CELL-TO-CELL INTERACTION IN THE IMMUNE RESPONSE

VI. Contribution of Thymus-Derived Cells and Antibody-Forming Cell Precursors to Immunological Memory*, ‡

By J. F. A. P. MILLER, F.R.S., and J. SPRENT, M.B.

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia)

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Interaction between thymus-derived (T)[1] and nonthymus-derived (B) cells occurs in the primary humoral antibody response of mice to heterologous erythrocytes (1–3) and serum proteins (4, 5). Unequivocal evidence has shown that the B cells are the antibody-forming cell precursors (AFCP) (6–8) and that immunological specificity characterizes both T and B cells before intentional antigenic stimulation (9). Although it is known that the faculty of immunological memory is carried by small lymphocytes (10), the relationship between memory and T and B cells is not clear. Answers to the following questions must be sought: Is collaboration between T and B cells obligatory for the generation of memory cells (in thymus-dependent humoral antibody responses)? Do both T and B cells carry memory and, if so, is the memory directed towards the same or different antigenic determinants? Is collaboration between T and B cells essential to activate memory cells in secondary antibody responses or does priming entail a qualitative change in B cells which permits them to interact directly with antigen without the need for the participation of T cells? Cell transfer experiments to date have led to different conclusions; in one case it appeared that T cells carried memory (11) whereas in another it seemed that memory was a function carried exclusively by B cells (12). In the secondary anti-hapten antibody response to hapten–protein conjugates, the data supports the concept that T cells are involved (13) and react to separate determinants on the carrier protein whereas B cells are responsible for producing antibody to the haptenic determinant (5, 14, 15).

In the present experiments, we have studied the capacity of mouse thoracic duct lymphocytes (TDL) to transfer memory adoptively. The transferred cell

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[1] *Abbreviations used in this paper:* AFCP, antibody-forming cell precursors; B, nonthymus-derived; FγG, fowl immunoglobulin G; HRBC, horse erythrocytes; NMS, normal mouse serum; NTx, neonatally thymectomized; PFC, plaque-forming cells; 19S PFC, direct plaque-forming cells; 7S PFC, indirect or developed plaque-forming cells; T, thymus-derived; TDL, thoracic duct lymphocytes; TxBM, adult thymectomized, irradiated, and marrow protected.

population was composed of T and B cells carrying different immunogenetic markers and was thus amenable to experimental manipulation so that the contribution of either cell type to immunological memory could be studied. The conclusion was reached that both T and B cells carried memory and that cell collaboration occurred in the secondary response.

Materials and Methods

Animals.—Male and female mice of the highly inbred CBA and C57BL strains were used. The CBA strain was originally obtained from Harwell, Didcot, Berkshire, England, and the C57BL strain from Dr. L. W. Law of the National Institutes of Health, Bethesda, Md. F_1 hydrid mice were obtained by crossing CBA and C57BL. AKR mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. The mice 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 (NTx) CBA 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/liter, was added to the drinking water of NTx mice each day.

White Leghorn fowls 6 months old were obtained from a commercial breeder and maintained in suitable poultry cages.

Cell Suspensions.—Suspensions of cells from thymus and spleen were prepared by teasing with fine forceps through an 80-mesh stainless steel sieve in cold Eisen's solution (16). Further disruption was achieved by gentle aspiration using 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 spleen. The cells were finally resuspended in Eisen's solution and counted in a hemocytometer, the volume being adjusted so as to have an appropriate cell concentration.

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. 5×10^6 cells were injected intravenously to protect adult thymectomized mice that had been irradiated.

TDL were obtained from mice cannulated and restrained in modified Bollman cages. The cells were collected in cold Dulbecco's solution (17) containing 10% fetal calf serum[2] and 50–100 IU of heparin[3]/ml. After gentle centrifugation the cells were resuspended in a volume suitable for injection.

Antigens.—Fowl immunoglobulin G (FγG) was prepared from normal adult fowl serum. The serum immunoglobulin was precipitated by adding saturated ammonium sulfate and was redissolved in phosphate-buffered saline so that the final volume was equal to that of the original serum sample. Reprecipitation was carried out as above and the precipitate washed twice in excess 50% saturated ammonium sulfate, dissolved in a minimum volume of phosphate-buffered saline, and dialyzed against normal saline in the cold overnight. The purity of the sample was checked by agar gel and acrylamide gel electrophoresis. All preparations used contained no more than 5–10% of other avian proteins. The protein concentration of the FγG solution was determined by the Lowry modification of the Folin assay (18). Small portions of the samples were kept frozen at −20°C until used. In some experiments mouse lymphocytes were incubated in vitro, at a concentration of 3×10^7 cell/ml at 37°C for 50–60 min with the

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

[3] Preservative-free heparin, Evans Medical Ltd., Liverpool, England.

required dose of fluid FγG. For this purpose, a batch of the antigen was absorbed with mouse spleen cells until all the lymphoagglutinating activity of the FγG preparation had been removed. Three such absorptions were usually required.

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 Alsever's solution for 1 wk before use. Horse erythrocytes (HRBC) were obtained from the Commonwealth Serum Laboratories, Melbourne, Australia and stored in citrate saline. When required, the erythrocytes were washed three times in saline and resuspended to an appropriate volume. The number of cells used for immunization was $2–5 \times 10^8$.

Injections.—Cell suspensions were injected into the tail vein or intraperitoneally as stated.

Operative Procedures.—Thymectomy was performed in newborn mice less than 36 hr old, or in 8–10-wk old mice, according to the method of Miller (19). Whenever thymectomized mice were sacrificed, the mediastinum was examined macroscopically and, in some cases, microscopically to check for the presence of thymus remnants. No such remnants were found. The technique used to establish a thoracic duct fistula has already been described in the first paper in this series (6).

Irradiation.—Mice were exposed to total body irradiation in a Perspex box. The dose given was 750 rads to midpoint with maximum backscatter conditions and the machine operated under conditions of 250 kv, 15 ma, and a half value layer (HVL) of 1 mm Cu. The focal skin distance was 50 cm and the absorbed dose rate was 170 R/min. All irradiated mice received polymyxin B (100,000 IU/liter) and neomycin (10 mg/liter) in the drinking water.

Immunization of Mice.—Mice were each given an intraperitoneal injection of alum-precipitated FγG together with 2×10^9 killed pertussis organisms,[4] or of fluid FγG which had not been ultracentrifuged. The dose given varied according to the experiment and is stated below. The technique of alum precipitation was that of Proom (20). For controls, mice were immunized to HRBC.

Reconstitution of NTx Mice.—Several intraperitoneal injections of (CBA × C57BL)F_1 thymus lymphocytes were given to NTx CBA mice from 1–4 wk after birth. The total number of cells given per mouse was $3–4 \times 10^8$. Such mice will be referred to as "reconstituted" NTx mice. For the experiments, they were used when 1–2 months old. The reason for this is that some of the NTx CBA recipients of F_1 thymus developed, usually after 2 months, a syndrome somewhat similar to "secondary disease" in allogeneic radiation chimeras.

Labeling Cells with ^{51}Cr.—In some experiments TDL were labeled with ^{51}Cr according to the technique described by Martin (21).

Preparation of Antisera.—Anti-H2 isoantisera were prepared by repeatedly injecting, at not less than two weekly intervals, 6 wk old CBA and C57BL mice with $2–10 \times 10^7$ cells from pooled thymus, spleen, and mesenteric lymph nodes of adult C57BL and CBA mice, respectively. The mice were bled 7–10 days after the last of six injections, the sera separated, inactivated at 56°C for 30 min, and stored in small portions 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 antisera.

Antiserum against θ-C3H was prepared in AKR/Jax mice by immunization with CBA thymus cells as described by Reif and Allen (22). Nine injections of thymus cells were given at weekly intervals. Individual AKR mice were bled and their sera titrated separately for cytotoxic activity against thymus lymphocytes and TDL. Only sera with cytotoxic titers above 1:1000 against thymus cells and 1:10 against TDL were pooled and used. The cytotoxic assays were performed according to the technique of Boyse et al. (23). Guinea pig serum served as a source of complement when this was required in vitro.

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[4] Pertussis vaccine (4×10^{10} organisms/ml), Commonwealth Serum Laboratories.

For incubation of lymphocytes with anti-H2 sera the following procedure was adopted: 0.1 ml of neat antiserum was added to 3×10^7 TDL in a volume of 1 ml and the mixture incubated at 37°C for 25–30 min. The cells were then washed twice and injected or incubated with fluid FγG before injection. In some experiments they were exposed for 30 min to guinea pig serum (approximately 1 ml of 1:5 dilution for 3×10^7 cells). In the case of anti-θ-serum the same procedure was adopted except that 0.1 ml of neat serum was added to 0.4 ml of cell suspension at a concentration of 5×10^6 cells/ml, the cells being then washed twice and incubated with complement.

Detection of Antibody-Forming Cells.—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 100–500 plaque-forming cells (PFC). The actual number of PFC was determined according to the method of Cunningham and Szenberg (24). In order to estimate the number of developed PFC a rabbit anti-mouse gamma globulin serum diluted appropriately (1:200) 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 (25, 26). The target erythrocytes used in the assays were sheep erythrocytes coated with FγG according to the technique of Miller and Warner (27). A standard fowl anti-sheep erythrocyte immunoglobulin preparation was used to sensitize the sheep erythrocytes (27).

Statistical Analysis.—Calculations of the geometric means, and upper and lower limits of the SE and P values according to the nonparametric rank test, were performed using an IBM 7044 computer. In comparison of the means of any two groups of observations, a significance level of 0.05 was chosen.

RESULTS

Transfer of Immune Reactivity by TDL from Primed Mice.—In order to determine whether lymphocytes could transfer immune capacity adoptively, CBA mice were lethally irradiated and injected intravenously with $5–10 \times 10^6$ TDL and intraperitoneally with 100 μg of fluid FγG. TDL from mice primed 4 wk before with 500 μg of alum-precipitated FγG enabled their hosts to produce antibody to FγG (Table I). 182,000 indirect (7S) PFC per spleen were obtained at 7 days when 5×10^6 cells from primed CBA mice were injected. The same dose of cells from primed (CBA × C57BL)F_1 mice gave a somewhat lower response. A mixture of cells from primed CBA and (CBA × C57BL)F_1 mice produced an intermediate response. By contrast, 5×10^6 TDL from unprimed mice failed to allow their hosts to respond to fluid FγG.

It was found that antigen need not be given in vivo to stimulate the primed cells. Thus TDL exposed in vitro for 50–60 min at 37°C to 100 μg of absorbed fluid FγG and then washed before transferring $5–10 \times 10^6$ cells produced a response of the same order of magnitude as when antigen had been given solely in vivo (cf. Tables I and IV). When incubation of TDL with antigen was preceded by exposure of the cells to anti-H2 serum, no significant adoptive transfer of immune reactivity occurred. This was observed whether or not guinea pig complement had been added after washing the anti-H2 serum off the cells (Table II).

It is evident therefore that primed TDL can be stimulated by antigen in vitro and that their capacity to transfer memory responses is impaired by preincu-

bation with anti-H2 serum even in the absence of added complement. The distribution of anti-H2–incubated TDL in mice was investigated by labeling the cells with ^{51}Cr, injecting them intravenously, and determining the radioactivity

TABLE I

PFC Response in Irradiated CBA Mice Given Normal or Primed TDL and Challenged In Vivo with FγG

TDL donors*	No. of TDL given	No. of lethally irradiated‡ recipients	7 day FγG PFC per spleen		*P* values for 7S PFC
			19S PFC§	7S PFC	
Normal (CBA × C57BL)F_1	5×10^6	9	10 (20–5)‖	30 (70–20)‖	<0.005
FγG-primed (CBA × C57BL)F_1	5×10^6	9	1120 (1570–800)	24,370 (32,590–18,230)	
Normal CBA	5×10^6	6	2 (4–1)	25 (45–10)	<0.005
FγG-primed CBA	5×10^6	6	25 (90–5)	182,320 (226,510–146,750)	
FγG-primed CBA + FγG-primed (CBA × C57BL)F_1	5×10^6 from each	7	10 (28–3)	98,330 (113,580–79,720)	

* Priming dose: 500 μg of alum-precipitated FγG.
‡ Stimulating dose: 100 μg of fluid FγG in vivo.
§ Direct plaque-forming cells.
‖ Geometric mean, upper and lower limits of SE.

TABLE II

PFC Response in Irradiated CBA Mice Given Primed TDL Incubated In Vitro with Anti-H2 Serum

Group	In vitro incubation of TDL*		No. of irradiated recipients‡	7S FγG PFC per spleen at 7 days§	*P* values (cf. group 1)
	First 30 min	Next 30 min			
1	NMS	1:5 guinea pig serum	5	204,360 (231,950–180,040)	—
2	CBA anti-C57BL serum	Dulbecco's solution	4	300 (2000–50)	<0.005
3	CBA anti-C57BL serum	1:5 guinea pig serum	5	40 (110–20)	<0.005

* TDL from (CBA × C57BL)F_1 mice primed to FγG.
‡ Each recipient received 5×10^6 washed TDL intravenously and 100 μg of fluid FγG intraperitoneally.
§ Geometric means, upper and lower limits of SE.

in spleen and liver 4 hr later. The localization indices were calculated according to methods previously described (21). The results shown in Table III indicate that the majority of the cells incubated with specific anti-H2 sera were inhibited from migrating to the spleen and diverted to the liver. The failure of such treated cells to lodge in the spleen readily accounts for the low PFC response in irradiated recipients.

The specificity of the various anti-H2 sera is seen in Table IV. The 7 day indirect PFC response was severely reduced when CBA TDL were incubated with C57BL anti-CBA serum, but not significantly when incubated with CBA anti-C57BL serum. Both antisera were effective against (CBA × C57BL)F_1 TDL. When mixtures of CBA and F_1 TDL were exposed to anti-H2 sera, only C57BL anti-CBA abrogated the response; the response obtained with the mixed cell population exposed to CBA anti-C57BL serum was somewhat less than that given by normal mouse serum (NMS)-incubated cells, but the difference was not statistically significant.

The effect of varying the dose of antigen given in vitro was tested in preliminary experiments. It was found that 100 μg of absorbed fluid FγG/ml was quite sufficient to allow optimal stimulation of primed cells incubated at a con-

TABLE III

Diversion of Mouse TDL from Spleen to Liver after Incubation with Anti-H2 Sera

Strain providing TDL	Serum used for incubation of TDL	Per cent TDL diverted to liver (in excess of normal percentage)*
CBA	CBA anti-C57BL	0
CBA	C57BL anti-CBA	72
(CBA × C57BL)F_1	CBA anti-C57BL	87
(CBA × C57BL)F_1	C57BL anti-CBA	60

* For calculations see reference 21.

centration of 3×10^7 cells/ml. For most experiments it was decided to use a standard dose of 5×10^6 cells for transfer and 100 μg of fluid-absorbed FγG/ml per 3×10^7 cells for in vitro stimulation.

The PFC response per spleen of groups of six irradiated recipients of 5×10^6 FγG-primed TDL stimulated with 100 μg of FγG was determined at days 3–7 posttransfer. The response was negligible before day 5 and was subsequently mainly an indirect PFC response, there being only few direct PFC. In this particular experiment, the number of 7S anti-FγG PFC per spleen averaged 2460 by day 5, 29,900 by day 6, and 81,800 by day 7. By day 8 many of the irradiated mice had become sick so that it was decided to determine the response at day 7 in all experiments.

Effect of Anti-θ Serum on the Capacity to Transfer Memory.—TDL from CBA mice, primed with 500 μg of alum-precipitated FγG or 100 μg of fluid FγG, were incubated with anti-θ serum and complement and transferred to irradiated mice. The PFC response of these mice was significantly lower than that of irradiated controls given NMS-incubated TDL (Table V). This implies that T cells must be involved in the adoptive memory response. On the other hand, the inability of anti-θ-serum to abrogate the response entirely (irrespective

TABLE IV

PFC Response in Spleens of Irradiated CBA Mice Given TDL Preincubated with Various Anti-H2 Sera

TDL donors*	Serum used for incubation of TDL‡	No. of washed TDL given	No. of irradiated CBA recipients	FγG PFC per spleen at 7 days		*P* values for 7S PFC (cf. NMS)
				19S	7S	
CBA primed to FγG	NMS	10^7	5	190 (500–70)§	216,970 (242,460–194,160)§	—
	C57BL anti-CBA	10^7	8	2 (3–1)	460 (1210–180)	<0.005‖
	CBA anti-C57BL	10^7	5	3 (5–2)	118,560 (160,730–87,460)	NS
(CBA × C57BL)F_1 primed to FγG	NMS	10^7	10	16,830 (18,850–15,030)	29,210 (36,260–23,530)	—
	C57BL anti-CBA	10^7	10	40 (80–20)	150 (190–120)	<0.005
	CBA anti-C57BL	10^7	10	10 (20–7)	40 (70–20)	<0.005
Both CBA and (CBA × C57BL)F_1 primed to FγG	NMS	10^7 from each	5	10 (30–5)	86,220 (91,950–80,850)	—
	C57BL anti-CBA	10^7 from each	8	0	220 (900–50)	<0.005
	CBA anti-C57BL	10^7 from each	5	1.5 (2–1)	59,010 (71,110–48,970)	NS

* Priming dose was 500 μg of alum-precipitated FγG.

‡ 0.1 ml of neat serum/ml of medium containing 3×10^7 TDL for 20 min at 37°C followed by addition of 100 μg of absorbed fluid FγG for a further 60 min.

§ Geometric means, upper and lower limits of SE.

‖ Not significant.

of the priming dose used) could be attributed either to lack of susceptibility of some T cells (28) or to the possibility that priming was associated with the production of memory B cells which do not require T cells for a response to antigen. In order to discriminate between these possibilities, it was considered necessary to devise a system in which the T cells would bear a stable antigenic marker, independent of the θ-antigen, rendering them distinguishable from B cells and allowing their elimination by an appropriate antiserum. As shown below, NTx CBA mice reconstituted with (CBA × C57BL)F_1 thymus cells provided such a system.

TABLE V

PFC Response in Irradiated CBA Mice Given Primed TDL Incubated In Vitro with Anti-θ-Serum

Priming dose	In vitro incubation of TDL		No. of irradiated recipients*	7S FγG PFC per spleen at 7 days‡	*P* values
	First 30 min	Next 30 min			
500 μg of alum-precipitated FγG	NMS	1:5 guinea pig serum	5	204,360 (231,950–180,040)§	<0.05
500 μg of alum-precipitated FγG	Anti-θ-serum	1:5 guinea pig serum	6	77,920 (91,090–56,650)	
100 μg of fluid FγG	NMS	1:5 guinea pig serum	5	81,950 (110,490–60,800)	<0.05
100 μg of fluid FγG	Anti-θ-serum	1:5 guinea pig serum	5	19,290 (24,900–14,940)	

* Each recipient received 5 × 10^6 washed TDL intravenously and 100 μg of fluid FγG intraperitoneally.
‡ Geometric means, upper and lower limits of SE.
§ Data from Table II.

Effect of Thymectomy on the Capacity to Transfer Memory.—Normal and NTx CBA mice or adult thymectomized, irradiated, and marrow-protected (TxBM) mice were primed with alum-precipitated FγG at 4 wk using doses of 50 and 500 μg. They were cannulated about 4 wk later and 5 × 10^6 TDL injected intravenously into irradiated recipients followed by an intraperitoneal injection of 100 μg of fluid FγG. The 7 day indirect PFC responses are given in Table VI. Normal mice were effectively primed by 50 and 500 μg. Only poor responses were obtained with 5 × 10^6 TDL from NTx mice primed with 50 μg. Increasing the priming dose to 500 μg in thymectomized mice allowed their TDL to give an adoptive response on the order of 1000–3000 indirect PFC per spleen (Table VI). The magnitude of this response was significantly below that given by an equivalent number of cells obtained from nonthymectomized mice primed with the same antigen dose.

Reconstitution of NTx Mice by Thymus Lymphocytes.—NTx CBA mice 4 wk old, reconstituted with thymus lymphocytes from normal 6 wk old (CBA × C57BL)F_1 donors, were given 500 μg of alum-precipitated FγG intraperito-

neally and boosted 2 wk later with 100 μg of fluid FγG. They were cannulated at 8 wk to provide TDL. 5×10^6 TDL were injected intravenously into irradiated CBA recipients which were challenged intraperitoneally with 100 μg of fluid FγG. As can be seen from Table VII, these TDL were capable of adoptively transferring good indirect PFC responses, of the same order of magnitude

TABLE VI

PFC Response in Spleens of Irradiated CBA Mice Given TDL from Primed Normal and NTx CBA Donors

Priming dose*	TDL donors	No. of TDL given	No. of irradiated recipients‡	7S FγG PFC per spleen at 7 days	*P* values	
(μg)						
50	Normal	5×10^6	10	96,450 (114,630–81,160)	<0.005	
	NTx	5×10^6	7	4 (9–2)		
500	Normal	5×10^6	10	222,420 (249,350–198,400)	<0.005	<0.05
	NTx	5×10^6	9	1050 (2820–390)		
	TxBM	5×10^6	5	2800 (5400–1450)		

* Alum-precipitated FγG.

‡ Stimulating dose 100 μg of fluid FγG intraperitoneally.

TABLE VII

PFC Response in Spleens of Irradiated CBA Mice Given TDL from Primed NTx CBA Mice Reconstituted with (CBA × C57BL)F_1 Thymus Lymphocytes

NTx TDL donors*	No. of irradiated recipients‡	7S FγG PFC per spleen at 7 days	*P* value
Nonreconstituted	9	1050 (2820–390)‡	<0.001
F_1 thymus cell reconstituted	7	189,000 (226,280–157,860)	

* Priming dose 500 μg of alum-precipitated FγG.

‡ Each received 5×10^6 TDL intravenously and 100 μg FγG intraperitoneally.

§ Data from Table V.

as those produced by TDL from primed intact mice, and in marked contrast to TDL from non-reconstituted NTx donors. The identity of the indirect PFC was determined by exposure of the spleen cell suspensions to anti-H2 sera as described before (6). It is evident that only C57BL anti-CBA serum impaired plaque formation (Table VIII). This indicates that the antibody-forming cells were derived from CBA cells present in the population of TDL obtained from the reconstituted NTx donors. The thymus-derived (CBA × C57BL)F_1 lymphocytes, known to be present in that population (28), did not evidently contribute directly to the antibody-forming cells produced on transfer.

The importance of the thymus-derived cell population was demonstrated in the following experiments. TDL from reconstituted NTx mice were incubated in vitro with NMS or CBA anti-C57BL serum and 100 μg of fluid FγG before transfer. Six separate experiments were performed and gave similar results. The data of one representative experiment are given in Table IX. The PFC response in irradiated recipients of anti-H2-incubated TDL was of the order of 1 $\log_{10}$ below that of recipients of NMS-incubated TDL. The thymus-derived (CBA × C57BL)F_1 cells present in the lymph of reconstituted NTx mice must

TABLE VIII

*Identity of 7S FγG PFC Arising in Irradiated CBA Recipients of TDL from Reconstituted NTx CBA Mice**

Serum used for incubation of spleen cells	No. of 7S PFC in sample	Reduction of 7S PFC
		%
NMS	147	—
CBA anti-C57BL	141	4
C57BL anti-CBA	3	98

* Reconstituted with 3–4 × 10^8 (CBA × C57BL)F_1 thymus lymphocytes.

TABLE IX

Effect of Incubating TDL from Reconstituted NTx FγG-Primed CBA Mice with Anti-H2 Sera

Serum used for incubation of TDL	Viable cells	No. of irradiated recipients*	7S FγG PFC per spleen at 7 days	*P* values
	%			
NMS and complement	95	8	277,050 (297,000–258,400)	<0.005
CBA anti-C57BL and complement	35	7	14,700 (17,170–12,590)	
Anti-θ and complement	47	6	29,570 (38,010–23,000)	=0.05

* 5 × 10^6 cells transferred.

thus be essential to allow the CBA cells, also present in the lymph, to transform to antibody-forming cells upon transfer to irradiated hosts. The response was not completely abolished by eliminating the F_1 cells presumably because the NTx CBA mice must have a small population of T cells that had migrated from the thymus before birth. These cells would not be susceptible to CBA anti-C57BL serum treatment and could thus have made some small contribution to the response. Treatment of the TDL population with anti-θ-serum would be expected to eliminate T cells, irrespective of whether these were CBA or F_1 and hence to produce a reduction in the response even more pronounced than that occurring after anti-H2 serum treatment. The anti-θ-serum did reduce the response but the extent of the reduction was not as great as that observed after incubation of TDL with anti-H2 serum (Table IX). It can be seen from Table

IX that anti-H2 treatment killed 65% of cells whereas anti-θ-treatment killed 53%. This corroborates previous findings that most, though by no means all, T cells are susceptible to the cytotoxic action of anti-θ-serum (28).

Could the antibody response that had been reduced after treatment of the transferred cells with anti-H2 or anti-θ-serum be restored to control levels by the addition of thymus-derived cells and, if so, would T cells from primed mice be more effective than T cells from normal mice? Since the percentage of T cells in TDL of nonthymectomized mice is of the order of 85% (28), we decided to use TDL from normal or FγG-primed mice as a source of T cells. When such TDL were given alone to irradiated mice, in doses ranging from 10^5–10^6, only low indirect PFC responses to fluid FγG were obtained (Table X). TDL from

TABLE X

PFC Response in Irradiated CBA Mice Given Various Doses of TDL from Normal or FγG-Primed Mice

Donors	No. of TDL given*	No. of irradiated recipients‡	7S FγG PFC per spleen at 7 days
Normal	5×10^6	6	170 (360–80)
FγG primed§	10^5	11	110 (190–70)
	10^6	5	4580 (7300–2870)
	5×10^6	10	222,420 (249,340–198,400)

* These same TDL populations were used as supplementing cells in the experiments shown in Table X.

‡ Priming dose was 500 μg of alum-precipitated FγG.

§ Each recipient received 100 μg of fluid FγG intraperitoneally.

reconstituted NTx mice primed to FγG were thus incubated in vitro with anti-H2 serum, as described above, and then given to irradiated mice together with TDL from either normal CBA mice, or from CBA mice primed to either FγG or HRBC. Five such experiments were performed and gave essentially similar results. The data pooled from two to three such experiments are given in Table XI. The adoptive indirect PFC response obtained by transferring anti-H2–incubated TDL could be elevated towards control levels by supplementing the cells with either normal or FγG-primed TDL. Cells from primed mice were at least 10 times as effective as cells from normal mice; thus, to achieve the same effect as 10^5 TDL from FγG-primed mice, 10^6 TDL from either normal CBA mice or from CBA primed to HRBC had to be given. It is interesting to record that TDL from (CBA $\times$ C57BL)F_1 mice could not supplement in this system, in marked contrast to CBA TDL. Thus, even though the T cells eliminated by antiserum incubation were (CBA $\times$ C57BL)F_1, the competence of the population could be restored only by CBA TDL, not by (CBA $\times$ C57BL)F_1 cells.

Effect of Transferring Primed T Cells and Unprimed B Cells.—It is clear from the above experiment that T-depleted TDL from primed reconstituted NTx mice could transfer good memory responses only if supplemented with T cells. Furthermore, as few as 10^5 TDL from primed intact mice contained sufficient numbers of T cells to achieve this effect. Such small numbers of primed TDL, when given alone to irradiated mice, produced only low responses (Table X) presumably because the number of B cells was limiting. The question was therefore asked: would the addition of unprimed B cells overcome this limitation

TABLE XI

Restorative Effect of TDL from Normal and Primed Mice on the Response Produced by TDL from Reconstituted NTx Mice Primed to FγG

Group	Serum used to incubate TDL from reconstituted NTx CBA*	Supplementary TDL		No. of irradiated recipients‡	7S FγG PFC per spleen at 7 days	*P* values	
		Donor	No. added			Cf. group 1	Cf. group 2
1	NMS	—	—	12	225,130 (255,050–198,710)	—	<0.005
2	CBA anti-C57BL	—	—	11	9720 (12,170–7770)	<0.005	—
3	CBA anti-C57BL	Normal CBA	10^5	11	13,070 (16,280–10,490)§	<0.005	NS
4	CBA anti-C57BL	Normal CBA	5×10^5	8	27,020 (35,660–20,480)	<0.005	=0.05
5	CBA anti-C57BL	Normal CBA	10^6	9	35,440 (43,220–29,070)	<0.005	<0.005
6	CBA anti-C57BL	Normal CBA	5×10^6	7	168,950 (197,450–144,570)	NS‖	<0.005
7	CBA anti-C57BL	FγG-primed CBA	10^4	4	14,840 (29,580–7450)	<0.05	NS
8	CBA anti-C57BL	FγG-primed CBA	10^5	10	93,720 (114,120–76,970)	<0.005	<0.005
9	CBA anti-C57BL	FγG-primed CBA	5×10^5	4	166,150 (194,080–142,240)	NS	<0.05
10	CBA anti-C57BL	HRBC-primed CBA	10^5	7	32,660 (37,350–28,560)‡	<0.005	<0.005
11	CBA anti-C57BL	HRBC-primed CBA	5×10^6	5	148,320 (172,040–127,870)	NS	<0.005

* 5×10^6 of these TDL given to each recipient.
‡ 100 μg of fluid FγG given intraperitoneally.
§ *P* value (group 3 compared to group 10) =0.05.
‖ Not significant.

and enable the T cells in the primed TDL population to express fully their collaborative potential? As can be seen from Table XII, the addition of B cells (either in the form of spleen cells from TxBM mice or of TDL from nonprimed, reconstituted NTx CBA preincubated with anti-H2 serum) did not produce good PFC responses in irradiated recipients. Increasing the dose of B cells from 5 to 50 million had no effect. Thus T cells from primed mice were unable to allow B cells from unprimed mice to produce significant responses.

DISCUSSION

The results of experiments presented here and of work performed in rats (10) indicate that the faculty of immunological memory is a property carried by recirculating lymphocytes. Since the TDL population contains both T and B cells (28), it is of importance to determine the relative contribution of these cells to the memory response. This can be done by using populations of T and B cells which bear different immunogenetic markers. Although the θ-C3H antigen

is a marker for thymus-derived cells, it has been demonstrated that a small proportion of T cells are not susceptible to the cytotoxic action of anti-θ-C3H serum (28). Consequently, the use of a population of T cells which carried a stable antigenic marker, independent of the θ-C3H antigen, would be advantageous in any experiment aimed at elucidating the function of T cells in a particular situation. NTx CBA mice were reconstituted by providing them with (CBA × C57BL)F_1 thymus lymphocytes. Such reconstitution enabled the mice

TABLE XII

PFC Response in Spleens of Irradiated CBA Mice Given T and B Cells from Primed and Unprimed Donors

Group	Source of T cells	Source of B cells	No. of irradiated recipients*	7S FγG PFC per spleen at 7 days	*P* values (cf. group 1)
1	10^5 TDL from CBA primed to FγG	5×10^6 TDL from reconstituted NTx CBA primed to FγG and preincubated with CBA anti-C57BL serum	10	93,720 (114,120–76,970)‡	—
2	10^5 TDL from CBA primed to FγG	5×10^6 TDL from reconstituted NTx CBA not primed to FγG but preincubated with CBA anti-C57BL serum	6	870 (1430–530)	<0.005
3	10^5 TDL from CBA primed to FγG	5×10^6 spleen cells from TxBM CBA	7	10 (20–4)	<0.005
4	10^5 TDL from CBA primed to FγG	5×10^7 spleen cells from TxBM CBA	6	60 (210–20)	<0.005
5	10^5 TDL from CBA primed to FγG	—	17	130 (190–90)	<0.005
6	—	5×10^7 spleen cells from TxBM CBA	9	7 (17–3)	<0.005

* 100 μg of fluid FγG given intraperitoneally.
‡ Data from Table XI.

to be successfully primed to FγG and to provide TDL that could adoptively transfer memory to FγG in irradiated recipients. Furthermore, the TDL population was composed of (CBA × C57BL)F_1 cells, which must be thymus-derived, and of host-derived CBA cells (28). The F_1 cells in such a population could thus be depleted by means of CBA anti-C57BL serum which eliminates virtually 100% of cells carrying the H2 antigens derived from C57BL (28). As has been demonstrated elsewhere, the elimination of cells known to be thymus-derived in such a chimeric population was more complete with anti-H2 serum than with anti-θ-serum (28). Elimination of F_1 cells by anti-H2 serum could also be carried out in the presence or absence of guinea pig complement. In the absence of complement, the cells were not killed but, upon injection into mice, failed to lodge in the spleen and were diverted to the liver. The TDL from

reconstituted NTx CBA mice primed to FγG produced excellent adoptive memory responses comparable in magnitude to those obtained after transferring TDL from intact primed mice. The contribution of T cells to immunological memory was studied by incubating TDL from reconstituted mice with various antisera in vitro before transfer. PFC responses on the order of 10^5 per spleen occurred when NMS-treated TDL were transferred and, in this case, the PFC were CBA-type and thus derived, not from F_1 cells, but from cells provided by the NTx CBA mouse. The PFC responses were lower by 1 $\log_{10}$ when T-depleted TDL, i.e. CBA anti-C57BL serum-treated TDL, were transferred. Since these experiments were concluded, the results of Takahashi et al. (29) were published. These investigators showed that anti-θ-treated immune spleen cells failed to transfer antibody responsiveness to sheep erythrocytes in irradiated mice.

The capacity of antigen to "educate" thymus cells (5) implies that the property of an enhanced antibody response can be linked to T cells. Likewise, the fact that the adoptive secondary antibody response to hapten–protein conjugates can be impaired by treatment of the carrier-primed cells with anti-θ-serum indicates that T cells from primed animals were involved in the secondary anti-hapten responses (13). The failure of T-depleted TDL in the present experiments, and of anti-θ-serum–treated immune spleen cells (29) to transfer secondary antibody responses also indicates that T cells must be essential for antigen-induced differentiation of AFCP to antibody-forming cells in a secondary response. These experimental results do not tell us, however, whether memory resides in T cells. Accordingly, we investigated whether the PFC response of irradiated recipients of T-depleted TDL could be augmented by adding T cells from normal mice or from mice primed either to the antigen in question or to an unrelated antigen. The source of T cells was TDL which contain only a minority population of B cells (28). They were used in doses low enough to ensure that, when given alone, low PFC responses were produced. A significant augmentation of the PFC response occurred when T-depleted TDL were supplemented with either normal or FγG-primed TDL. An important finding was that 10 times as many cells from normal as from FγG-primed mice had to be used to achieve the same effect. Takahashi et al. (29) failed in their attempts to reconstitute the immune capacity of their anti-θ-C3H–treated cells by either thymus or spleen cells. They did not, however, use a wide range of cell doses nor did they supplement with T cells from primed mice. Our results show that T cells from normal mice will reconstitute but that T cells from primed mice are much more effective. They show in addition that priming is specific since T cells from mice primed to HRBC were not much more effective than T cells from normal mice. Specific immunological memory must thus reside in the T cell population.

We also investigated whether memory was a function of the B cell population in this system. If only T cells carried memory, one would not expect any differ-

ence in the response of irradiated recipients of primed T cells supplemented with B cells from primed or unprimed mice. In fact, in marked contrast to primed B cells, unprimed B cells, even in large numbers, were unable to enhance the response of primed T cells.

In conclusion, it seems that memory is a property that can be linked to both T and B cells. Since normal T cells can substitute for T cells from primed mice, albeit only when larger numbers are used, it might be concluded that memory in T cells involves a quantitative change in that population, i.e., an increase in the number of T cells reactive to the antigenic determinants concerned. The possibility of a qualitative change in T cells is not, however, excluded by the present results.

In contrast to the situation with T cells, the deficiency in an unprimed population of B cells could not be overcome by the addition of normal B cells. It is possible, therefore, that priming entails, in the B cell population, a change which is fundamentally different from that produced in the T cell population. This may reflect selection (30), during the course of immunization, of those AFCP which can most readily be triggered by antigen to produce antibody of high avidity.

SUMMARY

Collaboration between thymus-derived lymphocytes and nonthymus-derived antibody-forming cell precursors occurs in the primary antibody response of mice to heterologous erythrocytes and serum proteins. The purpose of the experiments reported here was to determine whether collaboration took place in an adoptive secondary antibody response.

A chimeric population of lymphocytes was produced by reconstituting neonatally thymectomized CBA mice soon after birth with (CBA × C57BL)F_1 thymus lymphocytes. These mice could be effectively primed to fowl immunoglobulin G (FγG) and their thoracic duct lymphocytes adoptively transferred memory responses to irradiated mice. The activity of these cells was impaired markedly by preincubation with CBA anti-C57BL serum and to a lesser extent by anti-θ-serum. Reversal of this deficiency was obtained by adding T cells in the form of thoracic duct cells from normal CBA mice. Cells from FγG-primed mice were at least 10 times as effective as cells from normal mice or from CBA mice primed to horse erythrocytes. These results were considered to support the concept that memory resides in the T cell population and that collaboration between T and B cells is necessary for an optimal secondary antibody response.

Poor antibody responses were obtained in irradiated mice given mixtures of thoracic duct cells from primed mice and of B cells from unprimed mice (in the form of spleen or thoracic duct cells from thymectomized donors). In contrast to the situation with T cells, the deficiency in the B cell population could not be reversed by adding B cells from unprimed mice. It was considered that

memory resides in B cells as well as in T cells and that priming probably entails a change in the B cell population which is fundamentally different from that produced in the T cell population.

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