CELL TO CELL INTERACTION IN THE IMMUNE RESPONSE

I. HEMOLYSIN-FORMING CELLS IN NEONATALLY THYMECTOMIZED MICE RECONSTITUTED WITH THYMUS OR THORACIC DUCT LYMPHOCYTES*, ‡

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Neonatal thymectomy depresses the immune response of conventional and germfree mice to sheep erythrocytes (1, 2). The capacity to produce 19S hemolysin–forming cells develops later after thymectomy at birth and the number attained at the height of the response is approximately 1 $\log_{10}$ lower than in normal mice (1, 3). Thoracic duct cell output studies indicate that the number of recirculating small lymphocytes may be as low as 1% of normal (3). In addition, the thoracic duct cell population of neonatally thymectomized mice is markedly deficient in cells capable of transferring adoptively, to heavily irradiated mice, immunological responsiveness to sheep erythrocytes (3).

Studies with tritiated thymidine–labeling techniques (4, 5) indicate that some lymphocytes may leave the thymus and eventually appear in those areas of the lymph nodes and spleen known to be the site of traffic of recirculating small lymphocytes (6). The evidence obtained from the labeling and thymectomy experiments support the concept that the thymus may be the original source of some of these recirculating cells (7). Further support for this would be obtained if it could be shown that, under certain experimental conditions, thymus lymphocytes behaved and functioned exactly as thoracic duct lymphocytes.

The immunological responsiveness of neonatally thymectomized mice to sheep erythrocytes can be restored to normal by an injection of thoracic duct cells from normal donors (3). The exact relationship between the injected cells and the resulting hemolysin-forming cells is, however, unknown. The present series of papers deals with the capacity of thymus or thoracic duct cells to transfer responsiveness to sheep erythrocytes in mice crippled immunologically as a result of thymectomy, irradiation, or treatment with anti-lymphocyte serum. The relationship between the transferred cells and the precursors of the antibody-forming cells has been examined by the use of anti-H2 sera, anti-allotype

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sera, and chromosome markers. The first paper in the series deals with the identity of the hemolysin-forming cells produced in response to sheep erythrocytes in neonatally thymectomized mice reconstituted with thymus or thoracic duct cells. A preliminary report of this work has been published (8).

Materials and Methods

Animals.—Male and female mice of the highly inbred strains CBA (originally obtained from Harwell, Didcot, Berkshire, England), C57BL (originally obtained by Dr. R. Bradley of the University of Melbourne from Dr. L. W. Law of the National Institutes of Health, Bethesda, Md.), and the (CBA $\times$ C57BL)F$_1$ hybrids were used. The mice were raised and maintained at the Hall Institute and were fed Barastac 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 in the immediate postoperative period. Oxytetracycline,[1] at a dose level of 100 mg per liter, was added to the drinking water each day.

Preparation of Cell Suspensions.—Thymus lobes were removed from 6- to 8-wk-old mice, care being taken to avoid fascial tags which might contain mediastinal lymph nodes. The lobes were teased with fine forceps through an 80 mesh stainless steel sieve in cold medium 199.[2] Further disruption was achieved by gentle aspiration using a Pasteur pipette. The suspension of single cells was washed three times in medium 199 and the cells were finally resuspended in cold Dulbecco's phosphate-buffered saline. They were counted in a hemocytometer and the volume adjusted so that the number of cells required for injection was contained in 0.2–0.5 ml, depending on the experiment.

Bone marrow cells were expressed from the femurs and tibiae by means of a syringe and attached needle containing cold medium 199. The marrow plugs were gently disrupted by aspiration through a 25 gauge needle. The suspension of single cells was washed once, resuspended in cold phosphate-buffered saline, counted, and the volume adjusted so that the required dose could be injected in a suitable volume.

Thoracic duct lymphocytes were obtained as described below and the cell suspension washed and resuspended as mentioned above for marrow cells.

Sheep erythrocytes were obtained from a single animal assigned to this work. The jugular vein was punctured at weekly intervals, the blood collected and stored in Alsevers' solution for 1 wk prior to use. When required, the cells were washed three times in saline and finally resuspended to an appropriate volume for injection. The number of cells used for immunization purposes was 1–2 $\times$ 10^8 (0.1 ml of a 20% suspension).

Yeast cells were prepared by dissociating commercial baker's yeast into a single cell suspension.

Irradiation of Cell Suspensions.—Thoracic duct cells and thymus cells were X-irradiated in circular plastic dishes (30 mm diameter) with 1000 rads at a focal distance of 50 cm and a dose rate of 120 rads per minute using an X-ray machine operating under conditions of 250 kv, 15 ma, and an HVL (half-value layer) of 1 mm Cu. The cells were suspended in ice cold Eisen's balanced salt solution containing 10% fetal calf serum in a volume which completely filled the dish.

Thymus Extracts.—Cell-free saline thymus extracts were prepared from the thymuses of 6-wk-old mice according to the method of Metcalf (9). Each recipient mouse received the equivalent of one thymus lobe (approximately 10^8 cells) by intraperitoneal injection.

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[1] Terramycin, Pfizer Pty. Ltd., Sydney, Australia.

[2] Tissue culture medium 199 with 100 μg streptomycin and 100 units penicillin per milliliter was obtained from Commonwealth Serum Laboratories, Melbourne, Australia.

Injections.—All cell suspensions to be injected were given into the tail vein, unless otherwise stated. In the case of thymus cells to be given in doses exceeding 10 million per mouse, it was found absolutely essential to spread the injection over 1–2 min and give a large volume (0.4–0.5 ml) to prevent death from emboli. Mice did not have to be heparinized provided the injection was given slowly.

Operative Procedures.—Thymectomy or sham operation was performed in newborn mice, less than 36 hr old, according to the method of Miller (10). 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 they were discarded from the experiments.

The technique used to establish a thoracic duct fistula was similar to that described by Boak and Woodruff (11). The only modification was the method of inserting the Boak thoracic polythene cannula.[3] Instead of stabbing the thoracic duct with the pointed end of the cannula, a small sharp pointed jeweller's forceps was used to pierce the left lateral wall of the duct. If ventral tension was maintained on the aorta while puncturing the duct, air was drawn in and the chylous lymph displaced. This displacement clearly defined the position of the hole and the width of the duct. The bevelled end of the cannula could then be slipped into the duct and the site of insertion sealed with a drop of the monomeric tissue adhesive, isobutyl-2-cyanoacrylate.[4] This manipulation reduced the risk of lacerating the duct or of penetrating the right lateral wall which lies adjacent to the vena cava. 15–30 min prior to cannulation the mice were given 0.4 ml cream by intraesophageal instillation. The most satisfactory anesthetic agent was found to be avertin[5] and was given intraperitoneally as a 2% solution in 10% alcohol in doses of 0.1 ml/10 g body weight. The short duration of surgical anesthesia and the rapid return of muscular activity markedly increased the rate of lymph flow which assisted in the expulsion of clots from the cannula. During the collection of lymph, the mice were restrained in modified Bollman cages (12) and the lymph allowed to drain into siliconized glass tubes kept in ice water in vacuum flasks and containing 10% normal mouse serum or fetal calf serum in Dulbecco's phosphate-buffered saline and 50–100 IU of preservative-free heparin[6] per milliliter. A fine horse hair was passed up and down the cannula at regular intervals to clear lymph clots.

Hemolytic Plaque-Forming Cell Assays.—Spleen cell suspensions for assays were prepared in a manner similar to that described above, washed once, and diluted to an appropriate volume in medium 199 so that 0.1 ml contained an estimated number of 100–500 plaque-forming cells. The actual number of hemolysin-forming cells was determined on duplicate agar plates according to the method described by Jerne et al. (13).

Preparation of Anti-H2 Isoantisera.—Isoantisera were prepared by repeatedly injecting, at not less than 2-wk-intervals, 6-wk-old CBA mice and C57BL mice with 20–100 million cells from pooled thymus, spleen, and mesenteric lymph nodes of adult C57BL and CBA mice, respectively. All mice received C57BL or CBA allogeneic skin grafts between the 2nd and 3rd injection of lymphoid cells. The skin grafting technique used was that of Billingham and Medawar (14). The mice were bled 7–10 days after the last of three to six injections, the sera separated, inactivated at 56°C for 30 min, and stored in small aliquots at −20°C until required. Normal sera were obtained from CBA and C57BL mice of the same age as that of the mice used for the preparation of the isoantisera.

Incubation of Hemolysin-Forming Cells with Isoantisera.—Spleen cells were suspended in Eisen's balanced salt solution containing 10% fetal calf serum and 0.15-ml aliquots of approxi-

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[3] Portex, Portland Plastics Ltd., Hythe, Kent, England.

[4] Ethicon Inc., Somerville, N. J.

[5] Avertin with amylene hydrate, Winthrop Laboratories, N. Y.

[6] Heparin, B. P. without preservatives, Evans Medical Ltd., Liverpool, England.

mately 1-2 million cells or 100-1000 plaque-forming cells were incubated at 37°C for 30 min with 0.05 ml undiluted anti-C57BL serum, anti-CBA serum, normal C57BL serum, or normal CBA serum, and 0.05 ml undiluted guinea pig serum. The cells were washed once and resuspended to a volume of approximately 0.2 ml. This entire amount was plated onto two agar plates and developed according to the usual procedure. A similar technique for the inhibition of plaque-forming cells by isoantisera has recently been described (15).

Induction of Immunological Tolerance to Sheep Erythrocytes.—8-wk-old CBA mice were made tolerant to sheep erythrocytes according to the method of Dietrich and Dukor (16). They received one intraperitoneal injection of 0.5 ml packed sheep red cells and 24 hr later a subcutaneous injection of cyclophosphamide[7] in phosphate buffer in a dose of 1 mg/10 g body weight. Control mice were injected only with cyclophosphamide. Some mice from both groups were tested for specific immunological tolerance by challenging them 23-26 days later with either sheep or horse erythrocytes given intravenously (0.1 ml of a 20% suspension). At the same time, other mice had their thoracic duct cannulated and the lymphocytes collected for 12-16 hr. These donor mice were also tested individually for specific tolerance after closure of the fistula and removal from the restraining apparatus. Plaque-forming cell assays were performed on spleen cell suspensions 4-5 days after challenge. Since horse erythrocytes do not spread evenly in agar, the agar-free system described by Cunningham and Szenberg (17) was used to assay the number of plaque-forming cells in this case.

Statistical Analysis.—The standard errors of the means were calculated and *P* values determined by Student's *t* test.

RESULTS

Transfer of Syngeneic Thymus or Thoracic Duct Cells.—3- to 5-wk-old neonatally thymectomized CBA mice were injected intravenously with 10^8 SRBC[8] and either 10 or 50 million syngeneic thymus cells or 10 million syngeneic thoracic duct cells. Some neonatally thymectomized and sham-operated littermates were given SRBC alone. The PFC response per spleen, in mice of the various groups, was measured at 2,4,5,7, and 10 days. As shown in Fig. 1, sham-thymectomized mice produced a peak response of 32,000 PFC whereas neonatally thymectomized mice could produce only 2300 PFC. 10 million thymus cells and 10 million thoracic duct cells increased the peak response by approximately 20,000 PFC. 50 million thymus cells increased the number of PFC to levels obtained in sham-operated controls. In all groups, the peak PFC response occurred 4-5 days after challenge.

A statistical analysis of the 4-5 day PFC responses of mice of the various groups is given in Table I. Also included in this table is the background number of PFC in mice not injected with SRBC. It can be seen that the increased response of thymectomized mice given SRBC and 10 million thymus or thoracic duct cells or 50 million thymus cells is statistically significant. One and a half million thoracic duct cells, however, produced no significant increase. The

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[7] Endoxan Asta, Charles McDonald, Caringbah, Australia.

[8] The following abbreviations are used: SRBC, sheep erythrocytes; HRBC, horse erythrocytes; PFC, hemolysin plaque-forming cells; NS, not significant; and sE, standard error.

background number of PFC was similar in thymectomized and sham-operated mice as previously reported (18), and was slightly increased in thymectomized mice injected with 10 million thymus or thoracic duct cells.
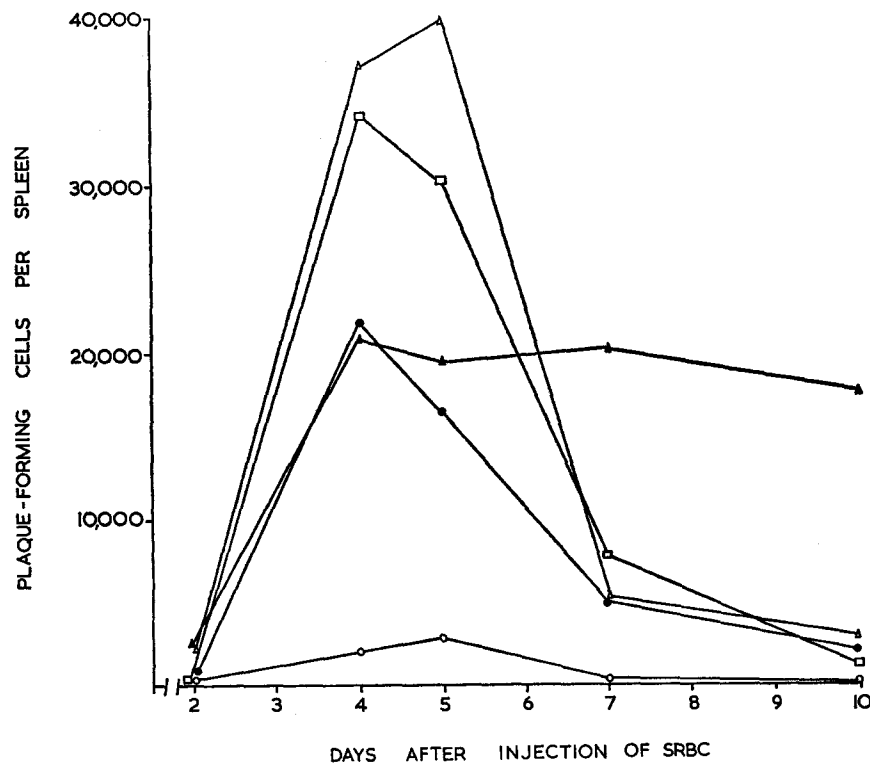

FIG. 1. PFC produced in the spleens of neonatally thymectomized CBA mice at various times after injection of SRBC and syngeneic thymus or thoracic duct cells. □——□, sham-operated controls given SRBC only; O——O, neonatally thymectomized mice given SRBC only; ●——●, neonatally thymectomized mice given 10 million CBA thymus cells and SRBC; △——△, neonatally thymectomized mice given 50 million CBA thymus cells and SRBC; ▲——▲, neonatally thymectomized mice given 10 million thoracic duct cells and SRBC. The number of mice per point was 3-20 with an average of 7.

During the course of these experiments, the spleens of neonatally thymec-tomized mice were found to be consistently enlarged and, at 4-5 days following an injection of SRBC and thoracic duct or thymus cells, contained up to 300 million nucleated cells. The spleen weights and number of nucleated cells per spleen were determined on a total of 31 uninjected thymectomized, sham-operated, and normal 3- to 4-wk-old mice. The size of the spleen in thymec-tomized mice was approximately twice that in sham-operated or normal mice

(Table II). Splenic enlargement in thymectomized mice has been previously reported and ascribed to hyperplasia of the myeloid and reticuloendothelial systems (19, 20).

TABLE I

PFC Produced in the Spleens of Neonatally Thymectomized CBA Mice after Injection of SRBC and Syngeneic Thymus or Thoracic Duct Cells

Group	Cells inoculated	No. of mice	Average PFC per spleen at 4–5 days (±SE)	P values (cf. group 1)
1. Thymectomized	SRBC	16	2,356 ± 537	—
2. Thymectomized	10×10^6 thymus cells + SRBC	20	19,160 ± 3,840	<0.01
3. Thymectomized	50×10^6 thymus cells + SRBC	23	38,855 ± 7,448	<0.01
4. Thymectomized	1.5×10^6 thoracic duct cells + SRBC	5	3,510 ± 1,040	N.S.
5. Thymectomized	10×10^6 thoracic duct cells + SRBC	12	20,254 ± 3,646	<0.01
6. Thymectomized	10×10^6 thymus cells only	6	241 ± 55	—
7. Thymectomized	10×10^6 thoracic duct cells only	4	323 ± 87	—
8. Thymectomized	Nil	5	106 ± 24	—
9. Sham-operated	Nil	9	123 ± 29	—
10. Sham-operated	SRBC	36	32,177 ± 3,550	<0.01

TABLE II

Spleen Size of Neonatally Thymectomized and Intact CBA Mice

Mice	Spleen cell No.		Spleen weight		
	No. of mice	Average No. of nucleated cells per spleen (±SE)	No. of mice	Average body weight (±SE)	Average weight of spleen per 10 g body weight(±SE)
		$\times 10^{-6}$		*g*	*mg*
Neonatally thymectomized	17	148.0 ± 12.2	7	8.5 ± 0.7	122.7 ± 9.7
Sham-thymectomized and unoperated	14	78.6 ± 10.2	5	8.6 ± 0.6	53.8 ± 2.1

The neonatally thymectomized mice used in our experiments had not yet shown any of the outward signs of wasting disease. In Fig. 2 are shown plots of the cumulative percentage mortality for neonatally thymectomized CBA mice which were either uninjected or which received 10 million syngeneic thoracic duct cells or 50 million syngeneic thymus cells at 3–4 wk of age. Deaths were first recorded at 6 wk of age and at 12 wk, 14 out of 15 (93%) uninjected

thymectomized mice had died in contrast to only 4 out of 12 (33%) mice in the group receiving thoracic duct cells and 8 out of 14 (57%) mice that were given thymus cells. 10 million thoracic duct cells thus prolonged survival of many thymectomized mice but five times as many thymus cells protected fewer mice.
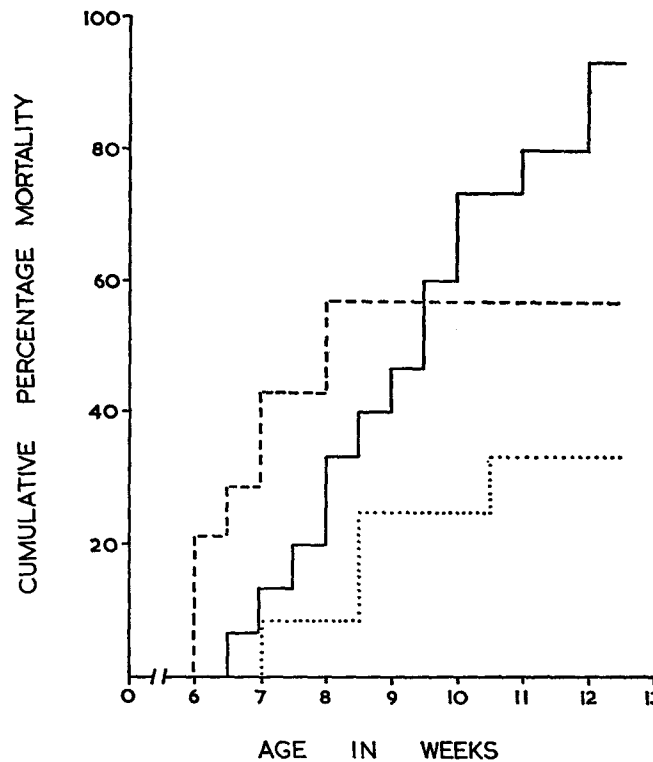
Fig. 2. Cumulative percentage mortality of neonatally thymectomized mice not given lymphoid cells (———), or injected at 3–4 wk of age with 50 million syngeneic thymus cells (-----), or 10 million syngeneic thoracic duct cells (. . . .).

Transfer of Allogeneic Thymus or Thoracic Duct Cells.—The PFC responses of neonatally thymectomized CBA mice given SRBC and 10 million (CBA X C57BL)F_1 thymus or thoracic duct cells are shown in Fig. 3. It can be seen that both these inocula were effective in elevating the response of their thymectomized hosts but, in the case of F_1 thoracic duct cells, the PFC response was prolonged with a peak at 7 days.

When C57BL thymus or thoracic duct cells were given to neonatally thymectomized CBA mice, the condition of the recipients progressively deteriorated after 5–6 days due to the development of a graft versus host reaction.

Hence the PFC response of these mice was measured not later than 4–5 days after challenge with SRBC and inoculation of C57BL cells. The results are given in Table III and compared with the 4–5 day responses of mice receiving
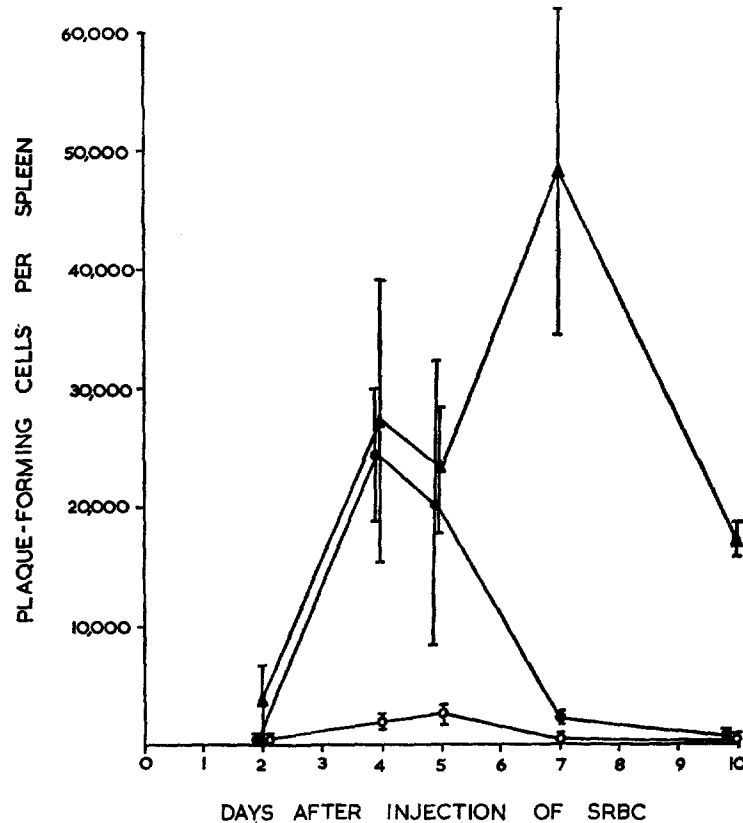
FIG. 3. PFC produced in the spleens of neonatally thymectomized CBA mice at various times after injection of SRBC and (CBA × C57BL)F_1 thymus or thoracic duct cells; O——O, neonatally thymectomized mice given SRBC only; ●——●, neonatally thymectomized mice given 10 million F_1 thymus cells and SRBC; ▲——▲, neonatally thymectomized mice given 10 million F_1 thoracic duct cells and SRBC. The magnitude of twice the standard errors is shown by the vertical bars. The number of mice per point was 2–8 with an average of 5.

SRBC and F_1 cells. It can be seen that the increased response produced as a result of these inocula is statistically significant. Furthermore, for a given number of cells transferred, F_1 thymus cells were as effective as C57BL or F_1 thoracic duct cells. A comparison of the results in Tables I and III shows that syngeneic cells produced the same effect as allogeneic or semiallogeneic cells with the exception of C57BL thymus cells which were less effective.

Transfer of Other Cells, Irradiated Cells, and Extracts.—Various cellular and noncellular inocula were tested for their capacity to augment the immune response of neonatally thymectomized mice to SRBC. Thymus and thoracic duct cells were irradiated in vitro in the cold with 1000 rads and injected into

TABLE III

PFC Produced in the Spleens of Neonatally Thymectomized CBA Mice after Injection of SRBC and C57BL or (CBA × C57BL)F_1 Thymus or Thoracic Duct Cells

Cells inoculated	No. of mice	Average PFC per spleen at 4-5 days (±SE)	P values*
SRBC	16	2,356 ± 537	—
10 × 10⁶ F_1 thymus cells + SRBC	10	22,380 ± 6,285	<0.01
10 × 10⁶ F_1 thoracic duct cells + SRBC	12	24,537 ± 5,519	<0.01
50 × 10⁶ C57BL thymus cells + SRBC	11	17,448 ± 3,901	<0.01
10 × 10⁶ C57BL thoracic duct cells + SRBC	10	30,120 ± 7,791	<0.01

* P values are compared with the average of the group of mice receiving SRBC only.

TABLE IV

PFC Produced in the Spleens of Neonatally Thymectomized CBA Mice after Various Inocula

Inoculum	No. of mice	Average PFC per spleen at 4-5 days (± SE)
SRBC	7	1840 ± 702*
50 × 10⁶ irradiated CBA thymus cells + SRBC	4	1160 ± 615
10 × 10⁶ irradiated CBA thoracic duct cells + SRBC	5	3776 ± 979
10 × 10⁶ irradiated (CBA × C57BL)F_1 thoracic duct cells + SRBC	6	4344 ± 2540
Thymus extract + SRBC	6	1802 ± 1155
50 × 10⁶ yeast cells + SRBC	6	2270 ± 450
50 × 10⁶ bone marrow cells + SRBC	11	3814 ± 2189

* This value does not differ significantly from the average value of all other groups.

neonatally thymectomized CBA mice with SRBC. As seen in Table IV, irradiation abolished the capacity of these cells to elevate the 4-5 day PFC response. A cell-free saline extract of thymus was injected intraperitoneally into neonatally thymectomized mice at the time of intravenous challenge with SRBC. Each mouse received the equivalent of one thymus lobe (approximately 100 million cells) but its response was not elevated. 50 million yeast cells, injected together with SRBC, were also ineffective. 50 million syngeneic bone marrow cells caused a slight but insignificant elevation. The large standard

error of the mean of this group of 11 mice was due to the response of one mouse which had no detectable thymus remnant, but which produced 25,050 PFC, this value exceeding all others in the group by at least 18,600.

The results obtained with irradiated cells and with thymus extracts suggest that the reconstitutive capacity of normal thymus and thoracic duct cells is dependent upon intact cells capable of division. Since bone marrow and yeast cells were ineffective, it is unlikely that the immunological capacity of thymectomized mice can be nonspecifically improved simply by the injection of large numbers of cells of any type.

TABLE V

PFC from Spleens of Normal Immunized Mice Remaining after Incubation with Isoantisera

Spleen cell donor	No. of PFC per aliquot remaining after incubation with:		
	Normal mouse serum	Anti-CBA serum	Anti-C57BL serum
CBA	310	50 (84%)*	407 (0%)
CBA	118	17 (86%)	131 (0%)
CBA	662	68 (90%)	550 (17%)
C57BL	317	360 (0%)	17 (95%)
C57BL	442	367 (17%)	74 (83%)
(CBA × C57BL)F₁	2481	16 (99%)	41 (98%)
(CBA × C57BL)F₁	815	31 (96%)	44 (95%)

* Number in brackets refers to per cent reduction.

Identity of the Hemolysin Plaque-Forming Cells.—Since allogeneic or semiallogeneic thymus or thoracic duct cells elevated the PFC response of thymectomized CBA mice, anti-H2 isoantisera can be used to identify the cells producing 19S hemolysins. If the PFC were the direct descendants of C57BL or F₁ cells, treatment with anti-C57BL serum would be expected to reduce the number of plaques appearing in agar containing SRBC. The specificity of the isoantisera and their effectiveness in inhibiting plaques are shown in Table V. Aliquots of spleen cells from normal CBA, C57BL, and (CBA × C57BL)F₁ mice, challenged 4 days before with SRBC, were incubated with normal CBA serum, normal C57BL serum, anti-CBA serum, or anti-C57BL serum, in the presence of complement. The reduction following specific or nonspecific isoantiserum treatment is expressed as a percentage of the number of PFC following incubation with normal mouse serum. Incubation in the presence of normal CBA and normal C57BL serum was associated with an average reduction, in the original number of PFC, of 29% (22 determinations). Presumably these losses were the result of nonspecific factors operating during the 30 min incubation

period and subsequent washing and plating procedures. It can be seen in Table V, that anti-CBA serum reduced the number of CBA PFC by 84–90 %, that anti-C57BL serum reduced the number of C57BL PFC by 83–95 %, and that both antisera reduced the number of F_1 PFC by 95–99 %. Incubation of CBA PFC with anti-C57BL serum or of C57BL PFC with anti-CBA serum was associated with a loss ranging from 0-17 %. The capacity of specific isoantisera to affect PFC in a mixed population of CBA and C57BL spleen cells was next

TABLE VI

PFC from Spleens of Reconstituted Neonatally Thymectomized CBA Mice Remaining after Incubation with Isoantisera

Cells used for reconstitution	No. of spleens in pool	No. of PFC per aliquot remaining after incubation with:		
		Normal mouse serum	Anti-CBA serum	Anti-C57BL serum
C57BL thymus cells	5	460	37 (92%)*	407 (12%)
C57BL thymus cells	3	363	36 (90%)	334 (8%)
C57BL thoracic duct cells	4	1612	60 (96%)	1930 (0%)
C57BL thoracic duct cells	6	142	20 (86%)	140 (1%)
C57BL spleen cells	2	1536	126 (92%)	1512 (2%)
(CBA × C57BL)F_1 thymus cells	3	609	20 (97%)	571 (6%)
(CBA × C57BL)F_1 thoracic duct cells	6	130	7 (95%)	108 (17%)
(CBA × C57BL)F_1 thoracic duct cells	4	744	84 (89%)	958 (0%)
(CBA × C57BL)F_1 thoracic duct cells	2‡	1174	29 (98%)	1454 (0%)
(CBA × C57BL)F_1 thoracic duct cells	3‡	90	0 (100%)	80 (11%)
(CBA × C57BL)F_1 thoracic duct cells	3‡	817	7 (99%)	796 (3%)

* Number in brackets refers to per cent reduction.

‡ Spleens assayed 7 days after thoracic duct cells were inoculated; all others were assayed 4–5 days after cell inoculation.

tested. A total of 551 PFC consisting of 221 C57BL PFC and 330 CBA PFC were incubated with anti-CBA or anti-C57BL sera. The number remaining after anti-CBA serum was 205 and after anti-C57BL serum was 285. Thus these antisera caused a reduction commensurate with the known content of PFC of one or other type in the mixture.

The results of 11 experiments involving 41 reconstituted mice are shown in Table VI. Spleens from thymectomized CBA mice reconstituted with either C57BL thymus or thoracic duct cells were taken 4–5 days after challenge with SRBC and aliquots of the cell suspensions were incubated with anti-C57BL serum. The reduction in the number of PFC was 0–12 %. On the other hand, incubation with anti-CBA serum reduced the number of PFC by 86–96 %. When F_1 thymus or thoracic duct cells were used to reconstitute, anti-CBA

serum (which is directed against both donor and host cell types) reduced the number of PFC by 89–97%. On the other hand, anti-C57BL serum (which is directed only against the donor cell type) caused a reduction in PFC of 0–17%. Since the peak response of thymectomized mice reconstituted with F_1 thoracic duct cells occurs 7 days after challenge (Fig. 3), spleen cells were taken at that time and incubated with isoantisera. The results show that anti-CBA serum reduced the number of PFC by 98–100% and anti-C57BL serum by 0–11%. When 50 million C57BL spleen cells were injected into thymectomized CBA mice the PFC response was increased to 23,875. Anti-CBA serum reduced the number of PFC by 92% and anti-C57BL serum by only 2%. These data

TABLE VII

PFC Produced in Response to SRBC and HRBC in the Spleens of CBA Mice* Treated 23–26 Days before with SRBC and Cyclophosphamide

Treatment	Erythrocytes used for challenge and assay	No. of mice	Average PFC per spleen at 4–5 days (±SE)
SRBC and cyclophosphamide	SRBC	9	2,766 ± 585‡
	HRBC	3	47,833 ± 4,110§
Cyclophosphamide only	SRBC	12	144,650 ± 16,562‡
	HRBC	3	72,600 ± 14,440§

* Some of these mice were used as donors of thoracic duct lymphocytes which were transferred to neonatally thymectomized mice (Table VIII).

‡ $P < 0.001$.

§ Not significant.

indicate that the majority, if not all of the PFC arising in reconstituted thymectomized mice were derived not from the inoculated cells but from the thymectomized host.

Transfer of Thoracic Duct Cells from Mice Tolerant of Sheep Erythrocytes.— Since thoracic duct lymphocytes could restore the capacity of neonatally thymectomized mice to respond to SRBC, it was of interest to determine whether this might also be achieved by thoracic duct cells from donors specifically tolerant of SRBC. It is evident from the results shown in Table VII that the lymphocyte donors used were specifically tolerant. Those treated with SRBC and cyclophosphamide 3–4 wk before challenge, produced in their spleens only 2766 PFC in response to SRBC in contrast to 47,833 PFC in response to HRBC. The cyclophosphamide-treated controls gave, in response to SRBC, 144,650 PFC per spleen, which exceeds what is generally seen in normal 6- to 8-wk-old CBA mice used in this laboratory (3). 10 million thoracic duct cells from tolerant donors or from cyclophosphamide-treated controls were injected intravenously together with SRBC into neonatally thymectomized

CBA mice and the PFC response determined 4–5 days later. The results, shown in Table VIII, indicate that the response was much lower in recipients of cells from tolerant donors than from cyclophosphamide-treated controls. The response in the latter was higher than that in recipients of the same number of normal thoracic duct cells obtained from 6- to 8-wk-old donor mice (Table I). This may be a reflection of the age of the donors or of factors associated with the recovery from the effects of cyclophosphamide. From a comparison of the data given in Table I and Table VIII it can be seen that the PFC produced in response to SRBC in the spleens of neonatally thymectomized CBA was increased slightly by an injection of "tolerant" cells. The fact that tolerance was not complete in the donors (Table VII) may account for this.

TABLE VIII

PFC Produced in the Spleens of Neonatally Thymectomized CBA Mice after Injection of SRBC and Syngeneic Thoracic Duct Cells from Mice Specifically Tolerant of SRBC

Cells Inoculated	No. of mice	Average PFC per spleen at 4–5 days (±SE)	P value
SRBC + 10^7 thoracic duct cells from cyclophosphamide-treated donors	8	41,600 ± 13,436	<0.05
SRBC + 10^7 thoracic duct cells from tolerant donors (treated with SRBC and cyclophosphamide)	8	7,331 ± 2,276	

DISCUSSION

In many experimental situations, the immunological performance of thymus cells was found to be far inferior to that of cells from spleen or lymph nodes (1). Thus, for instance, when relatively small numbers of thymus cells (up to 20 million) were injected into thymectomized mice soon after birth, the response to certain antigens given in later life was not improved and wasting disease was not prevented. By contrast, 5–20 million spleen or lymph node cells were effective (21, 22). Huge numbers of thymus cells (100–300 million) could however, reverse the effects of neonatal thymectomy (23,24) although no comparison was made between spleen and lymph node cells on the one hand, and thymus cells on the other, with respect to the minimal dose required to achieve an effect. The results reported here indicate that thymus cells were as good as thoracic duct cells in enabling neonatally thymectomized mice to respond to sheep erythrocytes when the antigen was given together with the cells, but 50 million thymus cells were inferior to 10 million thoracic duct cells in preventing wasting disease.

In previous studies, sheep erythrocytes were injected some weeks after thymus cells (25). The failure to achieve an effect in these experiments may be ascribed to the possibility that thymus cells were no longer available in adequate

numbers at the time of antigenic challenge owing to some previous immuno-
logical commitment or to a limited lifespan in the absence of specific antigenic
stimulation. These possibilities may also be invoked to explain the reduced
effectiveness of thymus cells in preventing wasting disease. In experiments in
which bovine serum albumin was injected into the foot-pad of neonatally
thymectomized rats at the same time as an intravenous injection of lympho-
cytes, cells from lymph nodes but not from thymus restored the capacity to
express delayed hypersensitivity (26). There seems to be some discrepancy be-
tween these results and those presented here as well as those reported by Taylor
(27). This might, however, reflect differences in the capacity of thymus and
lymph node cells to reach the sites in which the effector cells of the immune
response are being generated. The reaction to a foot-pad injection of bovine
serum albumin is probably mediated in the regional lymph nodes in contrast to
the antibody response to sheep erythrocytes which occurs in the spleen (28).
Intravenously injected thymus cells recirculate to a far lesser extent than
thoracic duct cells (and presumably lymph node cells) (29), and migrate in
larger numbers to the spleen than to the lymph nodes (30, 31). This differential
homing of thymus cells may be further exaggerated in the neonatally thymec-
tomized mice used in this study, as a result of their greatly enlarged spleens.

It is highly probable that the effective cells in the thymus cell suspensions
used in our experiments were lymphocytes, since these cells predominate and
since smears of the suspensions revealed only few epithelial cells or macrophages.
An inoculum of 50 million thymus cells is probably contaminated by some
blood-borne lymphocytes. It is unlikely that this contaminant population
accounts for the effects observed even if we assume that the proportion of blood
lymphocytes in the cell suspension used was as much as 10%. Thus 1.5 million
thoracic duct cells produced by no means as great an effect as 10 million
thymus cells.

The kinetics of the PFC response in thymectomized mice injected with
thymus or thoracic duct cells exhibited some differences. In the case of neona-
tally thymectomized mice given thymus cells the time-response curve was
similar to that of sham-operated controls. In the case of recipients of thoracic
duct cells the number of PFC produced remained elevated at a time when it
was falling in controls or in thymus-injected mice. This was evident whether
syngeneic or F_1 cells were used. It is not known to what extent these features
reflect differences in the population of cells inoculated. The prolonged response
may be associated with the capacity of thoracic duct lymphocytes to recirculate
and to be recruited continually into the antigenically stimulated spleen (32)
thus allowing further production of PFC. Thymus cells, on the other hand, may
not recirculate to the same extent (29). Alternatively, some delay in feedback
inhibition may have occurred in recipients of thoracic duct cells.

The capacity of anti-H2 isoantisera to discriminate between cells bearing

different H2 antigenic complexes has been documented in the experiments in which hemolysin-forming cells from CBA and C57BL donors were mixed in known proportions and exposed to either anti-CBA or anti-C57BL serum. The reduction in number of PFC obtained in either case could be ascribed solely to an effect of the specific isoantiserum used. Furthermore, hemolysin-forming cells from F_1 mice were reduced significantly with either anti-CBA or anti-C57BL serum indicating that both parental H2 antigens were expressed on the heterozygous cells as has been previously reported (33). From the data obtained when spleens from normal immunized mice were incubated with specific isoantisera, it is evident that the efficiency of these sera is such that a loss ranging from 80–100% can be achieved. These considerations enable one to conclude that at least 80% of the PFC in spleens of neonatally thymectomized CBA mice reconstituted with F_1 cells were derived from the host. It is unlikely that, in the environment of a CBA host, PFC of F_1 origin would express only one of the parental H2 complexes. Complete loss of an entire H2 complex has been recorded in heterozygous tumor cells but only after exposure, through serial transplantation, to considerable immunological pressure against the complex (34). Furthermore, as will be evident in the second paper in this series (35), F_1-type PFC were detected in the environment of an irradiated CBA host. In the case of CBA mice receiving C57BL cells, the possibility of antigenic exclusion cannot be entertained as complete loss of all H2 complexes has never been achieved, even in tumor cells (34).

It might be suggested that F_1 or C57BL cells were either rejected or inhibited from differentiating in neonatally thymectomized CBA mice, thus accounting for the failure to detect PFC of donor origin. If this is the case, then one must surmise that the restoration of responsiveness to SRBC is the result of a concomitant host *versus* graft reaction which nonspecifically boosted the host's response. This is most unlikely since syngeneic cells were restorative and since irradiated F_1 cells were ineffective. Furthermore, rejection of F_1 or C57BL tissues does not occur following neonatal thymectomy. Thus all the mice in a group of 12 neonatally thymectomized mice used in the present experiments and grafted with C57BL skin at 1 or 3 wk of age died of wasting disease with their grafts intact.

No donor-type PFC were detected in the spleens of thymectomized mice reconstituted with C57BL spleen cells. This suggests that PFC precursors of host origin are stimulated to differentiate in preference to precursor cells which must have been present in the inoculum of normal spleen cells. Presumably the host precursor cells are readily available in strategic sites within the recipient's spleen and are already in a state when their differentiation to PFC can readily be induced. Hence the fact that no donor-type PFC were detected in thymectomized mice reconstituted with either thymus or thoracic duct cells does not preclude the possibility that PFC precursors existed in these cell populations. It

is probable that, in order to detect them, a recipient lacking precursors, such as a heavily irradiated host, has to be used. This will be the subject of the investigations in the next paper (35).

The above considerations lead us to the conclusion that the immunologically deficient neonatally thymectomized host provides PFC precursors, but that these can differentiate to PFC in response to SRBC only in the presence of thymus or thoracic duct cells. The questions may now be asked: from what cell type does the PFC precursor arise and what is the role of the thymus or thoracic duct cell in this reaction? As will be seen in the next paper (35), a cell type in bone marrow, presumably after undergoing some differentiation, provides the precursors of the PFC. This is supported by previous work in which it was shown that the bone marrow of neonatally thymectomized mice was as effective as that of normal mice in restoring immunological responsiveness to nonthymectomized, heavily irradiated mice (36).

The role of thymus or thoracic duct lymphocytes in reconstituting thymectomized mice could be to provide nonspecific factors which enhance the reactivity of their hosts. It has been shown, for instance, that various cellular and noncellular inocula, including yeast, could elevate the immune response of irradiated rabbits (37, 38) and that oligodeoxyribonucleotides were capable of stimulating the early rate of increase of PFC in spleens of mice challenged with antigens such as SRBC (39). In the present study, however, attempts to restore thymectomized mice by using irradiated syngeneic or semiallogeneic cells, bone marrow cells, yeast, or thymus extracts have failed. This suggests that the activity of intact thymus or thoracic duct lymphocytes may be dependent on cells capable of division (presumably in response to antigenic stimulation) and not on the provision of nonspecific trephocytic, adjuvant, or hormonal factors. A discussion of the possible role of a humoral thymus factor presumed to be a product of the *epithelial* cells (21, 40), is beyond the scope of the present studies in which thymus *lymphocytes* were used to reconstitute mice.

Perhaps thymus or thoracic duct cells act as precursors of specialized cells which process antigen and focus a "super-antigen" to the sites where PFC precursors reside, or perhaps they play a more specific role in the response to SRBC. It has been shown that both tolerance and immunological memory are functions of the lymphocyte population of thoracic duct lymph (41, 42). Preliminary results obtained here indicate that thoracic duct cells from donors immunologically tolerant of SRBC had a reduced restorative capacity in neonatally thymectomized recipients of SRBC. The host, in this situation, was capable of providing PFC precursors, so that is is unlikely that specific tolerance is a property carried exclusively by the PFC precursor cell line. It is more plausible to suggest that the specific reactivity, which is abolished by the induction of immunological tolerance, is linked to the cell types, in thymus or thoracic duct lymph, which are essential to induce PFC precursors to differenti-

ate in response to SRBC. These cell types might then play a highly specific role in the immune response, viz. antigen recognition and the carriage of long-term immunological memory, and would thus deserve the name "antigen-reactive cells." If this is the case, then the question must be raised as to which cell type—the antigen-reactive cell or the antibody-forming cell precursor—is involved in the genetic control of antibody synthesis. Experiments employing allotype markers may help to resolve this question and will be the subject of a subsequent paper in the present series.[9]

SUMMARY

An injection of viable thymus or thoracic duct lymphocytes was absolutely essential to enable a normal or near-normal 19S hemolysin–forming cell response in the spleens of neonatally thymectomized mice challenged with sheep erythrocytes. Syngeneic thymus lymphocytes were as effective as thoracic duct lymphocytes in this system and allogeneic or semiallogeneic cells could also reconstitute their hosts. No significant elevation of the response was achieved by giving either bone marrow cells, irradiated thymus or thoracic duct cells, thymus extracts or yeast. Spleen cells from reconstituted mice were exposed to anti-H2 sera directed against either the donor of the thymus or thoracic duct cells, or against the neonatally thymectomized host. Only isoantisera directed against the host could significantly reduce the number of hemolysin-forming cells present in the spleen cell suspensions. It is concluded that these antibody-forming cells are derived, not from the inoculated thymus or thoracic duct lymphocytes, but from the host. Thoracic duct cells from donors specifically immunologically tolerant of sheep erythrocytes had a markedly reduced restorative capacity in neonatally thymectomized recipients challenged with sheep erythrocytes. These results have suggested that there are cell types, in thymus or thoracic duct lymph, with capacities to react specifically with antigen and to induce the differentiation, to antibody-forming cells, of hemolysin-forming cell precursors derived from a separate cell line present in the neonatally thymectomized hosts.

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