

## IMMUNOLOGICAL MEMORY IN MICE

### III. MEMORY TO HETEROLOGOUS ERYTHROCYTES IN BOTH T CELL AND B CELL POPULATIONS AND REQUIREMENT FOR T CELLS IN EXPRESSION OF B CELL MEMORY. EVIDENCE USING IMMUNOGLOBULIN ALLOTYPE AND MOUSE ALLOANTIGEN THETA MARKERS WITH CONGENIC MICE\*

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(Received for publication 2 September 1971)

In several experimental systems the full expression of the antibody response to certain antigens includes the participation of at least two cell types (e.g., 1-4). The impaired response of thymectomized mice to sheep erythrocytes (SRBC)<sup>1</sup> is restored to normal by injections of thymus or thoracic duct cells yet the reconstitutive inoculum (T cell population) does not contribute the precursors of detectable direct plaque-forming cells (PFC) in the spleen. Such precursors (B cell population) may be provided directly, or after some period of maturation, by cells contained in the bone marrow (1). Furthermore, low levels of anti SRBC antibody or small numbers of direct PFC are produced by mixed inocula of thymus and marrow cells in lethally irradiated mice (3). Responses in these instances do not approach those in normal mice but here again the marrow cells provide the majority, if not all, the precursors of direct PFC (5, 6). Thus, antibody production by B cells against antigens such as SRBC is facilitated by the presence of T cells which themselves are likely to have had their ancestry in the cell population of the bone marrow (7).

Understanding the mechanism of interaction between T and B cells would be aided

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\* Supported by U. S. Public Health Service grants AI 18817-02, AI 08917-06, AI 120, and AI 109072-02 and Cancer Research Coordinating Committee.

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<sup>1</sup> *Abbreviations used in this paper:* anti a, antiserum to Ig1<sup>a</sup>, Ig4<sup>a</sup> allotypes; anti b, antiserum to Ig1<sup>b</sup>, Ig4<sup>b</sup> allotypes; Anti  $\theta$ , AKR/J anti C3H/HeJ thymus cells; B cells, bone marrow-derived; CSM, cell-suspending medium (see footnote 12); FCS, fetal calf serum; GPS, guinea pig serum; HRBC, horse red blood cells; MEM-PM, Eagle's minimal essential medium (see footnote 8); NMS, normal AKR/J mouse serum; PFC, plaque-forming cells; RAM $\gamma$ G, rabbit anti-mouse gamma globulin serum; SRBC, sheep red blood cells; STx, sham thymectomized; T cells, thymus-derived; TDC, thoracic duct cells; Tx, thymectomized.

by clear evidence regarding specificity in both cell lines. Specific immunological tolerance appears to be a property of both cell types (8, 9) even though many systems have provided evidence for tolerance in T cells only (4, 10, 11). Only suggestive evidence is available on the question of whether adoptive immunological memory is carried by both cell populations. Specific immunological memory, involving heightened indirect PFC production, cannot be a property confined to T cells (12) yet the results of Cunningham (6), Raff (13), and evidence for "education" (14, 15) and antigen-mediated proliferation of T cells (2), suggest that these cells, as well as B cells, may exhibit secondary responsiveness.

The mouse alloantigen theta ( $\theta$ ) is expressed maximally on thymus lymphocytes and in brain tissue and to a lesser extent on lymphocytes in peripheral lymphoid organs such as the spleen and lymph nodes (16, 17). A clear-cut reduction in the anti- $\theta$  serum sensitivity of peripheral lymphocytes from thymectomized or athymic "nude" mice (18, 19) strongly suggests, but certainly does not prove, that  $\theta$  may be used as a marker for lymphoid cells previously influenced by the thymus, i.e., T cells.

Previous work in these laboratories has demonstrated that the *in vitro* immune response of normal mouse spleen cells to SRBC is reduced dramatically by anti- $\theta$  serum treatment in the presence of complement and that this response can be restored to control levels by *in vivo* incubated (educated) thymus cells (20). Anti- $\theta$  serum and complement did not affect the number of splenic hemopoietic stem cells, direct and indirect PFC themselves, or the precursors of direct PFC generated *in vitro*. The availability of cytotoxic Anti  $\theta$ , congenic C3H.SW and CWB mice (which as far as can be determined differ genetically in immunoglobulin allotype only) (21), and specific anti-allotype sera, led to attempts to dissect the cellular events of adoptive secondary responses in mice. The results of this study indicate that both T and B cells behave as specifically hyperreactive cell populations, and thus carry memory, after exposure to heterologous erythrocyte antigens.

#### *Materials and Methods*

*Mice.*—Male and female mice of the C3H.SW/SnHz strain ( $H-2^b$ ,  $Ig^a$ ,  $\theta C3H$ ) and the congenic partner, C3H.SW- $Ig^b$ (CWB) derived from either the 8th (CWB/8) or 13th (CWB/13) backcross generations (21), were used in most experiments. AKR/J and C3H/HeJ male mice, used for the production of AKR anti  $\theta$  C3H sera, were purchased from the Jackson Laboratories, Bar Harbor, Maine, and maintained in Berkeley. BALB/cN and C57BL/10J- $b/b$ ,  $p/p$ , a strain designated BCT, were raised and maintained in the colony of Dr. L. A. Herzenberg at Stanford. All mice were fed Mouse Chow<sup>2</sup> and water *ad libitum* and, in the case of mice at Stanford, were vaccinated with mouse pox vaccine<sup>3</sup> soon after weaning. Donors of thymus cells were always 4–6 wk old, and mice used for irradiation varied in age from 10 wk to 6 months.

*Irradiation.*—Irradiation procedures have been described in a previous paper (22), the

<sup>2</sup> Dean's Animal Feed, San Carlos, Calif.

<sup>3</sup> Mouse Pox Service Laboratory (US Public Health Service), New Jersey College of Medicine, Jersey City, N. J.

dose being 720–850 rads depending on the age and the size of the mice. 1 day before irradiation, and for the duration of life of the irradiated mice, antibiotics were given in the drinking water at doses of 1.13 g of neomycin sulfate and 113 mg of polymyxin B sulfate/liter.

*Cell Suspensions.*—Single cell suspensions of spleen and thymus were prepared and counted as described previously except that organs were disrupted and teased through 50-mesh stainless steel screens rather than pressed through with the rounded end of a test tube (22). Care was taken to ensure that all thymuses were removed from the mice in a bloodless field and all fascial tags dissected away from the lobes. Thoracic duct cells were collected into Dulbecco's phosphate-buffered saline (23) containing 5% fetal calf serum (FCS)<sup>4</sup> and 50–100 IU preservative-free heparin.<sup>5</sup> Cannulation of mouse thoracic ducts was performed using a modified method of Boak and Woodruff (24) and the mice restrained postoperatively on exercise wheels.

*Antigens and Immunization.*—The dose of SRBC and horse erythrocytes (HRBC) used most frequently was  $4 \times 10^8$  cells. SRBC in Alsever's solution<sup>6</sup> and HRBC in Alsever's solution<sup>7</sup> were washed three times in saline and resuspended finally in MEM-PM.<sup>8</sup> Most of the immune mice to be used as spleen cell donors were injected with SRBC or HRBC intravenously and/or intraperitoneally followed, on the same day, by 10 or 20  $\mu$ g of Piromen endotoxin<sup>9</sup> intraperitoneally.

*Assay for PFC.*—The modified assay of Jerne et al. (25) for detecting hemolytic antibody-producing cells in agarose has been described in detail in previous papers (22, 26). Indirect PFC were developed with anti-allotype sera as well as a polyvalent rabbit anti-mouse gamma globulin serum (RAM $\gamma$ G). The anti-allotype sera used in this study (anti Ig1<sup>a</sup>, anti Ig4<sup>a</sup> and anti Ig1<sup>b</sup>, anti Ig4<sup>b</sup> hereafter referred to as anti a and anti b) reacted with both  $\gamma$ G<sub>2a</sub> and  $\gamma$ G<sub>1</sub> immunoglobulins in assays involving precipitation of <sup>125</sup>I proteins (22). The number of indirect PFC was estimated by subtracting the number of PFC on slides incubated with normal mouse serum (1:150) from the number on slides incubated with the developing anti-allotype sera (1:300 for anti a and 1:200 for anti b) or RAM $\gamma$ G serum (1:1000). The average number of plaques on two slides incubated with developing sera (indirect PFC) was not considered to be above the average number on two slides incubated with normal mouse serum (direct PFC) unless the indirect PFC number on both of the slides was greater than the higher number of direct PFC.

*Production of Anti- $\theta$  Serum.*—AKR/J mice were injected intraperitoneally with dissociated C3H/HeJ thymus cells in balanced salt solution (23). The first bleedings were made 7 and 14 days after six weekly injections of  $2 \times 10^7$  thymus cells. Subsequent bleedings were performed 7 and 14 days after single booster injections given at 3-wk intervals. Pooled sera obtained at each bleeding were tested for their capacity to inhibit an in vitro primary response to SRBC and for lack of cytotoxicity against direct PFC (20). AKR and C3H strains possess

<sup>4</sup> Immunoprecipitin-tested fetal calf serum, Grand Island Biological Company, Grand Island, N. Y., and Berkeley, Calif.

<sup>5</sup> Hepathrom, Fellows Medical Manufacturing Company, Inc., Anaheim, Calif.

<sup>6</sup> Sterile sheep blood in modified Alsever's solution, W. T. Bennett Ranch Laboratory, Woodland, Calif.

<sup>7</sup> Palomino sterile horse blood in modified Alsever's solution, Colorado Serum Company Laboratories, Denver, Colo.

<sup>8</sup> Eagle's minimal essential medium, catalogue No. F-12. Instant tissue culture powder medium without NaHCO<sub>3</sub> (Grand Island Biological Company, Oakland, Calif.) made up with Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O (0.001 M) and MgCl<sub>2</sub>·6H<sub>2</sub>O (0.001 M) instead of bicarbonate in deionized water.

<sup>9</sup> *Pseudomonas* polysaccharide (Piromen) Flint Laboratories, Morton Grove, Ill.

different alleles at the *Ly-A* locus (27) and AKR anti C3H thymus cell sera (Anti  $\theta$ ) could contain anti-*Ly-A.1* antibodies (19). Anti  $\theta$  prepared as indicated above was absorbed with BALB/c ( $\theta$ C3H, *Ly-A.2*) thymus cells and tested against  $^{51}\text{Cr}$ -labeled thymus cells (see below) from C3H.SW and C57BL/10 (BCT) mice. As seen in Table I, BALB/c thymus cells (but not AKR/J thymus cells) absorbed out the cytotoxic activity of our Anti  $\theta$  for thymus cells. Thus, the cytotoxicity of this antiserum is not due to the presence of anti-*Ly-A.1* antibodies.

*In Vitro Treatment of Cells with Anti- $\theta$  Serum.*—Single cell suspensions at a final cell concentration of  $1 \times 10^7$  nucleated cells/ml were incubated with Anti  $\theta$  or normal AKR/J mouse

TABLE I  
*Absorption of Cytotoxic Activity of Anti  $\theta$  with Thymus Cells*

Donor of $^{51}\text{Cr}$ thymus cells	Donor of thymus cells used for absorption of Anti $\theta$	Cytotoxic index* (%)
C3H.SW	—	77.5
	BALB/c‡	0.3
	AKR/J	69.7
C57BL/10 (BCT)	—	72.2
	BALB/c	0.9
	AKR/J	64.0

\* Ratio, expressed as a percentage, of the number of counts released with Anti  $\theta$  (1:80 final dilution) and GPS (1:10 final dilution) minus the number of counts released with GPS to the number of counts released by freezing and thawing minus the number of counts released with GPS. Indices are calculated from the average of supernatant counts from two duplicate tubes. Treatment of labeled C3H.SW and C57BL/10 (BCT) thymus cells with normal AKR/J serum and GPS resulted in cytotoxic indices of 0 and 0.9%, respectively. Labeled cells were incubated with Anti  $\theta$  or AKR/J serum before adding GPS.

‡ Anti  $\theta$  was absorbed three times with an equal volume of packed thymus cells. The serum and cells were mixed at each step, and the plastic tubes left for 20 min in an ice bucket before centrifugation and removal of supernatant.

sera (NMS) (at a final concentration of 1:40) and guinea pig serum (GPS)<sup>10</sup> (1:10 final) in 5% FCS in MEM-PM for 45 min at 37°C. After centrifugation the cells were suspended and washed in a large volume of 5% FCS in MEM-PM. The cells were finally resuspended in MEM-PM, counted, and the volume adjusted to provide the required number of cells in 0.1–0.5 ml. The GPS, used as a source of complement, was absorbed immediately before use with either acetone-treated liver and lymphoid tissue powder (20 mg/ml) or agarose<sup>11</sup> (80 mg/ml) for periods of 1–3 hr in an ice bath (the latter procedure was suggested to us by Dr. M. Schlesinger).

<sup>51</sup>Cr Release Assay.—Thymus cells, suspended in 5% FCS in MEM-PM or cell-suspending medium (CSM)<sup>12</sup> at a concentration of  $10^8$  nucleated cells/ml, were incubated with occasional shaking at 37°C for 30–60 min with 100  $\mu\text{Ci}$   $\text{Na}_2^{51}\text{CrO}_4$ <sup>13</sup>/ml. Cells were washed three

<sup>10</sup> Dried complement (guinea pig), Hyland Laboratories, Costa Mesa, Calif.

<sup>11</sup> SeaKem, Marine Colloids Inc., Springfield, N. J.

<sup>12</sup> Cell-suspending medium consisting of Grand Island Biological Company medium 199, catalogue No. E12, plus 0.35 g/liter  $\text{NaHCO}_3$  and penicillin and streptomycin (100 IU/ml).

<sup>13</sup> New England Nuclear Corporation, Boston, Mass. Specific activity of 80–400 mCi/mg.

times in excess CSM or MEM-PM containing FCS and suspended finally at a concentration of  $10^8$  cells/ml. 1 million cells ( $10 \mu\text{l}$ ) were added to each tube in the assay together with  $80 \mu\text{l}$  of CSM or MEM-PM plus FCS and  $10 \mu\text{l}$  of the test antiserum dilution. The mixture was incubated for 30–45 min at  $37^\circ\text{C}$ , spun in a Beckman serofuge (Beckman Instruments, Inc., Fullerton, Calif.) for 10 sec, the supernatant discarded, and  $100 \mu\text{l}$  of 1:6 or 1:10 agarose-absorbed GPS added to each tube. After a further incubation of 30 min at  $37^\circ\text{C}$ ,  $25 \mu\text{l}$  was sampled into tubes, in some cases containing 0.2 ml of saline, and counted in a well-type gamma scintillation counter. Controls, run in parallel, were cells incubated without GPS, the appropriate dilution of NMS, or with NMS plus GPS. Other aliquots of  $10^6$  cells were frozen and thawed three times in a freezing mixture of acetone-dry ice. The cytotoxic index was computed using the formula:

$$\frac{\text{counts released with Anti } \theta \text{ and GPS} - \text{counts released with GPS}}{\text{counts released with freezing and thawing} - \text{counts released with GPS}} \times 100.$$

*Statistical Analysis.*—The geometric means and limits of the standard error of the means were determined from  $\log_{10}$  transformed data using a program on the Stanford Acme computer. *P* values were determined by Student's *t* test using a formula for small group sizes and a one-tailed test. When comparing the values of two means in this test, a figure of 0.05 was chosen as the limit of statistical significance.

*Experimental Design.*—The overall plan of the experiments is indicated in Fig. 1. Spleen cells from immunized C3H.SW mice, after incubation with Anti  $\theta$  + GPS, NMS + GPS, or after no incubation, were transferred to heavily irradiated CWB recipients together with heterologous erythrocytes by intravenous injection. Splenic PFC numbers were determined 7 days later, the indirect PFC being developed with three antisera: anti a allotype, anti b allotype, and RAM $\gamma$ G. In some cases, inocula of spleen cells treated in vitro with Anti  $\theta$  + GPS were supplemented with dissociated thymus or thoracic duct cells from CWB mice.

## RESULTS

### *Preliminary Experiment.*—

*Specificity of the Adoptive Secondary Response to Heterologous Erythrocytes.*—Lethally irradiated CWB mice were injected with either  $4 \times 10^8$  SRBC or HRBC together with a small number (4 million) of spleen cells from uninjected CWB mice or mice injected 12 wk previously with either  $4 \times 10^8$  SRBC or HRBC intravenously and  $10 \mu\text{g}$  of endotoxin intraperitoneally. PFC assays were performed on pooled spleen cells 7 days later and results are presented in Table II. Indirect PFC were developed with RAM $\gamma$ G. It is apparent that 4 million spleen cells from SRBC-immune mice gave rise to a large number of indirect anti SRBC PFC, but not anti HRBC PFC, in irradiated recipients injected with SRBC. Neither indirect anti SRBC nor anti HRBC PFC were apparent in the spleens of irradiated recipients injected with HRBC and spleen cells from SRBC-immune mice. Similarly, spleen cells from HRBC-immune mice transferred an impressive indirect PFC response to HRBC and not to SRBC. Thus, in all groups of irradiated recipients of cells from immunized donors, significant numbers of indirect PFC were obtained only when spleen cells were injected with the antigen against which the donors had been im-

munized and only when the recipient spleens were assayed against that particular antigen.

4 million spleen cells from uninjected CWB mice did not give rise to indirect PFC in recipient spleens. Some direct PFC were detected in spleens of recipients

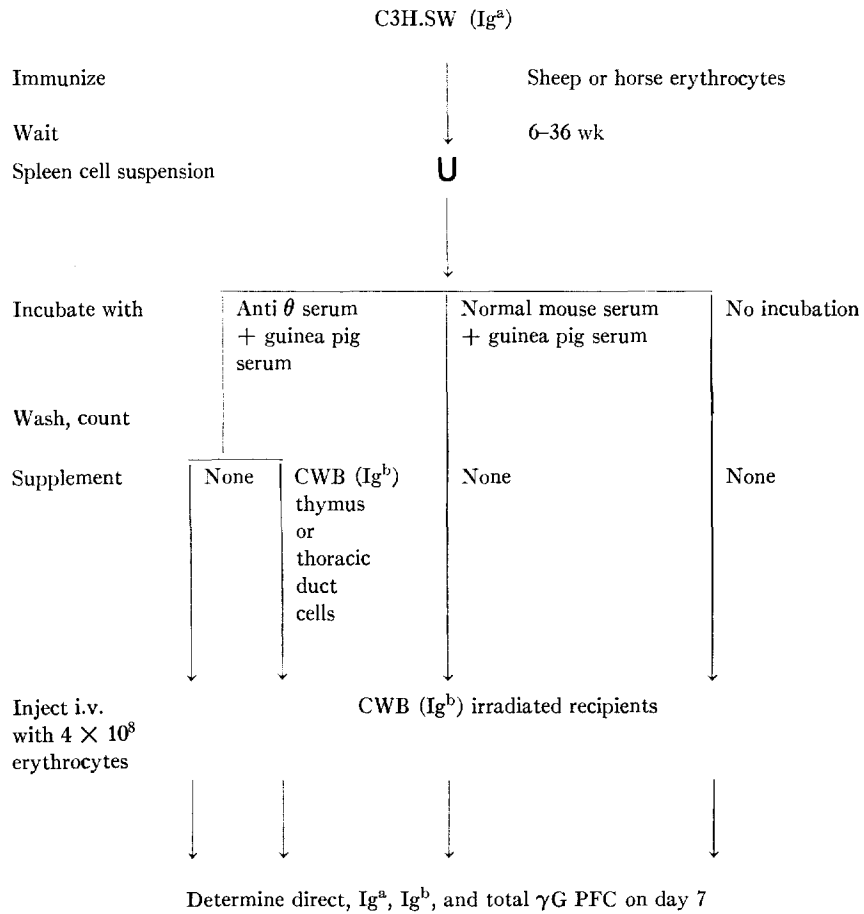


FIG. 1. Experimental plan to test for inhibition of adoptive secondary response with Anti  $\theta$  serum and restoration by thymus and thoracic duct cells.

of spleen cells and SRBC, but not in recipients of spleen cells and HRBC unless the donors had been immunized against HRBC. These direct PFC responses are low and will not be discussed further in this consideration of the adoptive secondary, indirect PFC response. In two other experiments (results not shown) 3-5 million spleen cells from uninjected C3H.SW mice also resulted in direct PFC (<400/spleen) but not indirect PFC to SRBC. Thus, the

impressive 7-day indirect PFC response, after transfer of a limited number of spleen cells from primed donors, is indeed a specific, adoptive secondary response.

*Preliminary Experiment.*—

*Dose Effect of Spleen Cells in the Transfer System.*—Spleen cells from C3H.SW mice injected 28 wk previously with  $4 \times 10^8$  SRBC and 20  $\mu$ g of endotoxin

TABLE II  
*Specificity of Adoptive Secondary Response to Heterologous Erythrocytes*

Spleen cell donor Primary erythrocyte challenge*	Spleen cell recipients†		PFC ( $\times 10^{-2}$ ) per spleen at 7 days‡			
	Secondary erythrocyte challenge	No. of recipients	Anti SRBC		Anti HRBC	
			Direct	Indirect	Direct	Indirect
Sheep (SRBC)	Sheep	4	4.4	30	<1¶	<1
	Horse	4	<1	<1	<1	<1
Horse (HRBC)	Horse	4	<1	1.9**	2.6	87
	Sheep	4	2.8	<1	<1	<1
None	Sheep	4	<1	<1	<1	<1
	Horse	4	<1	<1	<1	<1

\* Primed donor (CWB) mice were injected 12 wk before transfer with ( $4 \times 10^8$ ) sheep or horse erythrocytes and 10  $\mu$ g of endotoxin. Spleens from three mice were pooled for each transfer.

†  $4 \times 10^6$  nucleated spleen cells were injected i.v. together with  $4 \times 10^8$  erythrocytes from sheep or horse.

‡ PFC: Direct plaques require no developing antisera. The rabbit anti-mouse  $\gamma$ G antiserum used to develop indirect plaques is described in Materials and Methods Section.

|| Spleens of recipients were pooled before plaque assay.

¶ No PFC on slides when 1% of nucleated spleen cells in the suspension were plated, i.e., <100 PFC/spleen. In these experiments, pools of three to five spleens from irradiated mice injected with SRBC or HRBC alone contained an average of <10 direct and indirect PFC per spleen.

\*\* These plaques were relatively large areas of partial hemolysis.

intraperitoneally were incubated with Anti  $\theta$  or NMS together with absorbed GPS as a source of complement. The incubated cells were washed, counted, and 1–16 million nucleated cells injected into groups of three lethally irradiated CWB mice together with  $4 \times 10^8$  SRBC intravenously. The results of PFC assays performed 7 days later are shown in Fig. 2. Treatment with Anti  $\theta$  + GPS (cf. treatment with NMS + GPS) reduced direct (not shown) and indirect PFC responses to a constant dose of SRBC regardless of the number of treated spleen cells injected. The number of nucleated cells per spleen was decreased in recipients of low numbers of spleen cells treated with Anti  $\theta$  +

GPS (Fig. 2). This effect was also noticed in several subsequent experiments in which cell counts were performed at the time of PFC assay. The number of cells in spleens of recipients of 3 million spleen cells treated with NMS + GPS was  $26.3 \pm 1.7$  million and  $15.7 \pm 1.7$  million (six experiments) in the case of recipients of cells treated with Anti  $\theta$  + GPS ( $P < 0.005$ ).

In another experiment, spleen cells from two normal C3H.SW mice were

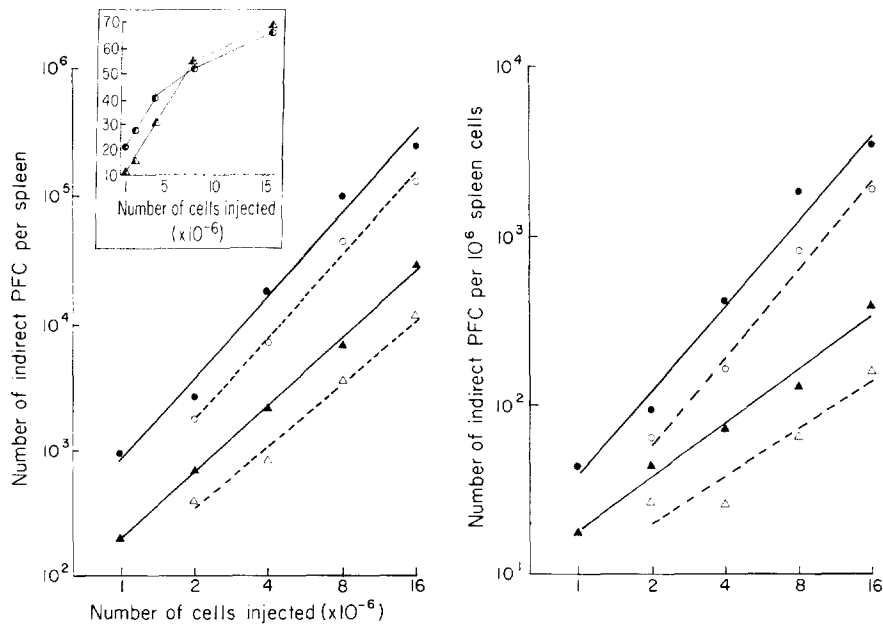


FIG. 2. Cell dose dependence of 7-day adoptive secondary PFC response. Spleen cells from SRBC-primed C3H.SW mice were treated with NMS + GPS (*circles*) or Anti  $\theta$  + GPS (*triangles*) and injected into irradiated CWB recipients. The points are mean responses of groups of three mice; *solid symbols* represent total indirect SRBC PFC developed with RAM $\gamma$ G; *open symbols* represent indirect SRBC PFC developed with anti a (cell donor) allotype sera. *Inset*: the mean number of nucleated cells per spleen ( $\times 10^{-6}$ ) at 7 days after transfer of treated spleen cells.

pooled, treated with NMS + GPS or Anti  $\theta$  + GPS, and 1 million washed cells injected into irradiated CWB recipients. Hemopoietic colonies in recipient spleens were counted 8 days later after fixation of the spleens in Bouin's solution (28). The number of splenic colonies in recipients of spleen cells treated with NMS + GPS was  $5.3 \pm 1.0$  (four mice); the number in recipients of cells treated with Anti  $\theta$  + GPS was  $9.2 \pm 1.7$  (five mice). This difference is not significant. The spleens of three irradiated control mice, not injected with spleen cells, contained a single colony. Hence, splenic hemopoietic stem cells of C3H.SW mice like those of C57BL mice (20) are not detectably sensitive to



treatment with Anti  $\theta$  + GPS. In all experiments to be described, doses of treated spleen cells ranging in number from 1–5 million were used in order to conserve anti- $\theta$  serum.

*Inhibition of the Adoptive Secondary Response to SRBC Using Anti  $\theta$  + GPS.*—C3H.SW mice, injected 9–36 wk previously with SRBC and endotoxin, were killed, their spleens removed, and the cells incubated in vitro with NMS + GPS or Anti  $\theta$  + GPS. After washing and counting, 1–5 million nucleated cells were injected intravenously into lethally irradiated CWB mice together with  $4 \times 10^8$  SRBC. The numbers of direct and indirect PFC in the spleens of these recipients, at 7 days after irradiation, are shown in Table III. In all six experiments, treatment of cells with Anti  $\theta$  + GPS markedly reduced 7-day indirect PFC responses when compared with responses in recipients of spleen cells treated in vitro with NMS + GPS or left untreated. The magnitude of the reduction in indirect PFC number varied from 1–3  $\log_{10}$  units and, in the one case in which statistical analysis could be applied, the differences in mean numbers of PFC developed with both anti a allotype and RAM $\gamma$ G were significant ( $P < 0.01$ ). The irradiated CWB hosts did not contribute PFC precursors (b allotype) and b-allotype PFC were detected only in the one experiment in which spleen cells from SRBC-immune CWB mice were transferred. Again, Anti  $\theta$  + GPS treatment reduced the adoptive secondary response by a factor of more than 1  $\log_{10}$ . In these experiments, as well as others (12, 22), RAM $\gamma$ G was more effective at developing PFC than anti-allotype sera. This probably reflects the contribution of a higher titer, or wider spectrum, of rabbit antibodies directed against immunoglobulin products of PFC. Emphasis in the analyses of the results of these and subsequent experiments will be on allotype PFC since these identify PFC as being of either C3H.SW or CWB type (see Discussion).

*Reconstitution of the Impaired Adoptive Secondary Response Using Thymus Cells.*—A reduction in the response of antiserum-treated spleen cells does not discriminate between an effect of the treatment on T cells or an effect on B cells. To determine whether PFC precursors (B cells) were affected by Anti  $\theta$  + GPS treatment, attempts were made to reconstitute the adoptive response of spleen cells from mice of one immunoglobulin allotype using thymus cells from normal donors of the other allotype. The results of four experiments are shown in Table IV: two using spleen cells from SRBC-immune C3H.SW and thymus cells from normal CWB, one using spleen cells from SRBC-immune CWB and thymus cells from normal C3H.SW, and another using spleen cells and thymus cells from (C57BL/10 [BCT]  $\times$  BALB/c) $F_1$  mice. In the three experiments involving congenic mice, (in which antisera against allotypic immunoglobulin markers could be used) it is clear that the reduced indirect PFC response of Anti  $\theta$ -treated spleen cells can be restored to control levels by thymus cells which do not themselves develop into detectable indirect PFC. In the cases in

which PFC determinations were performed on individual spleens, significant differences were observed between the mean numbers of indirect PFC in recipients of spleen cells after Anti  $\theta$  + GPS treatment and either NMS + GPS

TABLE III  
*Inhibition of Adoptive Secondary SRBC Response in Mice Receiving Primed Spleen Cells Depleted of  $\theta$ -Positive Cells*

Spleen cell donors (SRBC primed)*	After primary injection of SRBC	In vitro treatment†	Spleen cells injected ( $\times 10^{-6}$ )	No. of irradiated CWB (Ig <sup>b</sup> ) recipients§	Anti sheep PFC ( $\times 10^{-2}$ ) per spleen at 7 days			
					Direct	Allotype Ig <sup>a</sup>	Allotype Ig <sup>b</sup>	Total $\gamma$ G
C3H.SW (Ig <sup>a</sup> )	9	None	3.0	4	<1¶	12	<1	24
		NMS	"	4	3.5	41	<1	51
		Anti $\theta$	"	4	<1	<1	<1	1.2
C3H.SW (Ig <sup>a</sup> )	17	None	1.5	5	<1	48	<1	99
		NMS	"	4	9.6	70	<1	120
		Anti $\theta$	"	5	<1	<1	<1	2 (0.6-8.4)**
C3H.SW (Ig <sup>a</sup> )	18	None	1.0	3	4.2	24	<1	25
		NMS	"	3	5	51	<1	58
		Anti $\theta$	"	4	<1	<1	<1	<1
C3H.SW (Ig <sup>a</sup> )	19	None	2.0	4	1.8	62	<1	103
		NMS	"	5	7.7	102	<1	233
		Anti $\theta$	"	5	<1	6 (3.7-9.8)	<1	9 (6.8-12)
CWB (Ig <sup>b</sup> )	32	NMS	3.0	4	37	<1	61	273
		Anti $\theta$	"	4	1.3	<1	2	5
C3H.SW (Ig <sup>a</sup> )	36	None	5.0	4	10 (7.7-14)	285 (201-403)	<1	371 (318-432)
		NMS	"	3	16 (12-20)	136 (94-198)	<1	207 (158-271)
		Anti $\theta$	"	5	<1	18 (12-26)	<1	24 (15-38)

\* Donor mice received  $4-8 \times 10^8$  SRBC i.v. and/or i.p. and 20  $\mu$ g of endotoxin i.p. Pools of spleen cells from two or three mice were used for transfer in each experiment.

† When cells were incubated with NMS (normal AKR/J serum) or Anti  $\theta$  (AKR/J anti C3H/HeJ thymus cells), guinea pig serum (complement source) was always present.

§ Spleens of recipients were pooled before plaque assay except where standard error limits appear in parenthesis next to the number of PFC per spleen.

|| PFC: Direct plaques require no developing antisera. The anti-allotype and rabbit anti-mouse  $\gamma$ G antisera used to develop indirect plaques are described in Materials and Methods Section.

¶ No PFC on slides when 1% of nucleated spleen cells in the suspension were plated, i.e., <100 PFC/spleen. In these experiments, pools of three to five spleens from irradiated mice injected with SRBC alone contained an average of <100 direct and indirect PFC per spleen.

\*\* Geometric mean ( $\pm$  standard error). Where standard error appears, spleens of animals in the group were tested individually.

treatment or Anti  $\theta$  + GPS treatment but with a supplement of thymus cells ( $P < 0.025$ ). In the experiment involving F<sub>1</sub> mice, the donors were immunized with low dose SRBC in the absence of endotoxin and the spleen cells used 4 wk later. Although allotypic markers could not be used to trace presumed PFC

derivation, the results of PFC determinations parallel results in the experiments using congenic strains of mice and a priming regime of high-dose SRBC plus endotoxin. In one experiment (results not shown) the response of CWB spleen cells treated with NMS + GPS was not increased by a supplement of

TABLE IV  
Restoration, by Thymus Cells, of the Adoptive Secondary SRBC Response of Primed Spleen Cells Depleted of  $\theta$ -Positive Cells

Spleen cell donors (SRBC primed)*	After primary injection	In vitro treatment†	Spleen cells injected ( $\times 10^{-6}$ )	No. of irradiated CWB ( $Ig^b$ ) recipients‡	Thymus cell supplement		Anti sheep PFC ( $\times 10^{-2}$ ) per spleen at 7 days			
					No. ( $\times 10^{-6}$ )	Donor (allotype)	Direct	Allotype $Ig^a$	Allotype $Ig^b$	Total $\gamma G$
A C3H.SW ( $Ig^a$ )	15	NMS Anti $\theta$ " "	1.0	4	None	CWB " ( $Ig^b$ )	2.5 (2-4)**	50 (40-63)	<1¶	59 (37-93)
			"	5	"		<1	2.5 (2-3)	<1	2.6 (2-4)
			"	5	100		3.2 (2-4)	56 (47-66)	<1	108 (88-133)
			None	4	100		<1	<1	<1	<1
B C3H.SW ( $Ig^a$ )	21	NMS Anti $\theta$ " "	3.0	3	None	CWB " ( $Ig^b$ )	1.5§	18	<1	30
			"	3	"		<1	1.3	<1	2.5
			"	3	20		2	8.5	<1	16
			None	3	20		<1	<1	<1	<1
C CWB ( $Ig^b$ )	21	NMS Anti $\theta$ " "	3.0	4	None	C3H.SW " ( $Ig^a$ )	5.6	<1	7.1	48
			"	4	"		<1	<1	<1	3.7
			"	4	30		1	<1	3.8	55
			None	3	30		<1	<1	<1	<1
D (BCT $\times$ BALB/c)F <sub>1</sub> ( $Ig^b/Ig^a$ )	4	NMS Anti $\theta$ " "	5.0	4††	None	" (F <sub>1</sub> )	15 (13-18)	—	—	98 (70-129)
			"	6	"		<1	—	—	<1
			"	5	70		3 (7-12)	—	—	82 (57-119)
			None	3	70		<1	—	—	<1

Same as Table III except:

\* Primary injection, group A =  $8 \times 10^8$  SRBC i.v. and i.p. and 20  $\mu g$  of endotoxin i.p.

B =  $4 \times 10^8$  SRBC i.p. and 10  $\mu g$  of endotoxin i.p.

C =  $4 \times 10^7$  SRBC i.p. and 20  $\mu g$  of endotoxin i.p.

D =  $4 \times 10^8$  SRBC i.v. and i.p.

†† BCT  $\times$  BALB/c F<sub>1</sub> ( $Ig^b/Ig^a$ )-irradiated recipients (syngeneic to thymus cell donors).

$70 \times 10^6$  C3H.SW thymus cells. The results of these experiments suggest that the precursors of PFC (B cells) carry immunological memory but that their expression as indirect PFC requires the presence of a cell population sensitive to Anti  $\theta$  + GPS and which can be replaced functionally by T cells.

*Reconstitution of the Impaired Adoptive Secondary Response Using Thoracic Duct Cells.*—Moderately large doses of thymus cells from normal mice restored the secondary responsiveness of treated cells from immunized mice (*vide*

*supra*). It was of interest to determine whether a smaller number of T cells from primed mice could restore PFC production by spleen cells treated with

TABLE V  
*Specificity of Restoration by Thoracic Duct Cells of Adoptive Secondary Response to Horse Erythrocytes*

Spleen cell donors (HRBC primed)*	After primary injection of HRBC	In vitro treatment†	Spleen cells injected ( $\times 10^{-6}$ )	No. of irradiated CWB (Ig <sup>b</sup> ) recipients‡	Thoracic duct cell supplement (CWB donors)‡‡		Anti-horse PFC ( $\times 10^{-2}$ ) per spleen at 6 days			
					No. ( $\times 10^{-6}$ )	Primed with	Direct	Allo-type Ig <sup>a</sup>	Allo-type Ig <sup>b</sup>	Total $\gamma$ G <sub>2</sub>
A C3H.SW (Ig <sup>a</sup> )	6	None	2.0	3	None		8.7	41	<1¶	55
		NMS	"	3	"		2	43	<1	56
		Anti $\theta$	"	3	"		<1	<1	<1	<1
B C3H.SW (Ig <sup>a</sup> )	7	NMS	3.0	3	None		4.7	219	<1	409
		Anti $\theta$	"	2	"		<1	4.3	<1	16
		"	"	3	2.0	HRBC	4.3	124	15	272
		"	"	2	"	S "	1	19	<1	53
		"	"	3	"	H "	<1	<1	7	28
C C3H.SW (Ig <sup>a</sup> )	9	—	—	3	"	S "	<1	<1	<1	3
		NMS	3.0	4	None		<1	81	<1	143
		Anti $\theta$	"	4	"		<1	<1	<1	<1
		"	"	4	2.0	HRBC	4.4	113	10	367
		"	"	4	"	S "	1.8	11	<1	21
D C3H.SW (Ig <sup>a</sup> )	11	—	—	4	"	H "	5.2	<1	7	33
		—	—	4	"	S "	<1	<1	<1	2
		NMS	3.0	4	None		<1	20	<1	31
		Anti $\theta$	"	4	"		1	1	<1	1.2
		"	"	4	2.5	HRBC	<1	92	8.3	136
"	"	4	0.5	H "	<1	10	<1	22		
"	"	4	2.5	S "	<1	6	<1	13		
"	"	4	7.5	S "	1.9	51	7.4	10		
—	—	4	2.5	H "	<1	<1	1.3	3		
—	—	4	2.5	S "	<1	<1	<1	<1		

Same as Table III except:

\* All animals received a primary injection of  $4 \times 10^8$  horse erythrocytes (HRBC) i.p.; groups A, B, and C received 20  $\mu$ g of endotoxin at the same time while group D received 10  $\mu$ g of endotoxin.

¶ In these experiments, pools of three or four spleens from irradiated mice injected with HRBC alone contained <20 direct PFC per spleen and no indirect PFC.

‡‡ Donors of thoracic duct cells were primed with either  $4 \times 10^8$  HRBC or SRBC and endotoxin at the same time as the spleen cell donors received HRBC.

Anti  $\theta$  + GPS. For these experiments, C3H.SW mice, injected 7, 9, or 11 wk previously with  $4 \times 10^8$  HRBC and 10 or 20  $\mu$ g of endotoxin intraperitoneally, were killed and pools of spleen cells incubated with Anti  $\theta$  + GPS or NMS + GPS. In three experiments (Table V) spleen cells treated with Anti  $\theta$  + GPS

were supplemented at the time of transfer with thoracic duct cells (TDC) from CWB mice injected 7, 9, or 11 wk previously with either  $4 \times 10^8$  SRBC or HRBC and 10 or 20  $\mu\text{g}$  of endotoxin intraperitoneally. In all experiments Anti  $\theta$  + GPS treatment reduced the adoptive indirect PFC response to very low levels. A supplement of TDC, whether from SRBC-immune or HRBC-immune mice increased the response of spleen cells treated with Anti  $\theta$  + GPS (as measured by the number of indirect PFC developed with anti a allotype sera). However, TDC from HRBC-immune mice resulted in approximately ten times the number of allotype-marked PFC as did TDC from SRBC-immune mice. Moreover, in one experiment, inocula of 0.5 and  $2.5 \times 10^6$  TDC from HRBC-immune mice were more effective than 2.5 and  $7.5 \times 10^6$  TDC from SRBC-immune mice, respectively. As might be anticipated, TDC from HRBC-immune mice gave rise to a few allotype-marked (b-type) PFC whether or not they were injected together with treated spleen cells. The experiments clearly indicate that thoracic duct cells, when assayed for their T-cell activity, carry specific immunological memory in that they are able to facilitate indirect PFC expression by B cells contained in a suspension of Anti  $\theta$ -treated spleen cells from primed mice.

In two experiments of the type described above (results not shown), groups of irradiated mice were injected with Anti  $\theta$ -treated spleen cells from immune mice of a allotype together with TDC from mice of the b allotype immunized with the other erythrocyte type. These recipient mice were injected with SRBC + HRBC. There was no significant increase in either a-allotype PFC or b-allotype PFC, when compared with the responses in mice injected with one erythrocyte type and a combination of spleen cells and TDC from mice immunized with different erythrocyte types. Thus, T cells from mice primed to one erythrocyte type when confronted with the antigen could not facilitate indirect PFC production by B cells from mice primed to the other erythrocyte type and confronted with that particular antigen.

*Susceptibility of a Supplement to Treatment with Anti  $\theta$  + GPS.*—The finding that a cell population with T cell activity is reconstitutive in this system indicates that cells sensitive to Anti  $\theta$  + GPS are replaced functionally by T cells. It becomes important, however, to determine whether or not treatment with Anti  $\theta$  + GPS will reduce the T cell activity of a cell supplement. We chose to use spleen cells as the treated supplement simply because our incubation procedure had been developed with spleen cells and not thymus or thoracic duct cells. Spleen cells from SRBC-immune C3H.SW mice were treated in vitro with Anti  $\theta$  + GPS and mixed with spleen cells from SRBC-immune CWB mice treated with either Anti  $\theta$  + GPS or NMS + GPS. In the control groups, treatment with Anti  $\theta$  + GPS reduced the adoptive response of 3 million spleen cells from either immune C3H.SW or immune CWB mice by 15- to 20-fold. In the experimental groups, CWB spleen cells treated with NMS + GPS were six times better than CWB spleen cells treated with Anti  $\theta$  +

GPS at restoring the a-type indirect PFC response of C3H.SW spleen cells treated with Anti  $\theta$  + GPS.

The above experiment indicates that cells sensitive to Anti  $\theta$  + GPS in a supplement of spleen cells are required for the full expression of T cell cooperating activity of that supplement. This is also the case in an in vitro system where treatment with Anti  $\theta$  + GPS markedly reduced the ability of in vivo educated thymus cells to reconstitute the response of normal spleen cells treated with Anti  $\theta$  + GPS (20).

#### DISCUSSION

Numerous studies have been addressed to the question of the cellular events of secondary antibody responses to erythrocyte antigens (e.g. 6, 22, 29-34). Speculations on the life history of the cell which carries immunological memory must now take into account the finding of interacting cell types in the fully reconstituted primary response to SRBC in thymectomized mice (1). Is specific immunological memory a property which can be linked to both antibody-forming cell precursors (B cells) and thymus-influenced cells (T cells)? Results of the present study indicate that this is so in the case of the adoptive secondary response to heterologous erythrocytes in mice.

The primary in vitro (20, 35) and secondary in vivo (36) and in vitro (20) responses to SRBC can be inhibited by treatment of spleen cells with anti- $\theta$  serum and guinea pig complement (Anti  $\theta$  + GPS). Reconstitution with thymus cells [cf. educated thymus cells (20)] has not been reported. Before any statement can be made on the cellular site of action of the serum treatment, it is imperative to achieve reconstitution. Interpretations are restricted if it can be shown that reconstitution is possible with a cell type presumed to be related to that in the spleen cell population which is sensitive to Anti  $\theta$  + GPS and that, in turn, this reconstitutive inoculum is itself sensitive to Anti  $\theta$  + GPS. The data in Table IV indicates that thymus cells from normal mice reconstitute the secondary responsiveness of spleen cells treated with Anti  $\theta$  + GPS. Hence, a primed B cell population (B' cells) must be present in spleen cell suspensions treated with Anti  $\theta$  + GPS but this population is unable to express itself, in terms of indirect (7S) PFC production, in the absence of another population sensitive to Anti  $\theta$  + GPS. The cells of the latter population presumably bear the  $\theta$  surface alloantigen, are functionally inhibited or killed by Anti  $\theta$  + GPS, and are functionally replaced by thymus cells. The adoptive secondary serum antibody response to polymerized flagellin from *Salmonella adelaide* is markedly impaired by treatment of spleen cells with Anti  $\theta$  + GPS.<sup>14</sup> In this system we have been unable to restore the response with large numbers of thymus cells, and therefore, it is not possible to say that Anti  $\theta$  + GPS has no detectable effect on B cells primed to flagellin.

<sup>14</sup> Mitchell, G. F., E. L. Chan, and A. A. Amkraut. Unpublished data.

Thoracic duct cells from mice primed to HRBC were much better than an equivalent number of thoracic duct cells from mice primed to SRBC in reconstituting the B cell response of treated spleen cells from HRBC-immune mice (Table V). Thus, the T cell population, like the B cell population, must be altered by prior contact with antigen and carry specific immunological memory (T' cells). Jacobson et al. (12) demonstrated that, in a mixture of spleen cells from primed and unprimed mice, the indirect PFC were always of primed donor allotype. From this data and that in Table V, it is clear that primed T cell activity in the present system is not expressed in terms of 7S B cell memory [cf. 19S B cell memory (6)] unless the latter cell population is also primed.

With respect to the "factor of immunization" in each cell population, it is clear that moderately large numbers of thymus cells from unprimed mice and small numbers of thoracic duct cells from nonspecifically primed mice are reconstitutive in the transfer system. By contrast, spleen cells, when transferred in small numbers must be obtained from specifically primed mice in order for any indirect PFC to be detected in irradiated recipients at day 7 after transfer. Hence, it may be that the "potency" of T cells is increased after priming by a factor of approximately 5- to 10-fold (Table V), whereas in B cells this factor may be much greater. However, a meaningful factor of immunization figure is impossible to calculate because of multiple uncontrolled variables such as time of PFC assay and doses of antigen and endotoxin used. Moreover, it is not possible to control for complexities such as a disproportionate T cell priming vs. B cell priming by contact with environmental antigens before introducing the heterologous erythrocytes.

Anti  $\theta$  + GPS does not affect the number of C3H.SW splenic hemopoietic stem cells, but with small numbers of transferred cells the sizes of recipient spleens are significantly reduced. This difference in spleen cell number (twice at most) does not account for the difference in PFC number in the spleens of recipients of cells treated with NMS + GPS and Anti  $\theta$  + GPS (ten times at least). The differential effect of treatment with Anti  $\theta$  + GPS on splenic regeneration compared with the number of hemopoietic colony-forming units may be related to the observation that injected thymus cells facilitate erythropoietic regeneration in irradiated mice (37). Alternatively, or in addition,  $\theta$ -bearing cells may make up a large proportion of the cells in regenerating spleens of recipients of cells treated with NMS + GPS. The increased spleen size in recipients of NMS + GPS treated cells (cf. Anti  $\theta$  + GPS treated cells) suggests that an interaction between T cells, B cells, and antigen enhances cell proliferation in general.

No evidence could be obtained in the present study for a nonspecific influence of primed T cells, injected with the appropriate antigen, on the antibody-producing capacity of primed B cells. For example, thoracic duct cells from HRBC-immune CWB mice when injected with SRBC and HRBC, did not

result in increased numbers of anti SRBC (a allotype) PFC produced by Anti  $\theta$  + GPS-treated spleen cells from SRBC-immune C3H.SW mice. Results obtained in an in vitro system [cf. an in vivo system (11)], similar in design to that described above, have been interpreted as evidence for a nonspecific factor in interacting cell systems (38).

Many of the above conclusions and considerations are dependent on several assumptions: (a) The assumption that cells of a particular Fc immunoglobulin allotype will continue to express that allotype in irradiated recipients and not transfer allotype information to another, genetically dissimilar, cell type (39). (b) The assumptions that thymus-influenced cells (T cells) are susceptible to treatment with Anti  $\theta$  + GPS, and that the relevant antibodies in AKR anti C3H thymus cell sera are the anti  $\theta$ C3H antibodies (19; Table I). Our AKR/J anti C3H/HeJ thymus cell antiserum may contain some antibodies against immunoglobulins of the a type as determined by facilitation and inhibition of passive hemagglutination (40). No antiallotype antibody is detectable using  $^{125}\text{I}$ -labeled  $\gamma\text{G}_{2a}$  and  $\gamma\text{G}_1$  proteins of the a or b type.<sup>15</sup> Certainly, in the present system the activity, if any, of anti a allotype antibody is unnoticed since full reconstitution of a B cell-mediated response of C3H.SW is achieved with T cells. Moreover, the results are similar whether cells from C3H.SW or CWB are treated with Anti  $\theta$  + GPS. These or other antibodies, however, even if present in low amounts, may be of importance in fluorescent antibody sandwich techniques.<sup>16</sup>

If, in the future, assumption (a) is proven to be incorrect then the interpretations of the present study, like many others, must be altered profoundly. In the absence of evidence to the contrary we have assumed that the genes coding for the Fc immunoglobulin allotype markers (and therefore the genes coding for the H chain variable region if they are closely linked) are not transferred between cell types in an artificial mixture of cells. On this point, preliminary results of Hattis and Wegmann using allophenic mice indicate that spleen cells of a particular *H-2* type in the chimaera will produce the predicted allotype-marked anti SRBC immunoglobulin.

There is only indirect evidence available on the question of whether  $\theta$ -bearing peripheral lymphocytes are thymus-derived cells, or at least thymus-influenced cells. Athymic and surgically thymectomized mice contain relatively few  $\theta$ -bearing cells (41) or cells sensitive to Anti  $\theta$  + GPS (18, 19). This could be a primary consequence of removal of the source of the influence required for the generation of  $\theta$ -bearing cells or a secondary consequence dependent upon some other factor. The results of the present study indicate that spleen cells sensitive to Anti  $\theta$  + GPS can be replaced by thymus cells, a cell population known to be rich in  $\theta$ -bearing cells (16, 17).

<sup>15</sup> Ravitch, M., and G. F. Mitchell. Unpublished data.

<sup>16</sup> Masuda, T., and G. F. Mitchell. Unpublished data.



The present data does not provide obvious clues on the role of T cells in primary and secondary hemolytic antibody responses. Results of Miller et al. (42) and Taylor and Wortis (43) clearly indicate that indirect PFC production to SRBC is more thymus dependent than direct PFC production. This difference in thymus dependence vs. quality of antibody elicited is very obvious in the genetically controlled, *H-2*-linked antibody response to the synthetic polypeptide antigen (T,G)-A-L. Thymectomized (Tx) or sham thymectomized (STx) C3H.SW ("responders") and C3H ("nonresponders") produce similar titers of 19S IgM antibody in the 1st wk after injection of aqueous (T,G)-A-L. After booster immunizations, STx C3H.SW, unlike their Tx counterparts and either Tx or STx C3H, contain large amounts of 7S anti (T,G)-A-L antibody.<sup>17</sup> The possibility exists that an antigen-reactive B cell population in the absence of antigen-reactive T cells responds abortively by producing antibody to certain antigens. An abortive response may include a failure of recruitment of B cells capable of responding by secreting IgG antibodies (B<sup>7</sup> cells) or a failure of maturation of B<sup>7</sup>-cells from precursors synthesizing IgM antibodies (B<sup>u</sup> cells). Confining speculation on the role of T cells to two possibilities, it is conceivable that at least some types of B cell require either (a) antigen presentation in order to prevent tolerance induction or promote immune induction or (b) a secreted or surface substance of T cells to respond maximally by producing antibody. The data in this study show that in the absence of T cells even primed B cells respond feebly by producing IgG antibodies to foreign erythrocyte antigens.

#### SUMMARY

Using anti-allotype sera and AKR anti  $\theta$ C3H sera, a requirement for two cell types has been demonstrated in the adoptive secondary response of mice to heterologous erythrocytes. The cell types have been designated B cells [precursors of plaque-forming cells (PFC)] and T cells (thymus-influenced cells, not providing precursors of detectable PFC). The *in vivo* indirect PFC response of spleen cells from primed mice is markedly reduced by *in vitro* treatment of the cells with a mixture of anti- $\theta$  serum and guinea pig serum (Anti  $\theta$  + GPS). This B cell response is fully restored to control levels by thymus cells from normal mice which do not themselves provide precursors of indirect PFC. Thus memory is carried by the B cell lineage but the expression of this memory is dependent on the presence of a cell population which is sensitive to Anti  $\theta$  + GPS and which is replaced functionally by unprimed T cells. When assayed for T cell activity, thoracic duct cells from specifically primed mice are better than cells from nonspecifically primed mice in restoring the B cell

<sup>17</sup> Mitchell, G. F., F. C. Grumett, and H. O. McDevitt. 1971. Influence of thymectomy on the primary immune response to the synthetic polypeptide (T, G)-A-L. Manuscript in preparation.

response of spleen cells from immunized mice. Moreover, the T cell activity of a reconstitutive cell population from primed mice is reduced by incubation with Anti  $\theta$  + GPS. We conclude that memory to heterologous erythrocyte antigens is carried by the T cell lineage as well as the B cell lineage even though unprimed T cells are sufficient for expression of B cell memory.

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