

A RECEPTOR FOR ANTIBODY ON B LYMPHOCYTES

I. METHOD OF DETECTION AND FUNCTIONAL SIGNIFICANCE* †

BY A. BASTEN,§ J. F. A. P. MILLER, J. SPRENT, AND J. PYE

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

(Received for publication 1 November 1971)

Antibody-antigen complexes bind to the surface of some mouse lymphocytes (1-2). Recent evidence has suggested that this property may be characteristic of some nonthymus-derived "B" lymphocytes¹ (3), not of thymus-derived "T" lymphocytes (4). A similar conclusion has been reached independently in our laboratory from studies originally designed to examine the relationship between antigen-binding cells and "memory" cells in primed mice. In these experiments, use was made of the sensitive technique of radioautography to establish the presence of a receptor for antibody on lymphocytes. Thoracic duct lymphocytes (TDL) were selected as the main source of lymphoid cells because of their high viability and low contamination with other cell types.

The identity of lymphocytes binding antibody was established by examination of cell populations either enriched for T cells or B cells or containing the two cell types in known numbers. The T cell content was assessed by means of independent genetic markers such as θ C3H antigen or H-2 antigen in the case of chimeric mice. When this figure was compared with the proportion of antibody-binding lymphocytes, an inverse relationship was observed. An exact correlation was therefore demonstrable between the number of lymphocytes binding antibody and the number of B cells in the population. In other words, the receptor is carried by all B cells, not by just a subclass of B cells. The identification of a marker of this kind on all B cells has permitted the develop-

* This is publication No. 1619 from the Walter and Eliza Hall Institute of Medical Research.

† Supported by the National Health and Medical Research Council of Australia, the Australian Research Grants Committee, the Damon Runyon Memorial Fund for Cancer Research, the British Heart Foundation, and the National Heart Foundation of Australia.

§ Queen Elizabeth II Research Fellow. Present address: Department of Medicine, University of Sydney, Sydney, New South Wales, Australia.

¹ *Abbreviations used in this paper:* B, nonthymus-derived; CRL, complement receptor lymphocytes; FCS, fetal calf serum; F γ G, fowl immunoglobulin G; HGG, human gamma globulin; MBLA, mouse bone marrow-derived lymphocyte antigen; NCS, normal chicken serum; NMS, normal mouse serum; NTx, neonatally thymectomized; POL, polymerized flagellin; SRBC, sheep red blood cells; T, thymus-derived; TDL, thoracic duct lymphocytes; TxBM, adult thymectomized, irradiated, and marrow protected.

ment of a technique for separating T cells from B cells on antigen-coated columns (5).

The mechanism of attachment of antibody to receptor will be discussed in the subsequent paper (6). Although the binding process required an intact Fc piece, it was apparently complement independent. The conclusion is reached that the antibody-binding receptor and antigen-binding (immunoglobulin) receptor on B lymphocytes are distinct entities.

Materials and Methods

Animals.—Male and female mice of the highly inbred CBA strain and the (CBA × C57BL) F₁ hybrid were used in the majority of the experiments. Their origin and maintenance have been described previously (7). Athymic mice (nude; nu/nu) were obtained originally from the McMaster Laboratory, Commonwealth Scientific and Industrial Research Organization, Sydney, and maintained subsequently at the Hall Institute by Dr. M. Holmes.

Operative Procedures.—Thoracic duct cannulation was carried out by the method of Miller and Mitchell (7). Lymph was collected from each mouse at 4°C into 2 ml of Dulbecco's phosphate-buffered saline (8) containing 10% fetal calf serum (FCS) (Commonwealth Serum Laboratories, Melbourne, Australia) with 50–100 IU of preservative-free heparin (Evans Medical Ltd., Liverpool, England). Thymectomy was performed in young adult CBA mice, 4–7 wk old, as described by Miller (9). The mediastinum was examined at autopsy and any mice with thymus remnants discarded.

Preparation of Cell Suspensions.—Single cell suspensions of thymus, spleen, lymph nodes, and Peyer's patches were obtained by teasing the tissues through an 80 mesh stainless steel sieve in cold Eisen's solution (10). The cells were washed twice in the case of spleen, lymph nodes, and Peyer's patches and three times for thymus. Bone marrow cells were expressed from the femurs of mice by a syringe and attached needle containing cold Eisen's solution and washed once before counting.

TDL were collected as described previously. To wash them before incubation with iodinated antigen, suspensions were centrifuged through FCS gradients as described by Byrt and Ada (11). Lymphocytes were obtained from peripheral blood by sedimentation of heparinized samples on a methylcellulose-Urographin gradient as described by Hulliger and Blazhovec (12). They were washed three times before use.

Irradiation.—Intact or thymectomized mice were exposed to total body irradiation in a Philips (RT 250) X-ray machine (Philips Electronic Instruments, Mount Vernon, N.Y.). The dose used was 750–800 rads to midpoint with maximum back scatter at 15 ma and a half-value layer of 0.8 mm copper. Cell suspensions were injected within 3 hr of irradiation. In the case of thymectomized mice, irradiation was performed 2–4 wk postoperatively and reconstitution was achieved by injecting 3–5 × 10⁶ syngeneic bone marrow cells intravenously. After irradiation all mice were given polymyxin B (100,000 IU/liter) and neomycin (10 mg/liter) in their drinking water.

Antigens.—Fowl immunoglobulin G (F γ G) was obtained from chicken serum as described previously (13). Before iodination with ¹²⁵I (Radiochemical Centre, Amersham, England, catalogue No. IMS 3), it was absorbed extensively against mouse lymphocytes to remove non-specific anti-mouse activity. Human gamma globulin (HGG) was provided by the Commonwealth Serum Laboratories as a solution containing 160 mg protein/ml. Polymerized flagellin (POL) was prepared from the flagella of *Salmonella adelaide* by the method of Ada et al. (14).

Preparation of Iodinated Antigens.—The antigens, F γ G, HGG, and POL were iodinated with ¹²⁵I, according to the method of Byrt and Ada (11). Specific activities of approximately 50 and 150 μ Ci/ μ g F γ G and 50 μ Ci/ μ g HGG were used. Both levels of radioactivity gave the

same number of labeled cells if exposure times were adjusted appropriately. In the case of POL the specific activity was 30–40 $\mu\text{Ci}/\mu\text{g}$. F γG - ^{127}I was prepared in a similar way except that a trace amount of F γG - ^{131}I was used to monitor the iodination. For labeling of cells, the radioactive antigens were diluted in Dulbecco's solution (8) containing 10% FCS and 1.5×10^{-2} M sodium azide to a final concentration of 2 $\mu\text{g}/\text{ml}$. Azide at this concentration did not interfere with the viability of lymphocytes, but did reduce nonspecific uptake of labeled antigen by phagocytic cells (11).

Antisera.—Antisera to F γG were obtained by immunizing mice with 500 μg alum-precipitated antigen (13) together with 2×10^9 killed pertussis organisms (Commonwealth Serum Laboratories). 4–6 wk later, the mice were boosted with various amounts of fluid F γG ranging from 1 to 100 μg and bled at intervals to provide pools of antisera of various titers. Each batch was heat inactivated at 56°C for $\frac{1}{2}$ hr. Antibody levels were measured by a hemagglutination technique using sheep red blood cells (SRBC) coated with fowl anti-SRBC IgG (13). The method relies on the fact that chicken immunoglobulin does not fix mammalian complement (15).

In one set of experiments antisera to POL and HGG were required. Anti-HGG antibody was prepared as described for anti-F γG sera. Anti-flagellar sera were raised in rabbits and chickens as well as in mice. The former two were immunized with 500 μg POL in complete Freund's adjuvant (Difco Laboratories Inc., Detroit, Mich.) followed 3 wk later by a boost with 500 μg antigen in saline. Blood was then collected between days 7 and 10 and the sera inactivated as mentioned previously. Mice received 20 μg of POL in saline intraperitoneally on two occasions 4–6 wk apart and were then bled approximately 7 days after the second injection. Anti-HGG activity was assayed by microprecipitation (16) and anti-POL titers by the immobilization technique (14). When the effect of antisera from different sources was being compared, the dilutions were adjusted so that cells were exposed to comparable amounts of antibody activity.

Incubation of Cells with Radioactively Labeled Antigen.—Suspensions of lymphocytes prepared as described previously were divided into aliquots of 10^7 cells and centrifuged. To each cell button 400 ng of ^{125}I -labeled antigen in 0.2 ml of Dulbecco's solution containing 10% FCS and sodium azide was added. After resuspension the mixture was incubated in an ice bath for 30 min. Excess labeled antigen was then removed by centrifugation of the cells through 2–4 FCS gradients. Smears for radioautography were made in 100% FCS on gelatin-coated slides. They were dipped in Kodak NTB2 emulsion, developed using D19B, and stained with Giemsa. Clumped or damaged cells were ignored. Only cells carrying 10 or more grains were scored.

Incubation of Cells with Antibody.—Pretreatment of cell suspensions with antibody before exposure to radioactive antigen was performed by incubating for 30 min at 37°C in the appropriate dilution of antiserum. The suspension was then centrifuged, the supernatant removed, and the cell button either resuspended immediately in the antigen solution or washed through FCS gradients as described previously.

Preparation of Isoantisera.—Anti-H-2 isoantisera were prepared by repeatedly injecting CBA and C57BL mice with lymphocytes from C57BL and CBA donors, respectively, according to the schedule reported previously (7). Anti- θ C3H serum was raised in AKR mice by the method of Reif and Allen (17). After separation from the clot, serum samples were inactivated at 56°C for 30 min and stored in small aliquots at -20°C until required. The potency of both types of isoantisera was tested by cytotoxicity against TDL from the appropriate strain, as described by Miller and Sprent (18). Guinea pig serum was used as a source of complement. Control sera were obtained from normal CBA, C57BL, and AKR mice of comparable age and processed in identical fashion.

RESULTS

Binding of Antibody to Normal Lymphocytes In Vitro and In Vivo.—TDL from normal mice were exposed to either normal mouse serum (NMS) or

hyperimmune anti-F γ G serum (titer 1 in 512) diluted 1 in 10 followed by the appropriate antigen, F γ G-¹²⁵I (Table I). As expected, only occasional cells incubated in NMS (approximately 1 in 10⁴) bound antigen. In contrast, 12–20% TDL labeled with the immune complex provided they were not washed after incubation with antibody. Labeling was inhibited by preincubation of antibody-coated cells with excess F γ G-¹²⁷I. When the assay was performed in the presence of 0.01 M ethylenediaminetetraacetate (EDTA), which inhibits binding of complexes to phagocytic cells (2), no diminution in labeling was observed. Repeated washing of antibody-treated cells before exposure to radioactive antigen greatly reduced the number of labeled cells (to less than 1%) (Table II). No decrease, however, was observed when the TDL were washed two to four times after reaction with F γ G-¹²⁵I. These findings imply that the bond between

TABLE I
Proportion of Normal Thoracic Duct Lymphocytes Binding Antibody Given In Vitro

Pretreatment of TDL	Per cent labeled with F γ G- ¹²⁵ I
NMS	<1
Anti-F γ G serum	17 (12–20)*
Anti-F γ G serum + excess F γ G- ¹²⁷ I	<1
Anti-F γ G serum + 0.01 M EDTA	16 (15–18)*

* Results are expressed as arithmetic means of figures obtained from three to six experiments; range of values given in parenthesis.

cell and antibody is weak but that the formation of the antibody-antigen complex on the cell surface stabilizes the bond at least at 4°C.

When combinations other than anti-F γ G–F γ G were tested, e.g. anti-HGG–HGG or anti-POL–POL, similar results were obtained although insignificant binding occurred if an antigen not corresponding to the antibody was used (Table III). It can also be seen that antibodies to POL raised in rabbits and chickens bound to mouse lymphocytes as well as mouse antibody, provided the cells were incubated in antisera of similar potency. The ability of chicken anti-POL to adhere is particularly interesting since chicken immunoglobulin cannot fix mammalian complement (15). This point will be referred to again during the discussion on complement requirements (6).

To test whether antibody could attach to lymphocytes *in vivo* as well as *in vitro*, normal mice were passively immunized with hyperimmune anti-F γ G serum from the same pool used for the *in vitro* studies. 48 hr later the mice were cannulated and lymph was collected and centrifuged. The cell button was resuspended without further washing and the appropriate amount of radioactive antigen added immediately. Radioautographic examination revealed that 17% TDL from these mice were labeled. This was identical to the figure obtained *in vitro* (Table I).

Relationship between Antibody Titer and the Number of Lymphocytes Binding

Antibody.—TDL from normal mice were incubated with anti-F γ G antiserum of varying antibody titers (ranging from 1:2 to 1:2056) followed by F γ G-¹²⁵I as described previously. As shown in Table IV, the number of lymphocytes binding radioactive antigen rose with increasing antibody levels for titers

TABLE II
Effect of Washing on Proportion of Normal Thoracic Duct Lymphocytes Binding Antibody In Vitro

No. of washes		Percentage of TDL labeled with anti-F γ G-F γ G- ¹²⁵ I*
Before exposure to immune complexes	After exposure to immune complexes	
		%
0	—	18
1	—	2.4
3	—	0.2
5	—	0.1
—	2	17
—	3	15
—	4	17

* Figures obtained from one experiment. Two other experiments gave essentially the same results.

TABLE III
Capacity of Different Combinations of Antibody and Antigen to Bind to Normal Thoracic Duct Lymphocytes In Vitro

Treatment of TDL	Percentage labeled with*		
	F γ G- ¹²⁵ I	HGG- ¹²⁵ I	POL- ¹²⁵ I
Normal serum‡	<1	<1	<1
Anti-F γ G serum	12 (12-20)§	<1	<1
Anti-HGG serum	<1	15 (12-19)	<1
Mouse anti-POL	<1	<1	15 (12-17)
Rabbit anti-POL	—	—	16 (13-19)
Chicken anti-POL	—	—	17 (12-21)

* Figures obtained from two to six experiments.

‡ Obtained from normal mice, rabbits, or chickens. In all cases <1% TDL labeled with radioactive antigen.

§ Data from Table I.

ranging from 1 in 2 to 1 in 64, for the exposure time used. When, however, antisera of higher titer were used, no further increase in labeled cells was observed. Only a subpopulation of lymphocytes thus appears to bind antibody.

Identification of the Subpopulation of Lymphocytes Binding Antibody.—Mouse TDL are composed of both T and B cells. 75-85% have the characteristics of T cells in that they are susceptible to lysis with anti- θ serum and

complement (19). Those lymphocytes with the capacity to bind antibody (12–20%) might therefore be B cells or a subclass of T cells. This was investigated by using populations of lymphocytes enriched for either T or B cells, or containing the two cell types in known numbers. The T cell content was established with well-characterized markers such as θ C3H or H-2 isoantigens and then compared with the proportion of cells binding antibody.

TABLE IV
Relationship between Anti-F γ G Titer and the Number of TDL Binding F γ G-¹²⁵I

Anti-F γ G titer	Per cent of TDL labeled with F γ G- ¹²⁵ I*
	%
1:2	0.3
1:8	2
1:32	7
1:64	14
1:256	16
1:2048	15

* Results obtained from one particular experiment. Two other experiments gave similar results.

TABLE V
Selective Binding of Antibody to B Lymphocytes

Source of TDL	Per cent labeled with anti-F γ G-F γ G- ¹²⁵ I*	Per cent killed by anti- θ C3H serum*
Normal mice	16 (13–18)	80 (78–83)
Athymic mice‡	97 (95–98)	0
TxBM mice	73 (66–83)	15 (12–17)
Irradiated (CBA \times C57BL)F ₁ mice given CBA thymus cells (T.TDL)	0.3 (0.1–0.5)	94 (90–98)
Normal mice after prolonged thoracic duct drainage	45 (42–46)	50 (43–56)
TDL from normal mice following anti- θ serum and C'	75 (71–78)	–

* Results obtained from two to six experiments.

‡ The heterozygotes (nu/+) were shown to possess the θ C3H allele.

Thoracic duct cannulation of athymic mice or adult thymectomized, irradiated, and marrow-protected (TxBM) mice provided a source of lymphocytes markedly depleted of T cells (B.TDL). A pure population of T cells was obtained by the method of Sprent and Miller (20). For this purpose lethally irradiated (CBA \times C57BL)F₁ mice were given 2×10^8 CBA thymus cells and cannulated 4 days later. The TDL obtained were identified with anti-H-2 sera as CBA in origin and therefore derived from the original thymus cell inoculum. They will be referred to as T.TDL. Populations of lymphocytes containing identifiable numbers of T and B cells were obtained from chimeric donors prepared as described previously (21). CBA mice were neonatally thymectomized (NTx) and reconstituted to near normal immunological competence with repeated injections of (CBA \times C57BL)F₁ thymus cells. Their TDL were composed of T

cells, the great majority of which were derived from the F₁ thymus cell inoculum, and B cells of host (CBA) origin. The number of T and B cells present was then quantitated by cytotoxic assay (see Materials and Methods) with anti-C57BL serum and complement.

Two groups of experiments were performed to identify the subpopulation of lymphocytes binding antibody. In the first, various types of lymphocytes, viz. normal TDL, T.TDL, and B.TDL from both TxBM and athymic mice, were

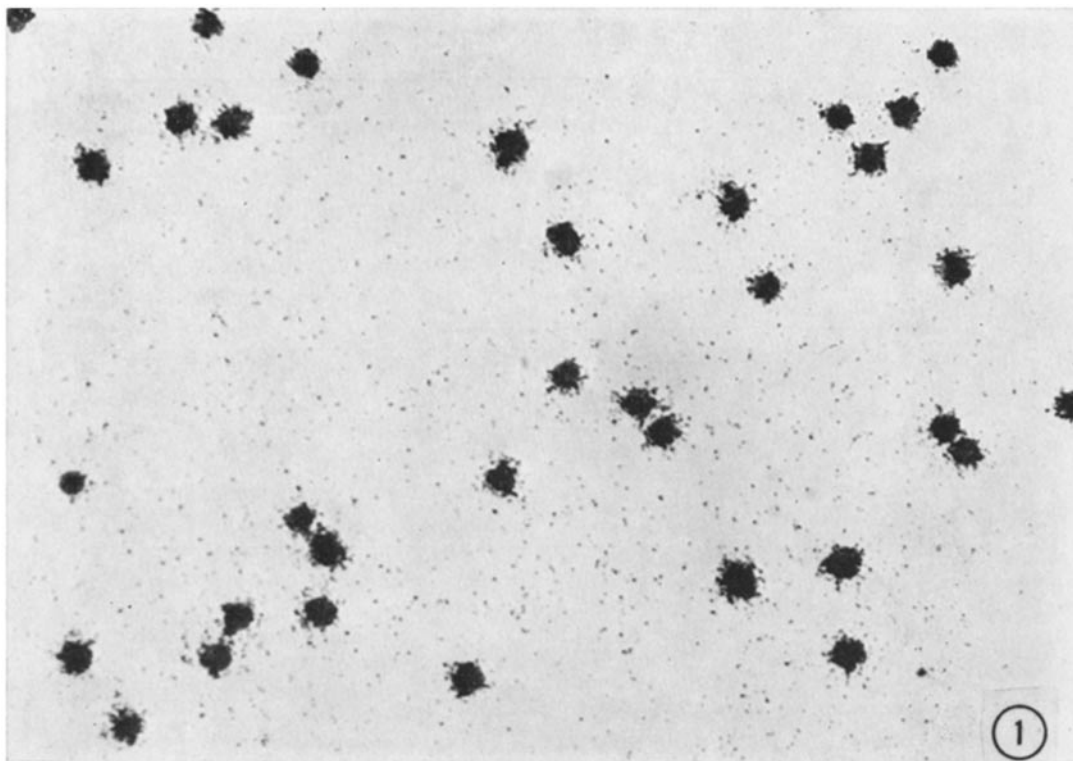


Fig. 1. Photomicrograph of thoracic duct lymphocytes from athymic (nu/nu) mice after treatment with anti-F γ G·F γ G-¹²⁵I. \times 480.

treated with either anti- θ C3H serum and complement or anti-F γ G·F γ G-¹²⁵I. As shown in Table V anti- θ serum killed approximately 80% of normal TDL, 94% of T.TDL, and 0% of B.TDL from athymic mice. The immune complex, on the other hand, bound to 16% of normal TDL, 97% of B.TDL from athymic mice (Fig. 1), and 73% of TDL from TxBM mice but less than 1% of T.TDL even after exposure of the radioautographs for 60 days. Furthermore, T cell depletion of normal mice by prolonged thoracic duct drainage and of normal TDL by in vitro treatment with anti- θ serum and complement, increased the proportion of lymphocytes binding antibody three- to fivefold (Table V).

For the second set of experiments, TDL were obtained from chimeric mice (NTx) CBA reconstituted with (CBA \times C57BL) F_1 thymus cells. Their T cell content was established by estimating the number of lymphocytes susceptible to lysis with anti-C57BL serum and complement and with anti- θ serum and complement (Table VI). Treatment of the same cell pools with anti-F γ G-F γ G- 125 I resulted in 19% labeling. Taken together these findings strongly suggest that B cells, not T cells, have a receptor on their surface for antibody.

Further evidence in support of this notion was sought by studying the effect of bursectomy on the number of antibody-binding lymphocytes in chickens.

TABLE VI
Evidence for Binding of Antibody to B Lymphocytes

Source of TDL	Per cent labeled with anti-F γ G-F γ G- 125 I	Per cent killed by*	
		CBA anti-C57BL	anti- θ
NTx CBA reconstituted with (CBA \times C57BL) F_1 thymus cells	19 (17-22)	79 (76-80)	69 (68-72)
Normal CBA	17 (12-20)‡	0	80 (75-84)

* Results obtained from three experiments.

‡ Data from Table I.

Fertile eggs were obtained from outbred White Leghorn chickens and incubated at 38°C. Hormonal bursectomy was carried out on the 11th day of incubation by injection of 5 mg testosterone propionate (Schering AG, Berlin) into the allantoic fluid (22). After hatching all birds were housed in cages and maintained on water and chick feed *ad libitum*. Blood was collected by venepuncture at approximately 2 months of age and lymphocytes separated as described previously (12). Bursectomized chickens without detectable circulating immunoglobulin were selected as donors.

Cells from the latter and from nonbursectomized controls were reacted with normal chicken serum (NCS) or chicken anti-POL serum followed by POL- 125 I. Radioautography revealed that only occasional cells (<1%) incubated with NCS and iodinated antigen were labeled (Table VII). When anti-POL serum was used, this figure rose to a mean of 22% in the case of control birds. In contrast, only 2-5% cells from bursectomized chickens bound antibody.

Proportion of Small Mononuclear Cells Binding Antibody in Various Lymphoid tissues.—If T cells do indeed lack a receptor for antibody, an inverse relationship between numbers of antibody-binding mononuclear cells resembling small lymphocytes and of θ -positive cells would be expected in the different lymphoid tissues. Radioautographic examination revealed that only occasional thymus cells bound anti-F γ G-F γ G- 125 I, whereas 40-45% splenic mononuclear cells, 20-25% lymph node cells, 57-68% Peyer's patch cells, and 19-23% blood mononuclear cells did so (Table VIII).

Distribution of Lymphocytes Binding Antibody.—Normal CBA mice were injected intravenously with 40×10^6 TDL from syngeneic donors. Before inoculation the cells were labeled

in vitro with anti-F γ G·F γ G-¹²⁵I. The recipients were killed ½, 4, 8, and 24 hr after injection and their spleens were removed and fixed in 10% formol saline. 5- μ sections were then prepared on gelatin-coated slides for radioautography and stained with methyl green-pyronin.

Examination of sections of spleen taken ½ hr after injection revealed the presence of numerous labeled cells in the marginal zones. By 4 hr, however,

TABLE VII
Frequency of Lymphocytes Binding Antibody in Blood from Normal and Bursectomized Chickens

Source of lymphocytes*	Pretreatment	Per cent labeled with anti-POL·POL- ¹²⁵ I
Normal	NCS	<1
	anti-POL	22 (21-24)
Bursectomized	NCS	<1
	anti-POL	3.5 (2-5)

* Obtained from eight normal and four bursectomized chickens.

TABLE VIII
Proportion of Small Lymphocyte-Like Cells Binding Antibody in Various Mouse Lymphoid Tissues*

Tissue	Per cent labeled with anti-F γ G·F γ G- ¹²⁵ I†
Thymus§	0.3 (0-1)
Spleen	42 (40-45)
Lymph node	21 (20-25)
Thoracic duct lymph	17 (12-20)
Peyer's patches	61 (58-67)
Bone marrow¶	15 (12-17)
Bone marrow	27 (21-33)
Blood	19 (15-23)

* Only small mononuclear cells were counted because of the difficulty in distinguishing between monocytes and some lymphocytes.

† Results obtained from two to four experiments.

§ When C57/6J thymus cells were examined instead of CBA, the number of labeled lymphocytes rose to 2-4%.

|| Data from Table I.

¶ Cells incubated with anti-F γ G + 0.01 M EDTA.

very few cells with attached label could be seen anywhere. This was due to elution of radioactive material into the surrounding white pulp where it was diffusely scattered presumably on dendritic processes of macrophages. The radioautographic appearances thus resembled those of previous studies on antigen localization (23). In a separate experiment, labeled TDL were injected into syngeneic mice with an established thoracic duct fistula. When smears of TDL collected 12-48 hr later were examined, no labeled cells were found. This

complemented the previous observation that antigen eluted from the injected lymphocytes soon after they had entered the lymphoid tissues.

The Relationship between Antigen-Binding Lymphocytes and Immunological Memory.—The ability of some lymphocytes to take up passively administered antibody in vivo (*vide supra*) made it important to test whether appreciable numbers of unwashed TDL from actively immunized mice would bind labeled antigen.

For this purpose CBA mice were immunized with 500 μg of alum-precipitated F γ G and 2×10^9 killed pertussis organisms. 4–6 wk later (at which time no antibody-forming cells to F γ G were detectable in the lymph) TDL were collected and divided into two batches, one of which was washed one to eight times and the other left unwashed. Lymphocytes from both batches were reacted in vitro with F γ G- ^{125}I (for radioautography) or F γ G- ^{127}I (for adoptive transfer). Those incubated with nonradioactive F γ G were then counted and 5×10^6 cells in-

TABLE IX
Effect of Washing on Number of Labeled TDL from Mice Primed to F γ G

No. of washes before exposure to labeled antigen	Per cent TDL labeled with F γ G- ^{125}I *
	%
0	17
1	3
3	0.6
5	0.2
8	28

* Data obtained from one experiment. Five other experiments gave similar results.

jected intravenously into heavily irradiated syngeneic recipients. The antibody response was measured 7 days later by the Cunningham and Szenberg modification (24) of the Jerne plaque assay (25).

Primed TDL exposed to F γ G- ^{125}I without either prior washing or addition of specific antibody contained 17% labeled lymphocytes (Table IX). Repeated washing, however, resulted in a rapid decline in this percentage which, even after five washes reached neither a plateau nor the level found in unprimed TDL (approximately 1 in 10^4). Further washing (e.g. eight times) resulted in a nonspecific rise in antigen-binding cells presumably due to damage to the cell membrane.

The relationship between the presence of antibody on lymphocytes and their capacity to express memory was tested with TDL from various sources in an adoptive transfer system. Unwashed primed TDL, of which 1 in 5 to 1 in 6, bound labeled antibody-antigen complexes, and thrice-washed primed TDL containing only 1 in 500 such labeled cells, elicited an equally good 7S memory response when incubated with F γ G in vitro before transfer (Table X). It was therefore not surprising that irradiated recipients of unprimed TDL, incubated with anti-F γ G antiserum and with or without antigen in vitro, failed to show

significant antibody production. In other words immunological memory did not appear to depend on the presence of antibody on circulating lymphocytes.

DISCUSSION

B lymphocytes differ from T lymphocytes in the high density of immunoglobulin on their surface (19, 26) and in the absence of the θ -alloantigen (19). Neither characteristic, however, provides an absolute way of distinguishing them from T cells. For example, some T cells may carry too little θ -antigen to be detectable in standard assay systems (18). Furthermore, immunoglobulin has recently been detected on T cells as well as on B cells by both direct (27) and indirect techniques (28). The demonstration of a distinctive marker for B

TABLE X
Relationship between Membrane-Associated Antibody on Lymphocytes and their Capacity to Carry Immunological Memory

Source of cells	No. of washes	Per cent TDL labeled with $F\gamma G-^{125}I$	Peak $7S F\gamma G$ PFC per spleen of irradiated recipients*
		%	
$F\gamma G$ -primed TDL	0	18	215,200 (234,735-197,295)‡
$F\gamma G$ -primed TDL	3	<1	201,265 (220,175-183,981)‡
Normal TDL	0	<1	21 (63-7)‡
Normal TDL + anti- $F\gamma G$ serum in vitro	0	16	92 (263-32)‡

* $F\gamma G$ given in vitro. Five to seven irradiated hosts per group.

‡ Geometric means, upper and lower limits of SE.

cells was therefore desirable. Initially the mouse bone marrow-derived lymphocyte antigen (MBLA) antigen (29) appeared to fulfill this role, but conflicting reports of the efficacy of anti-MBLA serum in specific elimination of B cells have now emerged.^{2, 3} Until these inconsistencies have been reconciled and the extent of contamination of effective anti-MBLA serum with antimacrophage or stem cell activity established, the value of this marker remains limited.

In the present communication, evidence has been advanced for the existence on all B cells, but not on T cells, of a receptor for antibody. The receptor was detected by a radioautographic technique in which lymphocytes were incubated with antibody followed by the corresponding radioiodinated antigen. The ease with which antisera eluted with washing indicated that the bond between antibody and cell was weak. The formation of an antibody-antigen complex on the surface, however, stabilized the bond and, provided the assay was performed

² Raff, M. C. Personal communication.

³ Niederhuber, J. E., and E. Möller. 1971. Antigenic markers on mouse lymphoid cells: the presence of MBLA on antibody forming cells and antigen binding cells. Submitted for publication.

in the cold, permitted accurate quantitation of cells with adherent antibody (Table II). Attachment of antibody to the lymphocyte required an intact Fc region as will be shown in a subsequent paper (6). Use of TDL and the failure of azide or EDTA to reduce the number of cells binding antibody tended to exclude significant labeling of macrophages or their precursors (Table I). The capacity of more than one antibody-antigen complex to adhere to the cells established the nonspecificity of the phenomenon (Table III).

Two approaches were adopted to show that this characteristic is a marker for B cells, not T cells. In the first, mouse lymphocyte populations greatly enriched for either T or B cells or containing T and B cells in known proportions were used. The number of T cells present was assessed by means of well-established markers namely θ C3H antigen, or H-2 antigen in the case of chimeric mice. This figure was then compared with the proportion of antibody-binding cells (Tables V and VI). In each instance, an inverse relationship between T cells and antibody-binding cells was observed which was particularly striking for TDL from athymic mice; 97% labeled with an immune complex, but none were θ positive. The variability in numbers of labeled TDL from TxBM mice was at first sight disturbing; however, electron microscope studies of these cells (6) revealed that up to 25% had the features characteristic of plasma cells, none of which bound antibody. Examination of the effect of bursectomy in the chicken provided a second approach to identification of the type of lymphocyte carrying this receptor. The frequency of antibody-binding cells among blood lymphocytes of bursectomized birds was remarkably reduced (Table VII). Since these birds have negligible quantities of circulating immunoglobulin presumably due to a deficiency in B cells, and yet are capable of mounting normal delayed hypersensitivity responses (22), these findings, together with those in the mouse, strongly suggest that B cells, not T cells, bind antibody.

The proportion of antibody-binding cells from various sources other than thoracic duct lymph was estimated and expressed as a percentage of the total number of small lymphocyte-like cells present. When these percentages are compared with reported figures for θ -positive cells in the same tissues, an inverse relationship is obtained for spleen, lymph node, thymus, Peyer's patches, and blood (19). Examination of small mononuclear cells from bone marrow revealed that approximately 15% labeled, provided the antiserum used was pretreated with EDTA to minimize binding to phagocytic cells. In the absence of EDTA the number of cells binding antibody increased to a mean of 27%. Thus the ability of effective anti-MBLA serum to kill up to 60% of bone marrow cells³ implies that it must possess nonspecific activity against cell types other than B lymphocytes.

Adherence of immune complexes to some lymphocytes had been demonstrated previously (1, 2), but the establishment of this property as a marker for all B lymphocytes had not. For example, complexes in the form of antibody-coated erythrocytes have, in the presence of C'3, been shown to make rosettes

(complement receptor lymphocytes, [CRL]) with B cells, but not with T cells (3, 4). However, only a minority of lymphocytes from thymectomized mice (e.g. 40–45% lymph node cells) displayed this ability (3). Two possible interpretations of the data suggest themselves; either CRL represent a subpopulation of B cells or the sensitivity of the technique does not permit detection of those lymphocytes with receptors of lower avidity. Evidence for the latter interpretation stems from recent experiments in which B cell rosettes were obtained in the absence of complement, provided the erythrocytes were sensitized by isologous antibody rather than heterologous antibody.⁴ In other words C'3 may simply have acted as an adjuvant to rosette formation. Furthermore, binding in our system in which soluble, not particulate, antigens were employed occurred with isologous or heterologous antibody and in the absence of complement (6). It is therefore reasonable to suggest that both methods detect the same type of receptor but with different degrees of sensitivity.

The existence of a marker of this kind for all B cells has permitted the development of a method for selectively depleting mixed lymphocyte populations of their B cell content (5). Plastic beads coated with an antigen such as HGG were poured into columns. Mouse lymphoid cells from spleen and thoracic duct were incubated with the corresponding antibody and then passed through the column. As predicted, B cells were retained while T cells were excluded. T cells obtained in this way have proved of considerable value in both *in vivo* and *in vitro* studies of T–B cell interaction. Furthermore, the demonstration of a subpopulation of human lymphoid cells with the capacity to bind antibody (5) may provide a way of separating T cells in man.

The fact that B lymphocytes have receptors on their surface for the Fc portion of antibody molecules as well as immunoglobulin receptors (19, 26) necessitates care in interpretation of antigen-binding studies particularly when cells from immunized animals are being used or when cross-reacting antibody might be present. It will be recalled that repeated washing of primed TDL (Table IX) before exposure to radioactive antigen, resulted in a rapid decline in the percentage of labeled cells. This figure, however, never reached a plateau even after five centrifugations while further washing resulted in progressive damage to the cell membrane and a nonspecific rise in antigen-binding cells. In our hands at least, it was not possible to establish, with this technique, whether a lymphocyte population carrying memory to a particular antigen was enriched for cells specifically binding that antigen.

Modabber and Sercarz (30) using a different assay system have also shown that the increase in antigen-binding cells detectable in primed animals can be explained in terms of cytophilic antibody. They did not, however, correlate their findings with the capacity of lymphocytes to carry memory. We have

⁴ Cline, M. J., J. Sprent, N. L. Warner, and A. W. Harris. 1971. Receptors for immunoglobulin on B lymphocytes and cells of a cultured plasma cell tumor. Submitted for publication.

tested this possibility in an adoptive transfer system by comparing the antibody response of TDL from primed mice washed zero to three times with that of normal TDL either coated or not coated with antibody (Table X). The results showed clearly that immunological memory is not related to cytophilic binding of antibody to B cells.

The precise physiological significance of this receptor has not been established. Some workers have already suggested that B cells as well as dendritic macrophages may play a role in antigen localization particularly during a secondary response when antibody is already present in the system (2, 31). Support for this possibility stems from our own studies (32) which utilized the ability of antibody-coated B cells to carry antigen into the spleen where, as shown here, it eluted rapidly into the surrounding white pulp. Opsonization of cells did not occur presumably because insignificant amounts of complement were fixed during binding. In these experiments B cells from TxBM donors were coated with the haptenic determinant, NIP, in the form of anti-F γ G · F γ G · NIP₁₀ complexes. If they were washed to remove excess antigen and then transferred into irradiated hosts together with NIP-HGG primed spleen cells, as a source of hapten-sensitive cells, an excellent anti-NIP antibody response ensued without further addition of antigen. In other words NIP was concentrated so effectively in the spleen that overriding of the carrier effect was achieved with very low doses of antigen. Experiments with this system are in progress to examine the effect of using a more highly substituted NIP conjugate. It is possible that a sufficiently high epitope density may be achieved in the microenvironment of the B cell to induce a state of tolerance rather than immunity (33). If that should prove to be the case, the receptor on B cells for the Fc fragment of the antibody molecule could possibly play a role in the genesis of *in vivo* antibody-mediated suppression (34, 35) or *in vitro* antibody-mediated tolerance (33).

The capacity of B cells to bind antibody in the absence of complement may in addition provide a rational explanation for antibody-dependent killing of target cells by nonsensitized lymphoid cells (36, 37). Here anti-target antibody which is known to inhibit the cytotoxic activity of T cells (38) would enhance contact between target cells and B cells thereby permitting killing by the latter. This notion is supported by recent evidence establishing that spleen cells from rats subjected to either prolonged thoracic duct drainage (39) or thymectomy (40) can still exert a cytotoxic effect.

SUMMARY

Evidence is presented for the existence on all B lymphocytes, but not on T lymphocytes, of a membrane-associated receptor for antibody. The receptor was detected by a radioautographic technique in which lymphoid cells were incubated with antibody followed by the corresponding radioiodinated antigen. The ease with which antibody eluted during washing indicated that the bond between antibody and cell was weak. The formation of an antibody-antigen

complex on the cell surface, however, stabilized the bond and permitted accurate quantitation of cells with adherent antibody.

The ability of several combinations of antibody and antigen to adhere to the cells demonstrated the nonspecificity of the phenomenon and emphasized the need for care in interpretation of antigen-binding studies particularly when immune cells are being used.

The identity of antibody-binding lymphocytes was established by two different approaches. In the first, mouse lymphocyte populations greatly enriched for either T cells or B cells were examined. Their T cell content was assessed by means of well-established markers such as the θ C3H isoantigen. When this was compared with the number of antibody-binding cells, an inverse relationship was obtained in each instance; thus almost all thoracic duct cells from athymic mice labeled with an immune complex although none were θ positive. The striking reduction in antibody-binding cells observed in bursectomized chickens provided a second and independent line of evidence suggesting that B cells, not T cells, bind antibody.

The ability of B cells from primed animals to bind antibody in vivo made it important to test whether this phenomenon was related to the carriage of immunological memory. No correlation was, however, found between membrane-bound antibody and memory.

It was proposed that the existence of a receptor of this kind may provide a rational explanation for antibody-dependent killing of target cells and may prove of importance in antigen concentration particularly during the secondary response.

The authors would like to thank Dr. M. Holmes for providing athymic mice; Dr. M. Feldmann for pools of anti-POL sera; Dr. B. Rouse for blood samples from normal and bursectomized chickens; and Misses Ludmila Ptschelinzew and Lorraine Evans for their excellent technical assistance.

BIBLIOGRAPHY

1. Uhr, J. W., and J. M. Phillips. 1966. In vitro sensitization of phagocytes and lymphocytes by antigen-antibody complexes. *Ann. N. Y. Acad. Sci.* **129**:792.
2. Bianco, C., R. Patrick, and V. Nussensweig. 1970. A population of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. I. Characterization and separation. *J. Exp. Med.* **132**:702.
3. Dukor, P., C. Bianco, and V. Nussensweig. 1971. Bone marrow origin of complement-receptor lymphocytes. *Eur. J. Immunol.* In press.
4. Bianco, C., and V. Nussensweig. 1971. Theta-bearing and complement-receptor lymphocytes are distinct populations of cells. *Science (Washington)*. **173**:154.
5. Basten, A., J. Sprent, and J. F. A. P. Miller. 1971. A receptor for antibody-antigen complexes on B cells: its value in separating T cells from lymphocyte populations. *Nature (New Biol.) (London)*. In press.
6. Basten, A., N. L. Warner, and T. Mandel. 1971. A receptor for antibody on B lymphocytes. II. Immunochemical and electron microscopy characteristics. *J. Exp. Med.* **135**:627.

7. Miller, J. F. A. P., and G. F. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:801.
8. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **99**:167.
9. Miller, J. F. A. P. 1960. Studies on mouse leukemia. II. The role of the thymus in leukaemogenesis by cell-free leukaemic filtrates. *Brit. J. Cancer.* **14**:93.
10. Kern, M., and H. N. Eisen. 1959. The effect of antigen stimulation on incorporation of phosphate and methionine into proteins and isolated lymph node cells. *J. Exp. Med.* **110**:207.
11. Byrt, P., and G. L. Ada. 1969. An in vitro reaction between labeled flagellin or haemocyanin and lymphocyte-like cells from normal animals. *Immunology.* **17**:503.
12. Hulliger, L., and A. A. Blazhovec. 1967. A simple and efficient method of separating peripheral blood leucocytes for in vitro studies. *Lancet* **1**:1304.
13. Miller, J. F. A. P., and N. L. Warner. 1971. The immune response of normal, irradiated and thymectomized mice to fowl immunoglobulin G as determined by a hemolytic plaque assay technique. *Int. Arch. Allergy Appl. Immunol.* **40**:59.
14. Ada, G. L., G. J. V. Nossal, J. Pye, and A. Abbot. 1964. Antigens in immunity. I. Preparation and properties of flagellar antigens from *Salmonella adelaide*. *Aust. J. Exp. Biol. Med. Sci.* **42**:267.
15. Cushing, J. E. 1945. A comparative study of complement. II. The interaction of components of different species. *J. Immunol.* **50**:75.
16. Herzenberg, L. A., and N. L. Warner. 1968. Genetic control of mouse immunoglobulins. *In* Regulation of the Antibody Response. B. Cinader, editor. Charles C Thomas, Publisher, Springfield, Ill. 322.
17. Reif, A. E., and J. M. V. Allen. 1964. The AKR thymic antigen and its distribution in leukemia and nervous tissues. *J. Exp. Med.* **120**:413.
18. Miller, J. F. A. P., and J. Sprent. 1971. Thymus derived cells in mouse thoracic duct lymph. *Nature (New Biol.) (London).* **230**:267.
19. Raff, M. C., and J. J. T. Owen. 1971. Thymus derived lymphocytes and their distribution and role in the development of peripheral lymphoid tissues of the mouse. *Eur. J. Immunol.* **1**:27.
20. Sprent, J., and J. F. A. P. Miller. 1971. Activation of thymus cells by histocompatibility antigens. *Nature (New Biol.) (London).* **234**:195.
21. Miller, J. F. A. P., and J. Sprent. 1971. Cell-to-cell interaction in the immune response. VI. Contribution of thymus-derived cells and antibody-forming cell precursors to immunological memory. *J. Exp. Med.* **134**:66.
22. Warner, N. L., Z. Ovary, and F. S. Kantor. 1971. Delayed hypersensitivity reactions in normal and bursectomized chickens. *Int. Arch. Allergy Appl. Immunol.* **40**:719.
23. Nossal, G. J. V., C. M. Austin, J. Pye, and J. Mitchell. 1966. Antigens in immunity. XII. Antigen trapping in the spleen. *Int. Arch. Allergy Appl. Immunol.* **29**:368.
24. Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology.* **14**:599.
25. Jerne, N. K., A. A. Nordin, and C. Henry. 1963. *In* Cell Bound Antibodies. B.

- Amos and H. Koprowski, editors. The Wistar Institute Press, Philadelphia, Pa. 109.
26. Rabellino, E., S. Colon, H. M. Grey, and E. R. Unanue. 1971. Immunoglobulins on the surface of lymphocytes. *J. Exp. Med.* **133**:156.
 27. Marchalonis, J. J., J. L. Atwell, and R. E. Cone. 1971. Isolation of surface immunoglobulin from lymphocytes from human and murine thymus. *Nature (New Biol.) (London)*. In press.
 28. Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye. 1971. Specific inactivation of thymus derived (T) and non thymus derived (B) lymphocytes by ¹²⁵I-labeled antigen. *Nature (New Biol.) (London)*. **231**:104.
 29. Raff, M. C., M. Nase, and N. A. Mitchison. 1971. A marker antigen for lymphocytes derived from bone marrow. *Nature (New Biol.) (London)*. **229**:50.
 30. Modabber, F., and E. E. Sercarz. 1970. Antigen binding and the immune response. II. The large number of antigen-binding cells in primed animals. *Proc. Soc. Exp. Biol. Med.* **135**:400.
 31. Nossal, G. J. V., G. L. Ada, C. M. Austin, and J. Pye. 1965. Antigens in immunity. VIII. Localization of ¹²⁵I-labeled antigens in the secondary response. *Immunology*. **9**:349.
 32. Miller, J. F. A. P., J. Sprent, A. Basten, N. L. Warner, J. C. S. Breitner, G. Rowland, J. Hamilton, H. Silver, and W. J. Martin. 1971. Cell-to-cell interaction in the immune response. VII. Requirement for differentiation of thymus-derived cells. *J. Exp. Med.* **134**:1266.
 33. Feldmann, M. 1971. Induction of immunity and tolerance to the dinitrophenyl determinant in vitro. *Nature (New Biol.) (London)*. **231**:21.
 34. Ivanyi, J. 1970. Early 7S antibody-antigen complexes with affinity to spleen cells in chickens immunized with a low dose of human serum albumin. *Immunology*. **19**:629.
 35. Sinclair, N. R. St. C., R. K. Lees, P. L. Chan, and R. H. Khan. 1970. Regulation of the immune response. II. Further studies on differences in ability of F(ab)₂ and 7S antibodies to inhibit an antibody response. *Immunology*. **19**:105.
 36. Perlmann, P., and H. Perlmann. 1970. Contactual lysis of antibody-coated chicken erythrocytes by purified lymphocytes. *Cell. Immunol.* **1**:300.
 37. MacLennan, I. C. M., G. Loewi, and B. Harding. 1970. The role of immunoglobulins in lymphocyte mediated target cell damage, in vitro. I. Comparison of the effects of target cell specific antibody and normal serum factors on cellular damage by immune and non-immune lymphocytes. *Immunology*. **18**:397.
 38. Brunner, K. T., J. Maul., H. Rudolf, and B. Chapuis. 1970. Studies of allograft immunity in mice. I. Induction, development and in vitro assay of cellular immunity. *Immunology*. **18**:50.
 39. MacLennan, I. C. M., and B. Harding. 1970. Failure of certain cytotoxic lymphocytes to respond mitotically to phytohaemagglutinin. *Nature (London)*. **227**:1246.
 40. Harding, B., D. J. Pudifin, F. Gotch, and I. C. M. MacLennan. 1971. Cytotoxic lymphocytes from rats depleted of thymus processed cells. *Nature (New Biol.) (London)*. **232**:80.