.

types of erythrocytes. The degree of cross-reactivity between SRBC and HRBC was reported to be slight (13), as is also evident from the results in Table I.

Effects of Lymphoid Cells on the Tolerant State.—Attempts were made to break tolerance to SRBC in mice pretreated 3 wk before with SRBC and cyclophosphamide, by giving normal lymphoid cells from various sources. The results of a time course study on the 19S and 7S PFC response of SRBC-tolerant recipients given lymphoid cells are shown in Fig. 4, and the peak responses are statistically analyzed in Table II. It can be seen that bone marrow did not break tolerance. On the other hand, a significant elevation of the anti-SRBC 19S PFC response was obtained in SRBC-tolerant recipients given either thymus, thoracic duct, or normal spleen cells, or given a mixture of thymus or thoracic duct lymphocytes and bone marrow. These responses were by no means restored to normal (Fig.

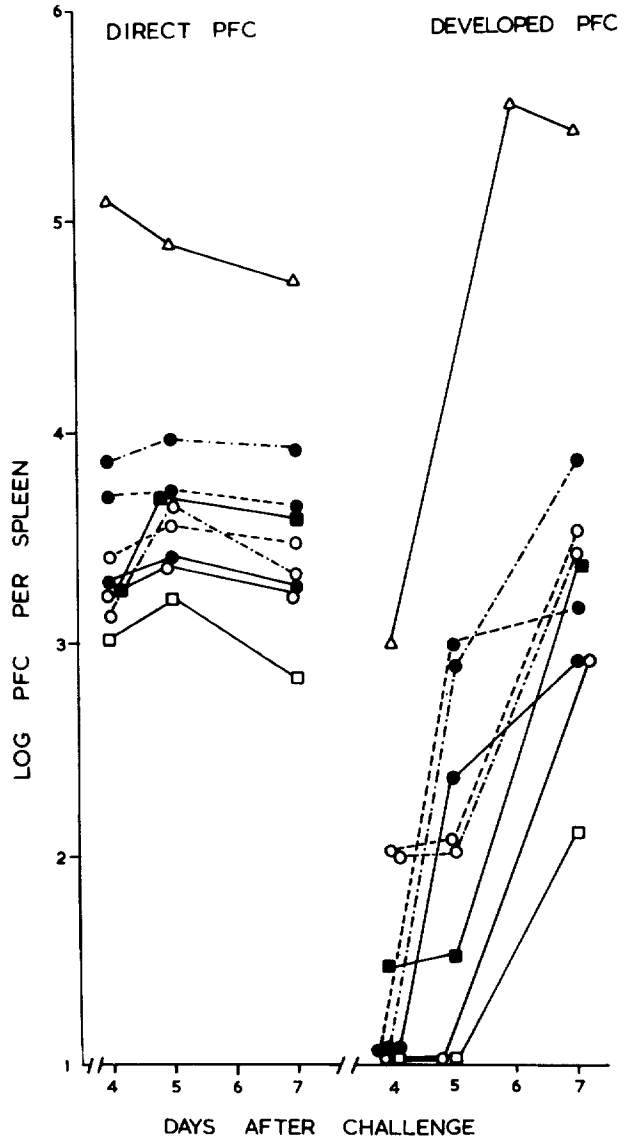

FIG. 4. Direct and developed PFC produced in the spleens of CBA mice challenged with SRBC 3 wk after pretreatment with SRBC and cyclophosphamide and given 2×10^7 marrow cells (□——□), 10^8 thymus cells (○——○), 2×10^7 thoracic duct cells (●——●), 4×10^7 normal spleen cells (■——■), or a mixture of 2×10^7 marrow and 10^8 thymus cells (○----○), 2×10^7 marrow and 2×10^7 thoracic duct cells (●----●), 10^8 thymus cells and 2×10^7 spleen cells from adult thymectomized, irradiated mice (○·---○) or 2×10^7 thoracic duct cells and 2×10^7 spleen cells from such mice (●·---●). △——△ PFC response of normal mice of the same age. The number of mice per point was from 3 to 15. The peak responses are analysed statistically in Table II.

4). Furthermore, only a few of the 7S PFC responses in the various groups were elevated significantly.

It is known that mice which have been thymectomized in adult life, irradiated with a lethal dose of X-ray, and protected with bone marrow cannot give a normal anti-SRBC response. Sham-thymectomized irradiated mice, by contrast, recover the capacity to produce a significant PFC response after 2–3 wk (14, and see below). Thymectomized irradiated, marrow-protected mice lack thymus-derived cells but can be reconstituted to give a normal PFC response to SRBC by an injection of thoracic duct lymphocytes (5). The PFC, in this system, are derived not from the injected thoracic duct lymphocytes, but from the

TABLE II

Peak PFC Response of CBA Mice Challenged with SRBC and Given Various Types of Lymphoid Cells 2–3 Wk after Pretreatment with SRBC and Cyclophosphamide

Group	Cells inoculated	Peak 19S PFC count (4–5 days after challenge)		*P* values, cf. group 1	Peak 7S PFC count (6–7 days after challenge)		*P* values, cf. group 1
		PFC/spleen			*PFC/spleen*		
1	SRBC	9,80(1,160–820)*	(22)‡	—	260(330–200)*	(5)	—
2	2×10^7 BM + SRBC	1,660(2,120–1,310)	(14)	NS	230(310–180)	(5)	NS
3	10^8 T + SRBC	2,550(3,030–2,140)	(14)	<0.05	830(1,030–670)	(7)	<0.05
4	10^8 T + 2×10^7BM + SRBC	3,700(4,650–2,940)	(14)	<0.005	3,260(4,480–2,380)	(8)	<0.05
5	10^8 T + 2×10^7TxS + SRBC	4,580(5,370–3,910)	(5)	<0.005	2,590(2,740–2,440)	(4)	<0.05
6	2×10^7 TDL + SRBC	2,580(2,970–2,240)	(16)	<0.005	820(1,170–580)	(4)	NS
7	2×10^7 TDL + 2×10^7 BM + SRBC	5,310(6,520–4,330)	(15)	<0.005	1,420(1,770–1,150)	(4)	NS
8	2×10^7 TDL + 2×10^7TxS + SRBC	9,260(1,1070–7,750)	(8)	<0.005	7,110(8,740–5,790)	(4)	NS
9	2×10^7 TxS + SRBC	2,200(2,490–1,960)	(7)	<0.05	190(220–170)	(4)	NS
10	4×10^7 normal S + SRBC	5,070(6,470–3,970)	(18)	<0.005	2,360(3,310–1,680)	(7)	<0.05

* Geometric mean, upper and lower limits of SE.

‡ Single number in brackets refers to number of mice involved in the assays.

marrow used to protect the mice from the lethal effects of irradiation. Hence, the cells which become 19S PFC are not thymus-derived but can respond to SRBC only after interacting with thymus-derived cells. It was thus of interest to show whether spleen cells of adult thymectomized irradiated donors could interact with thymus or thymus-derived cells to produce a response to SRBC in SRBC-tolerant mice. As can be seen from Table II and Fig. 4, such spleen cells increased the 19S PFC response of SRBC-tolerant mice slightly when given alone, but to a greater extent when given with thymus or thoracic duct cells. From a consideration of these results as a whole, the question may be raised as to whether target cells for tolerance induction are present in both thymus-derived and non–thymus-derived (marrow-derived) lymphoid cells.

Induction of Tolerance in Thymus-Derived Lymphoid Cells.—CBA mice, given SRBC and cyclophosphamide 3 wk before, were used as donors of thymus or

thoracic duct lymphocytes in cell transfer experiments. Mice pretreated with only cyclophosphamide served as control donors. Recipients were either neonatally thymectomized, or lethally irradiated and given bone marrow. 20 million thymus cells obtained from SRBC-tolerant donors were as effective as 2×10^7 thymus cells from cyclophosphamide-treated controls in elevating the 19S PFC response of neonatally thymectomized recipients challenged with SRBC (Table III). This suggests that the tolerization regime did not induce tolerance at the

TABLE III

19S PFC Produced in the Spleens of Neonatally Thymectomized CBA Mice after Injection of SRBC and Thymus Cells from Normal Donors or from Mice Pretreated 3 Wk Previously with Either Cyclophosphamide Alone or SRBC and Cyclophosphamide

Thymus cell donor	Cells inoculated	Number of neonatally thymectomized recipients	19S PFC count at 4–5 days after challenge	*P* values
			PFC/spleen	
—	SRBC	12	710 (940–540)*	<0.005
Normal	2×10^7 T + SRBC	12	15,540 (18,720–12,910)	NS
Cyclophosphamide pretreated	2×10^7 T + SRBC	6	13,350 (18,300–9,750)	NS
SRBC-cyclophosphamide pretreated	2×10^7 T + SRBC	12	12,610 (15,930–9,980)	

* Geometric mean, upper and lower limits of SE.

level of the lymphocytes differentiating within the thymus. The PFC response in neonatally thymectomized mice given 20 million thymus cells was slightly lower than that previously reported (4). This may be because the mice used here as donors of thymus cells were, on the average, 1 month older than those used in the previous studies.

The failure to achieve tolerance at the level of the lymphocyte population within the thymus was further substantiated in experiments with irradiated mice in which 5×10^7 thymus cells were given together with 2×10^7 marrow cells. The same PFC response was achieved whether the thymus cells were obtained from normal, cyclophosphamide-treated, or SRBC-tolerant donors (Table IV).

The failure to achieve specific tolerance at the level of the thymus lymphocyte population could result from insufficient penetration of the thymus by the SRBC antigens capable of inducing tolerance. We therefore devised experiments to determine whether the mobile pool of thymus-derived cells could behave as a tolerant population. The capacity of thoracic duct cells from SRBC-tolerant mice to transfer SRBC reactivity adoptively was tested by injecting 10 million cells into 3–5-wk old neonatally thymectomized mice. Other thymectomized

TABLE IV

19S PFC Produced in the Spleens of Heavily Irradiated CBA Mice after Injection of SRBC and HRBC, Normal Bone Marrow Cells, and Thymus Cells from Normal Donors or from Mice Pretreated 3 Wk Previously with Cyclophosphamide Alone or SRBC and Cyclophosphamide

Thymus cell donor	Cells inoculated	Number of irradiated recipients	19S PFC count at 8 days			
			Anti-SRBC	*P* values	Anti-HRBC	*P* values
			PFC/spleen		*PFC/spleen*	
—	2×10^7 BM + SRBC + HRBC	14	10(15–5)*	NS	5(8–1)*	NS
Normal	5×10^7 T + SRBC + HRBC	12	20(25–15)	<0.005	15(20–10)	<0.05
Normal	5×10^7 T + 2×10^7 BM + SRBC + HRBC	8	1270(1600–1010)	NS	100(165–65)	NS
Cyclophosphamide pretreated	5×10^7 T + 2×10^7 BM + SRBC + HRBC	8	1360(1810–970)	NS	120(170–90)	NS
SRBC-cyclophosphamide pretreated	5×10^7 T + 2×10^7 BM + SRBC + HRBC	7	1430(1680–1210)		270(350–210)	

* Geometric mean, upper and lower limits of SE.

mice of the same age received an equivalent number of cells from cyclophosphamide-treated control mice. The mice were challenged with either SRBC or HRBC or both antigens simultaneously. The number of 19S PFC in the spleens of the recipients was determined 4–5 days later and the results are shown in Table V. Thymectomized mice inoculated with 10 million thoracic duct cells from tolerant mice produced an average of 9,000–16,000 anti-SRBC PFC per spleen, whereas those receiving the same number of thoracic duct cells from control mice had an average response of 46,000–53,000 PFC. This difference is statistically significant ($P < 0.05$). On the other hand, 10 million thoracic duct cells from both tolerant and control mice increased the average response of thymectomized mice challenged with HRBC to values exceeding 25,000 PFC per spleen. The anti-SRBC response of recipients of SRBC and lymphocytes

from tolerant mice was increased slightly by a simultaneous injection of HRBC. This increase in the average number of PFC per spleen from approximately 9,000 to 16,000 is not statistically significant.

It is evident that thoracic duct cells from SRBC-tolerant mice were inferior to cells from nontolerant donors in adoptively transferring reactivity to the specific antigen used for tolerization. Nevertheless, they did increase the PFC response of neonatally thymectomized mice to SRBC. It may be that among the

TABLE V

19S PFC Produced in the Spleens of Neonatally Thymectomized CBA Mice after Injection of SRBC or HRBC and Thoracic Duct Lymphocytes from Mice Pretreated 3 Wk before with Either Cyclophosphamide Alone or SRBC and Cyclophosphamide

Thoracic duct cell donor	Cells inoculated	Number of neonatally thymectomized recipients	19S PFC count at 4–5 days	
			Anti-SRBC	Anti-HRBC
			PFC/spleen	*PFC/spleen*
—	SRBC	12	1,100(1,380–880)*	—
	HRBC	7	—	1,840(2,530–1,340)
	SRBC + HRBC	7	1,770(3,230–970)	620(1,030–370)
Cyclophosphamide pretreated	10^7 TDL + SRBC	9	53,310(66,780–42,550)‡	—
	10^7 TDL + HRBC	5	—	33,680(40,170–28,240)
	10^7 TDL + SRBC + HRBC	9	46,110(58,450–36,380)§	25,680(34,100–19,340)
SRBC cyclophosphamide pretreated	10^7 TDL + SRBC	10	9,200(12,160–6,950)‡,‖	—
	10^7 TDL + HRBC	4	—	28,750(37,980–21,760)
	10^7 TDL + SRBC + HRBC	17	15,930(19,190–13,220)§, ‖	26,540(31,320–22,480)

* Geometric mean, upper and lower limits of SE.
‡, § $P < 0.05$.
‖ N.S.

panel of donors cannulated to provide thoracic duct lymphocytes, a few were much less tolerant than others. This could account for the increase in the response of neonatally thymectomized recipients. It is also evident that the PFC response of neonatally-thymectomized mice given 10 million thoracic duct cells from 12-wk old cyclophosphamide-treated donors, was higher than that previously recorded in neonatally thymectomized recipients of normal thoracic duct lymphocytes from 6 to 8-wk old donors (4). This may be due to the age difference between the two sets of donors, or it may be a consequence of the recovery from cyclophosphamide.

Since the neonatally thymectomized hosts provide the PFC precursors (4), the above results indicate that tolerance is a property that can be linked specifi-

cally to thymus-derived cells. Attempts were next made to determine whether or not target cells for tolerance induction exist in the non–thymus-derived cell population.

Induction of Tolerance in Nonthymus-Derived (Marrow-Derived) Lymphoid Cells

In order to determine whether target cells for tolerance induction also exist in the nonthymus-derived cell population, two experimental designs were used. In the first, normal thymus cells were transferred into heavily irradiated mice together with either marrow cells or lymph node cells from normal donors or from mice pretreated with either cyclophosphamide alone or SRBC and cyclophosphamide 3 wk before. The PFC response of these mice to SRBC and HRBC was determined 8 days later. In the second system, attempts were made to produce a specific tolerance to SRBC in the lymphoid cell population of thymectomized irradiated, marrow-protected mice which contain PFC precursors (5).

Effects of Inoculating Irradiated Hosts with Mixtures of Normal Thymus or Thoracic Duct Cells and Marrow or Lymphoid Cells from Normal or Tolerant Donors.—Heavily irradiated mice were injected with SRBC, 5×10^7 normal thymus cells and 2×10^7 marrow cells from either normal, cyclophosphamide-pretreated donors, or SRBC-tolerant donors. The results in Table VI show that 2×10^7 marrow cells obtained from SRBC-tolerant donors were as effective as 2×10^7 marrow cells from cyclophosphamide-treated controls in elevating the 19S PFC response of heavily irradiated recipients. It is thus obvious that the tolerization regime had no effect upon those cells, present in bone marrow, which could, upon transfer to irradiated hosts, differentiate to cells which collaborate with thymus cells to produce a response to SRBC. The marrow is known to be a rich source of multipotential stem cells and may thus not be the ideal tissue in which to demonstrate tolerance at the level of the nonthymus-derived lymphocyte population. A tissue lacking stem cells, such as lymph node, was therefore examined. Irradiated mice received mixtures of lymph node and thymus cells or thoracic duct cells together with SRBC and HRBC. The donors of the thymus or thoracic duct cells were normal mice but the donors of the lymph node cells were either normal, SRBC-tolerant (normal mice pretreated 3 wk before with SRBC and cyclophosphamide), or mice subjected 3 wk before to thymectomy, irradiation, and marrow protection. Lymph node cells from mice of the latter group could be expected to lack thymus-derived cell. The results of these experiments are shown in Table VII. Lymph node cells from thymectomized irradiated mice failed to adoptively transfer immune reactivity to either SRBC or HRBC in irradiated recipients. Addition of thymus cells or of thoracic duct cells increased the response to about 1000 and 6000 PFC per spleen in the case of SRBC and to about 200 and 1000 in the case of HRBC. Lymph node cells from

SRBC-tolerant mice adoptively transferred immune reactivity to HRBC but not to SRBC. Normal lymph node cells, on the other hand, were capable of transferring reactivity to both SRBC and HRBC. A mixture of tolerant lymph node cells and thymus cells enabled irradiated recipients to respond to SRBC by producing some 600 PFC per spleen. This implies that collaboration between thymus cells and non–thymus-derived lymphocytes supplied from lymph nodes of SRBC-tolerant mice took place in the irradiated host. Furthermore, when a mixture of thoracic duct cells and lymph node cells was injected, the PFC response was essentially of the same order of magnitude whether the lymph node cells were derived from tolerant donors or from adult thymectomized irradiated mice. Clearly, therefore, non–thymus-derived cells in lymph nodes of SRBC-tolerant mice were capable of interacting effectively with thymus-derived cells in thoracic duct lymph to produce a response not only to HRBC but also to SRBC.

TABLE VI

19S PFC Produced in the Spleens of Heavily Irradiated CBA Mice after Injection of SRBC and HRBC, Normal Thymus Cells, and Bone Marrow Cells from Normal Donors or from Mice Pretreated 2 Wk Previously with Either Cyclophosphamide Alone or SRBC and Cyclophosphamide

Marrow cell donor	Cells inoculated	Number of irradiated recipients	19S PFC count at 8 days			
			Anti-SRBC	*P* values	Anti-HRBC	*P* values
			PFC/spleen		*PFC/spleen*	
—	5×10^7 T + SRBC + HRBC*	12	20(25–15)‡	NS	15(20–10)‡	NS
Normal	2×10^7 BM + SRBC + HRBC*	14	10(15–5)	<0.005	5(8–1)	<0.05
Normal	2×10^7 BM + 5×10^7 T + SRBC + HRBC*	8	1270(1600–1010)	NS	100(165–65)	NS
Cyclophosphamide pretreated	2×10^7 BM + 5×10^7 T + SRBC + HRBC	7	920(1100–770)	NS	200(330–130)	NS
SRBC-cyclophosphamide pretreated	2×10^7 BM + 5×10^7 T + SRBC + HRBC	8	1120(1380–910)		110(220–50)	

* Data from Table IV.

‡ Geometric mean, upper and lower limits of SE.

Attempts to Induce Tolerance in Thymectomized Irradiated, Marrow-Protected Hosts.—The above experimental design failed to demonstrate the existence of specific target cells for tolerance induction among the nonthymus-derived lymphocyte population of the lymph nodes. Therefore, either such cells do not exist so that the only cells capable of dictating the specificity of the response must be thymus-derived, or alternatively, tolerance had been achieved in

the non–thymus-derived cell line but, after transfer to irradiated hosts, cells with new reactivity patterns were generated from thymus-independent *lymphoid* stem cells. Attempts were thus made to induce tolerance in thymectomized irradiated, marrow-protected mice at a time after irradiation when regeneration of the thymus-independent lymphoid system could be expected to be complete.

TABLE VII

19S PFC Produced in the Spleens of Heavily Irradiated Mice after Injection of SRBC, HRBC, Normal Thymus, or Thoracic Duct Lymphocytes and Lymph Node Cells from Normal, SRBC-Tolerant or Thymectomized Irradiated Donors

LN cell donor	Cells inoculated	Number of irradiated recipients	19S PFC count at 7–8 days			
			Anti- SRBC	*P* values	Anti-HRBC	*P* values
			PFC/spleen		*PFC/spleen*	
TxBM	2×10^7 LN + SRBC + HRBC	5	4(7–2)*	<0.05	2(2.5–1)*	NS
	2×10^7 LN + 5×10^7 T + SRBC + HRBC	5	980(1,500–640)	<0.05	160(200–90)	<0.05
	2×10^7 LN + 10^7 TDL + SRBC + HRBC	5	5,510(7,090–4,280)		950(1,060–850)	
SRBC-tolerant	2×10^7 LN + SRBC + HRBC	12	50(70–40)	<0.005	360(440–290)	NS
	2×10^7 LN + 5×10^7 T + SRBC + HRBC	14	610(840–450)	<0.005	560(690–460)	<0.005
	2×10^7 LN + 10^7 TDL + SRBC + HRBC	14	6,970(9,320–5,220)		3,080(3,990–2,380)	
Normal	2×10^7 LN + SRBC + HRBC	17	1,600(1,870–1,360)	NS	400(490–330)	NS
	2×10^7 LN + 5×10^7 T + SRBC + HRBC	12	2,200(2,660–1,820)	<0.005	750(1,000–560)	<0.005
	2×10^7 LN + 10^7 TDL + SRBC + HRBC	18	8,350(10,080–6,920)		2,270(3,050–1,690)	
–	SRBC + HRBC	5	10(15–4)	<0.05	5(8–2)	<0.05
	10^7 TDL + SRBC + HRBC	5	1,130(1,350–900)		560(640–490)	

* Geometric mean, upper and lower limits of SE.

Mice thymectomized in adult life and subjected to irradiation and marrow protection were challenged with SRBC at various intervals of time after irradiation and the splenic PFC response was determined 5 days after challenge. The results shown in Fig. 5 clearly demonstrate that adult thymectomy prevents the postirradiation recovery of the capacity to produce a significant 19S PFC response to SRBC. For up to 10 wk after irradiation, groups of these thymectomized mice could not produce an average PFC response higher than 3×10^3 per spleen. It has been shown that in these mice, an injection of thoracic duct lymphocytes significantly elevated the PFC response and that the PFC's

were derived not from the inoculated lymphocytes, but from precursors derived from the marrow given after irradiation (5). Attempts to induce tolerance in this particular marrow-derived lymphoid cell population were made by treating adult thymectomized, irradiated, marrow-protected mice with SRBC and

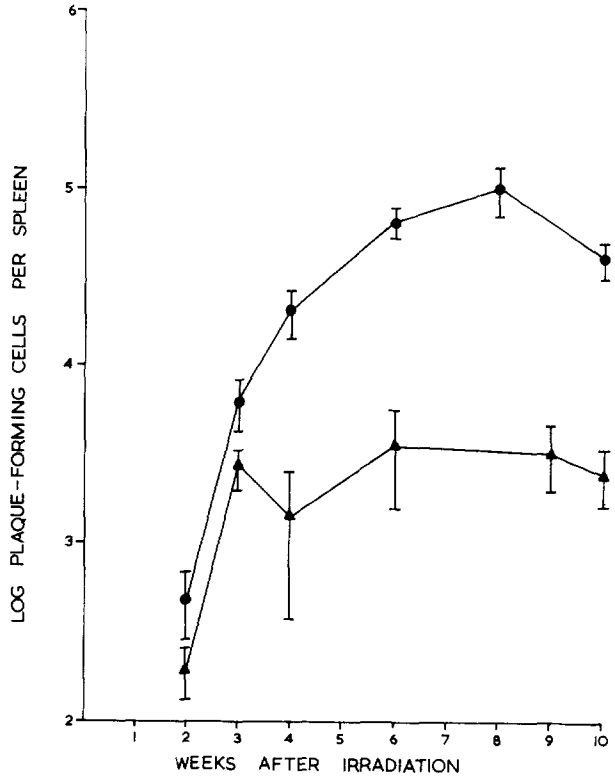

FIG. 5. PFC produced in the spleens of sham adult thymectomized (●) and adult thymectomized (▲) CBA mice at various times after receiving 900 rads total body irradiation and 10 million syngeneic bone marrow cells. Splenic PFC were enumerated 4–5 days after the challenge injection of SRBC. The number of mice per point was from 2 to 7 and the magnitude of twice the standard error is indicated by the length of the vertical bars.

cyclophosphamide 3–4 wk after irradiation. As these thymectomized irradiated mice are unresponsive to heterologous erythrocytes (since they lack thymus-derived cells), tests for specific tolerance to SRBC can be made only after providing thymus-derived cells. If specific tolerance had been induced to SRBC in the marrow-derived lymphoid cell population, it would be expected that no significant increase in the PFC response could be produced after an injection of thoracic duct lymphocytes in thymectomized irradiated mice pretreated 3 wk before with SRBC and cyclophosphamide. The results of such an experiment

are shown in Table VIII. It is evident that thoracic duct lymphocytes elevated the PFC response of thymectomized irradiated mice markedly and just as effectively whether the recipients had been pretreated with SRBC-cyclophosphamide or not. On the other hand, cells from the same thoracic duct lymphocyte pool increased only slightly the anti-SRBC PFC response of normal mice given the tolerization regime.

Hence, the data obtained in thymectomized irradiated mice do not determine whether tolerance was effectively induced and broken, or whether tolerance was

TABLE VIII

19S PFC Produced in the Spleens of Normal and Adult Thymectomized Irradiated Marrow-Protected CBA Mice Given Normal Thoracic Duct Lymphocytes and Challenged with SRBC and HRBC 3 Wks after Pretreatment with SRBC and Cyclophosphamide

Treatment of recipients	Cells inoculated	Number of mice per group	19S PFC count at 5 days			
			Anti-SRBC	*P* values	Anti-HRBC	*P* values
			PFC/spleen		*PFC/spleen*	
TxBM	SRBC + HRBC	5	560(680–470)*	<0.05	290(490–170)*	<0.05
	2×10^7 TDL + SRBC + HRBC	8	50,950(61,150–42,450)		34,780(38,460–31,450)	
TxBM followed 3 wks later by SRBC and cyclophosphamide	SRBC + HRBC	7	280(440–180)	<0.005	500(680–360)	<0.005
	2×10^7 TDL‡ + SRBC + HRBC	17	46,850(58,480–37,530)		27,070(32,700–22,400)	
Normal mice given SRBC and cyclophosphamide	SRBC + HRBC	8	2,250(2,430–2,080)	<0.05	52,430(62,020–44,320)	NS
	2×10^7 TDL‡ + SRBC + HRBC	6	4,800(5,340–4,320)		43,100(53,820–34,520)	

* Geometric mean, upper and lower limits of SE.

‡ The same pool of TDL was used for these recipients.

not achieved because it cannot be induced in the population of nonthymus-derived cells. The possibility that the induction of tolerance, like the induction of antibody formation in antibody-forming cell precursors, is facilitated by the presence of thymus-derived cells was investigated. Thymectomized irradiated mice protected with bone marrow were given 10^8 thymus cells intravenously and SRBC intraperitoneally 1 day later. 2 days after treatment with SRBC, they received cyclophosphamide subcutaneously. Controls received no thymus cells. 3 wk later, all mice received 15×10^6 thoracic duct lymphocytes, SRBC, and HRBC intravenously and the peak number of PFC in their spleens was determined. The results given in Table IX indicate that the PFC response was of the same order of magnitude in thymectomized irradiated mice whether or not they had received thymus cells before treatment with SRBC and cyclophos-

phamide. It appears, therefore, that induction of tolerance was not achieved in the presence of thymus cells.

DISCUSSION

It is clear from the experimental evidence given in earlier papers of this series (4, 5, 7) that the non-thymus-derived ("marrow-derived") lymphocyte can produce the hemolysin-forming cell in the response of mice to SRBC after interaction with thymus-derived cells. What then is the role of the thymus-derived lymphocytes—the cells which never become the antibody-forming cells that are

TABLE IX

PFC Response of Thymectomized Irradiated Mice Pretreated with SRBC and Cyclophosphamide

Day 1	Day 2	Day 4	Day 21*	Number of mice	19S PFC count			
					Anti-SRBC	*P* values	Anti-HRBC	*P* values
					PFC/spleen		*PFC/spleen*	
—	—	—	SRBC + HRBC	9	200(310–130)‡	<0.005	30(50–20)‡	<0.005
—	SRBC§	Cyclophosphamide	1.5×10^7 TDL + SRBC + HRBC	11	16,830(20,890–13,550)	NS	5,690(6,620–4,890)	NS
10^8 T	SRBC§	Cyclophosphamide	1.5×10^7 TDL + SRBC + HRBC	12	13,010(15,840–10,690)	<0.005	2,860(3,590–2,280)	<0.005
10^8 T	SRBC§	Cyclophosphamide	SRBC + HRBC	9	490(680–350)		170(290–100)	

* Similar results were obtained when challenge was performed on Day 12.
‡ Geometric mean, upper and lower limits of SE.
§ Tolerance-inducing dose.

detected? The role of the thymus-derived cell clearly hinges on whether its influence in the response is specific or not, and whether antigen recognition is a property that can be linked to thymus-derived cells, marrow-derived cells, or both. The data available to date suggests that the influence of the thymus-derived lymphocyte is specific. Two experimental approaches have shown specificity at the level of "activation" of thymus lymphocytes by antigen. (*a*) In the first, a double transfer system was used. A first set of irradiated hosts received either SRBC alone, thymus cells alone, or thymus cells together with SRBC or HRBC. 7 days later, a cell suspension from the spleens of the first irradiated hosts was injected into a second group of irradiated hosts together with marrow cells and SRBC and the response, in terms of PFC per spleen against SRBC was measured. Only those recipients of cells that had been "activated" with the same antigen—SRBC—could produce a significant PFC response (15). These results have been confirmed and extended in other investigations (3). (*b*) In the second set of experiments, published in this paper, thoracic duct cells (which behave essentially as thymus-derived lymphocytes in reconstitution exper-

iments (4)), were used. Cells from mice specifically tolerant to SRBC, adoptively transferred immune reactivity to HRBC but not to SRBC in neonatally thymectomized recipients which themselves can supply the nonthymus-derived PFC precursors (4). It is evident, therefore, that tolerance is a property that can be linked to thymus-derived cells and that the thymus must produce unispecific cells of the antigen-reactive type, each being sensitive to certain antigenic determinants and to the induction of specific tolerance.

No evidence could be obtained for the induction of tolerance to SRBC at the level of the lymphocyte population within the thymus itself. Thus thymus cells from SRBC-tolerant mice were as effective as thymus cells from controls in enabling neonatally thymectomized mice to respond to SRBC and in collaborating with marrow cells in irradiated mice. These results are in agreement with preliminary data published by Playfair (16) but not with results obtained from the experiments of Waksman and collaborators (17). These investigators, however, used soluble antigens such as bovine serum albumin (BSA) which presumably, and in contrast to SRBC, diffuse readily within the thymus. Further evidence that tolerance can be achieved at the level of the thymus lymphocyte population with soluble antigens, such as BSA, has recently been obtained by Taylor (8): thymus lymphocytes from mice treated with BSA were no longer capable of collaborating with marrow-derived cells in anti-BSA antibody production but were able to do so with respect to antibody production against human serum albumin. It is possible that if a soluble SRBC antigen were available, tolerance at the level of the cell population within the thymus could be demonstrated.

The indications that specific tolerance can be linked to thymus cells in the case of soluble antigens and to thymus-derived cells (in thoracic duct lymph) in the case of insoluble antigens such as SRBC, do not support the various hypotheses which would attribute to thymus-derived cells a passive or nonspecific role in the immune response, such as trephocytic or phagocytic.

If the provision of nucleosides from thymus-derived cells was the only essential function of these cells in enabling the differentiation and proliferation of marrow-derived PFC precursors in response to antigens, one would not expect a tolerant population of thymus-derived cells to lack the capacity to subserve such a nonspecific trophic function. Likewise, if thymus-derived cells were simply the precursors of macrophages required to process *nonspecifically* the antigens of SRBC, the population from tolerant animals should have performed just as effectively as that from controls.

The data given here and in other publications (8) indicate that the role of thymus-derived cells in the interaction is specific. A crucial question is: since the marrow-derived cells are the antibody-formers, are they also unispecific? Three experimental systems were used to provide an answer to this question: (*a*) attempts were made to break tolerance by injecting thymus-derived cells or

mixtures of thymus and non–thymus-derived cells; (*b*) the immune response of irradiated recipients of normal thymus cells and of marrow or marrow-derived lymphocytes from tolerant donors was studied, and (*c*) attempts were made to induce specific tolerance in thymectomized irradiated, marrow-protected mice which lack thymus-derived cells. Some increase in reactivity to SRBC was produced in tolerant mice by thymus cells, thoracic duct lymphocytes, spleen cells, or a mixed inoculum of thymus or thoracic duct cells and marrow or marrow-derived lymphoid cells (in the form of spleen cells from thymectomized irradiated mice). It is not possible, however, to claim that tolerance was effectively broken by such treatment. Clearly, some factor, or factors, other than both thymus and nonthymus cells, must be supplied to the tolerant animal in order to break tolerance more effectively.

The second attempt to demonstrate the existence of target cells for tolerance induction in marrow-derived cells was made by transferring mixtures of marrow cells from tolerant donors and normal thymus cells into irradiated mice. The results obtained do not agree with claims made by Playfair (16) on the basis of preliminary evidence. In our system marrow cells from SRBC-tolerant mice were as effective as marrow cells from cyclophosphamide-treated donors in collaborating with thymus cells in the response to SRBC in irradiated hosts. In Playfair's system marrow cells from SRBC-tolerant mice taken 3 wk after tolerance induction, when mixed with normal thymus cells, allowed a response to SRBC in irradiated hosts which was 15% of that expected between normal marrow and thymus cells. Unfortunately, neither the response to a non–cross-reacting antigen in that particular group (which showed the greatest depression) was determined nor was that of irradiated recipients of normal thymus and marrow from cyclophosphamide-treated controls. Furthermore, although tolerant donors still showed a response to SRBC between 8 and 15% of normal 6–10 wk after tolerance induction, mixtures of marrow from tolerant mice, 7 wk after tolerance induction, and normal thymus gave a response 86% of that expected in recipients of mixtures of normal cells in the case of SRBC and as low as 65% in the case of chicken erythrocytes. It is difficult to conclude from such data that target cells for tolerance induction have been demonstrated in the marrow population. It might be argued that tolerance cannot be demonstrated at the level of the marrow cell population simply because this populatioin contains multipotential stem cells which, upon transfer to heavily irradiated hosts, could differentiate to cells with new reactivity patterns. Our data, however, clearly demonstrate that tolerance cannot be revealed at the level of either the marrow cell population or the non–thymus-derived lymphoid cell population of the lymph nodes which lack such multipotential stem cells. Thus lymph node cells from either thymectomized irradiated mice (which lack thymus-derived cells) or from SRBC-tolerant mice (in which the population of thymus-derived cells must be presumed to be specifically tolerant) collaborated as effectively or

almost as effectively with normal thymus or thoracic duct cells in allowing a response to SRBC in irradiated recipients. The conclusion seems inescapable that the tolerization regime used induced tolerance only at the level of the thymus-derived cell population and not in the non–thymus-derived population.

There was no difference in the PFC response of adult thymectomized, irradiated recipients of normal thoracic duct cells whether the hosts had been pretreated with SRBC and cyclophosphamide or not. It is thus impossible to decide whether or not tolerance had been induced in the marrow-derived lymphocytes by such a regime. If it had, then, in contrast to the situation in normal animals, tolerance was effectively broken by thoracic duct cells. If this is so, it may be that failure to break tolerance in the normal animal is linked to the failure of adequate numbers of cells to penetrate the sites where antigen triggering occurs. Such a penetration would occur readily in an animal rendered lymphopenic by thymectomy and irradiation. It may well be, on the other hand, that tolerance could not be induced in the thymectomized irradiated animal, because there are no target cells for tolerance induction in the marrow-derived cell population. Alternatively, such cells might be present but induction of tolerance, like induction of immunity, might require the presence of thymus-derived cells. This suggestion receives support from findings obtained in thymectomized irradiated mice that were not treated with cyclophosphamide but given repeated doses of SRBC with or without thymus cells (18). In the present study, however, thymectomized irradiated mice pretreated with thymus cells immediately before the SRBC-cyclophosphamide regime, were not tolerant of SRBC when challenged 3 wk later. Similar results, not shown here, were obtained when challenge was made 12 days later, and when thymus cells were given instead of thoracic duct cells.

The experiments described here have failed to provide a satisfactory answer to the most crucial question: are non–thymus-derived cells capable of dictating the specificity of the response? If it turns out that they are not, the possibility of information transfer must seriously be considered. It may be, for instance, that the genetic control of antibody synthesis has to be shared by two cell types: the nonthymus-derived cell could be differentiated so as to be capable of expressing the information essential for the synthesis of that part of the immunoglobulin molecule which determines class specificity; the other cell could be thymus-derived and differentiated so that it could express the information necessary for the synthesis of only the variable part of the molecule in which the specificity of the antibody combining site resides. Fusion of the two parts could occur in the non–thymus-derived cell after its interaction with the antigen-activated, thymus-derived cell, at the level of DNA or mRNA, as can be envisaged by various models. The particular genetic region determining antibody specificity could exist in the episomal form. Once transferred to the marrow-derived cell, the episome could be inserted very quickly back into the chromosome. Great

difficulties are, however, encountered with such information transfer theories, particularly when we consider that most humoral antibody responses are not thymus-dependent. One would have to postulate mechanisms of information transfer from lymphocytes of the bursal equivalent system to marrow-derived cells. Evidence for cell interaction in those responses not dependent upon the thymus is to date not available.

Failure to demonstrate specific tolerance in the non–thymus-derived lymphoid cell lineage by no means precludes the existence of cells in this population, capable of dictating the specificity of the response. Treatment with SRBC and cyclophosphamide presumably leads to a specific deletion of those thymus-derived cells that expand mitotically as a result of exposure to SRBC. This could leave the marrow-derived lymphoid cell population intact and yet unable to respond since there are no specific thymus-derived cells with which to interact. Much recent evidence supports the concept that lymphocytes specifically absorb antigenic determinants (19, 20). It can thus be envisaged that thymus-derived cells specifically react with some of the antigenic determinants of sheep erythrocytes to carry these or other attached determinants into appropriate sites where PFC precursors can be stimulated to produce the hemolysin response. At least two mechanisms can be envisaged. The first is based on the cellular events occurring during bacterial resistance mediated by macrophages (21) and during delayed hypersensitivity. The activation of lymphocytes by certain antigens in delayed hypersensitivity reactions triggers the production of factors that have a multitude of biological activities—e.g. the migration inhibitory factor which acts on macrophages (22). Once elaborated, however, these factors have no immunological specificity. It may be, therefore, that in immune responses in which collaboration between thymus-derived and non–thymus-derived cells is required, the former are activated by antigen to produce factors which facilitate the response of the latter to other antigenic determinants. Facilitation may occur via the macrophage (possibly by enhancing the capacity of the macrophage to process the antigen) or by an effect on the antibody-forming cell precursors—e.g. by enhancing the recruitment of these into the area where the antigen is deposited. The second mechanism can be called antigen focussing. This has been implicated in the immune response to hapten-protein conjugates in which one cell is carrier-reactive and the other hapten-sensitive (23, 24). Antigen focussing has also been implicated as occurring during immune responses to heterologous erythrocytes and serum proteins, responses which require cell to cell interaction (1, 8, 25). The data presented in Table V do not support the contention that the production of an agent by activated thymus-derived cells is the sole mechanism involved in the collaboration between the two cell types. Thymus-derived cells from SRBC-tolerant donors, once activated by HRBC, might be expected to produce a factor capable of promoting a response by antibody-forming cell precursors not only to HRBC but also to SRBC in a host injected with both erythrocytes.

If non–thymus-derived lymphocytes are unispecific, and antigen-sensitive, some experimental system other than that involving tolerization by SRBC and cyclophosphamide, is required to demonstrate their existence. It is possible that induction of tolerance by some technique, other than the cyclophosphamide in vivo method, such as, for instance, the use of judicious doses of soluble antigen in vivo or in vitro, might lead to the demonstration of the existence of specific non–thymus-derived cells.

SUMMARY

Collaboration between thymus-derived lymphocytes, and nonthymus-derived antibody-forming cell precursors occurs during the immune response of mice to sheep erythrocytes (SRBC). The aim of the experiments reported here was to attempt to induce tolerance in each of the two cell populations to determine which cell type dictates the specificity of the response.

Adult mice were rendered specifically tolerant to SRBC by treatment with one large dose of SRBC followed by cyclophosphamide. Attempts to restore to normal their anti-SRBC response by injecting lymphoid cells from various sources were unsuccessful. A slight increase in the response was, however, obtained in recipients of thymus or thoracic duct lymphocytes and a more substantial increase in recipients of spleen cells or of a mixture of thymus or thoracic duct cells and normal marrow or spleen cells from thymectomized donors.

Thymus cells from tolerant mice were as effective as thymus cells from normal or cyclophosphamide-treated controls in enabling neonatally thymectomized recipients to respond to SRBC and in collaborating with normal marrow cells to allow a response to SRBC in irradiated mice. Tolerance was thus not achieved at the level of the lymphocyte population within the thymus, perhaps because of insufficient penetration of the thymus by the antigens concerned. By contrast, thoracic duct lymphocytes from tolerant mice failed to restore to normal the response of neonatally thymectomized recipients to SRBC. Tolerance is thus a property that can be linked specifically to thymus-derived cells as they exist in the mobile pool of recirculating lymphocytes outside the thymus. Thymus-derived cells are thus considered capable of recognizing and specifically reacting with antigenic determinants.

Marrow cells from tolerant mice were as effective as marrow cells from cyclophosphamide-treated or normal controls in collaborating with normal thymus cells to allow a response to SRBC in irradiated recipients. When a mixture of thymus or thoracic duct cells and lymph node cells was given to irradiated mice, the response to SRBC was essentially the same whether the lymph node cells were derived from tolerant donors or from thymectomized irradiated, marrow-protected donors. Attempts to induce tolerance to SRBC in adult thymectomized, irradiated mice 3–4 wk after marrow protection, by treatment with SRBC and cyclophosphamide, were unsuccessful: after injection of thoracic duct cells, a vigorous response to SRBC occurred. The magnitude of the response was the

same whether or not thymus cells had been given prior to the tolerization regime.

The various experimental designs have thus failed to demonstrate specific tolerance in the nonthymus-derived lymphocyte population. Several alternative possibilities were discussed. Perhaps such a population does not contain cells capable of dictating the specificity of the response. This was considered unlikely. Alternatively, tolerance may have been achieved but soon masked by a rapid, thymus-independent, differentiation of marrow-derived lymphoid stem cells. On the other hand, tolerance may not have occurred simply because the induction of tolerance, like the induction of antibody formation, requires the collaboration of thymus-derived cells. Finally, tolerance in the nonthymus-derived cell population may never be achieved because the SRBC-cyclophosphamide regime specifically eliminates thymus-derived cells leaving the antibody-forming cell precursors intact but unable to react with antigen as there are no thymus-derived cells with which to interact.

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BIBLIOGRAPHY

1. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen reactive cells. *Transplant. Rev.* **1**:3.
2. Davies, A. J. S. 1969. The thymus and the cellular basis of immunity. *Transplant. Rev.* **1**:43.
3. Claman, H. N., and E. A. Chaperon. 1969. Immunological complementation between thymus and marrow cells—A model for the two-cell theory of immunocompetence. *Transplant. Rev.* **1**:92.
4. Miller, J. F. A. P., and G. F. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:801.
5. Mitchell, G. F., and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune response. II. The source of the hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:821.
6. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Thymus-marrow cell combinations — synergism in antibody production; *Proc. Soc. Exp. Biol. Med.* **122**:1167.
7. Nossal, G. J. V., A. Cunningham, G. F. Mitchell, and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune response. III. Chromosomal marker analysis of single antibody-forming cells in reconstituted, irradiated, or thymectomized mice. *J. Exp. Med.* **128**:839.
8. Taylor, R. B. 1969. Cooperation in the antibody response of mice to two serum albumins: specific function of thymus cells. *Transplantation Revs.* **1**:114.
9. Miller, J. F. A. P. 1960. Studies on mouse leukaemia. The role of the thymus in leukaemogenesis by cell-free leukaemic filtrates. *Brit. J. Cancer.* **14**:93.
10. Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody-forming cell. *Immunology.* **14**:599.

11. Dietrich, F. M., and P. Dukor. 1967. The immune response to heterologous red cells in mice. III. Cyclophosphamide-induced tolerance to multispecies red cells. *Pathol. Microbiol.* **30:**909.
12. Aisenberg, A. C., and C. Davis. 1968. The thymus and recovery from cyclophosphamide-induced tolerance to sheep erythrocytes. *J. Exp. Med.* **128:**35.
13. Radovich, J., and D. W. Talmage. 1967. Antigenic competition: cellular or humoral. *Science (Washington).* **158:**512.
14. Cross, A. M., E. Leuchars, and J. F. A. P. Miller. 1964. Studies on the recovery of the immune response in irradiated mice thymectomized in adult life. *J. Exp. Med.* **119:**837.
15. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic duct lymphocytes. *Proc. Nat. Acad. Sci. U.S.A.* **59:**296.
16. Playfair, J. H. 1969. Specific tolerance to sheep erythrocytes in mouse bone marrow cells. *Nature (London).* **222:**882.
17. Waksman, B. H., K. Isakovic, and S. B. Smith. 1966. The thymus and the tolerance function. *Ann. N. Y. Acad. Sci.* **135:**479.
18. Gershon, R. K. 1969. Thymic dependency of tolerance to sheep red blood cells. *Fed. Proc.* **28:**376.
19. Sulitzeanu, D. 1968. Affinity of antigen for white cells and its relation to the induction of antibody formation. *Bacteriol. Rev.* **32:**404.
20. Byrt, P. N., and G. L. Ada. 1969. Antigens and lymphocytes *in vitro*: a reaction of labelled flagellin and haemocyanin with cells from normal animals. *Immunology.* **17:**501.
21. Mackaness, G. B. 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. *J. Exp. Med.* **129:**973.
22. David, J. R. 1968. Macrophage migration. *Fed. Proc.* **27:**6.
23. Mitchison, N. A. 1967. Antigen recognition responsible for the induction *in vitro* of the secondary response. *Cold Spring Harbor Symp. Quant. Biol.* **32:**431.
24. Rajewsky, K., V. Schirrmacher, S. Nase, and N. K. Jerne. 1969. The requirement of more than one antigenic determinant for immunogenicity. *J. Exp. Med.* **129:** 1131.
25. Miller, J. F. A. P., and G. F. Mitchell. 1969. Cell to cell interaction in the immune response. *Transplant. Proc.* **1:**535.