

## EXPRESSION OF IgD BY MURINE LYMPHOCYTES

### Loss of Surface IgD Indicates Maturation of Memory B Cells\*

BY SAMUEL J. BLACK,‡ WESSEL VAN DER LOO, MICHAEL R. LOKEN, AND LEONARD A. HERZENBERG

(From the Department of Genetics, Stanford University School of Medicine, Stanford, California, 94305)

Antibody-forming cell (AFC)<sup>1</sup> precursors arise from cells in the bone marrow and differentiate to antigen-specific B cells. On exposure to antigen, in the presence of T lymphocytes and macrophages, these B cells further differentiate to effector cells—AFC that secrete large amounts of immunoglobulin (Ig) with a specificity for antigen identical to that of the receptor on the stimulated cell, or to memory B cells—lymphocytes capable of rapid expansion and differentiation to effector cells upon re-exposure to antigen.

Antibody genes in mammalian cells comprise at least three families, one encoding for  $\kappa$ -light chains, another for  $\lambda$ -light chains, and a tightly linked cluster of genes encoding for heavy chains (1). The surface Ig on a single B cell is homogeneous with respect to the light chain family expressed and to the specificity for antigen (2, 3). Considerable evidence has now accumulated showing that Igs with two or more different Ig heavy chain (Ig-H) classes are present on the surface of a single B cell (4). The expression of different heavy (H) chains and combinations of H chains on B cells has been studied in an effort to chart the ontogeny of, and to distinguish between, functionally different B cells.

In newborn mice most B cells carry only surface IgM. From 4 to 6 wk after birth the majority of B cells in the immune system have both surface IgM and IgD. The developmental relationship between these two cell populations has not yet been established (5). Studies with anti-IgM treatment of newborns have shown that IgM-bearing cells are precursors of IgG and IgA-producing cells (6); however, these studies have not established whether the precursors are IgM only or IgM-IgD-bearing B cells.

In the adult mouse, several investigations have been conducted to establish which Ig classes (isotypes) are found on B cells responding in primary responses and which are found on B cells responding in secondary responses. In T-independent primary responses, both the IgM-only and IgM-IgD B-cell populations were shown to give rise to AFC (7). In T-dependent primary responses, Coffman and Cohn (7) found that only the IgM-IgD B

\* Supported, in part, by grants from the National Institute of General Medical Sciences (GM 17367), National Cancer Institute (CA-04681), and the National Institute of Child Health and Human Development (HD-01287).

‡ Junior Fellow of the American Cancer Society, California Division.

<sup>1</sup> Abbreviations used in this paper: AFC, antibody-forming cells; BRBC, burro erythrocytes; DNP-KLH, 2,4-dinitrophenyl conjugates of keyhole limpet hemocyanin; F\*, conjugated with fluorescein, fluoresceinated; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; Ig<sup>a</sup>, Ig with Ig-1a linked allotypes; Ig<sup>b</sup>, Ig with Ig-1b linked allotypes; Ig-5, locus for IgD heavy chain,  $\delta$ ; Ig-6, locus for IgM heavy chain,  $\mu$ ; Ig-H, Ig heavy chain; i.v., intravenous (via a lateral tail vein); MEM, minimum essential medium; PAGE, polyacrylamide gel electrophoresis; PFC, plaque-forming cells; SDS, sodium dodecyl sulfate.

cells responded; however, Zan-bar et al. (8) also obtained a small T-dependent response with IgD<sup>-</sup> (IgM only) B cells as well.

In secondary responses, most of the responding B cells (memory cells) carry IgG (7-11). Some of these IgG-bearing cells carry IgD as well (7). There are also apparently some IgG memory cells that carry little or no surface IgG (9, 10). Thus, the appearance of surface IgG on a B cell defines a major B-cell maturation step; however, the information presently available on expression of surface IgD is inadequate to fit the presence or absence of this Ig isotype into a scheme of B-cell maturation.

In this publication, we present data from a comprehensive study of the presence of IgD on B cells at different stages of maturation, from unprimed lymphocytes to AFC. We show that (a) T-dependent primary antibody responses are generated mostly, if not entirely, by IgD-bearing B cells; (b) early IgG memory cells carry surface IgD; (c) mature (later, higher avidity) IgG memory cells do not carry surface IgD; and (d) most IgM AFC carry IgD whereas few (if any) IgG AFC carry IgD. Thus evidence from this study indicates that, with respect to T-dependent-responding B cells, the presence of surface IgD distinguishes less mature from more mature B cells, and can, therefore, be used in conjunction with evidence from other studies to construct an outline for a B-cell maturation pathway.

## Materials and Methods

*Experimental Approach.* Alloantisera with specificity for mouse IgD were used to identify splenic B cells with surface IgD (12). The reaction of such sera with their target cells was visualized by indirect immunofluorescence microscopy and analysis on the fluorescence-activated cell sorter (FACS) (13). IgD<sup>+</sup> (stained) and IgD<sup>-</sup> (unstained) populations in the suspension were then isolated with the FACS and tested for functional activity in an adoptive transfer assay. The isolated populations were supplemented with an appropriate source of T cells in these assays to allow full expression of B-cell activity. Three functionally distinct populations of cells involved in antibody responses were examined for expression of surface IgD: unprimed B cells, primed (memory) B cells, and IgM and IgG plaque-forming cells (PFC).

*Mice.* Two pairs of Ig-H chain congenic mouse strains were used for this work: SJL/JHz, which carries the *Ig<sup>b</sup>* haplotype, and SJA/9Hz, which carries the *Ig<sup>a</sup>* haplotype derived from BALB/c; and BALB/cNHx (*Ig<sup>a</sup>* haplotype) and BAB/14Hz, which carries the *Ig<sup>b</sup>* haplotype of C57BL/Ka (14). Male or female mice between 3 and 6 mo of age were used in these experiments.

*Antisera.* Mouse anti-Ig-5b (anti- $\delta$  of the *b* allotype) was raised by injecting SJA female mice (Ig<sup>a</sup>) with 10<sup>7</sup> washed spleen cells from female BAB/14 mice (Ig<sup>b</sup>). The BAB/14 spleen cells were administered i.p. on five occasions each separated by 1 wk. Recipient mice were bled 7 days after the last injection of antigen and serum pools were made on the basis of anti-Ig-5b titer. Serum producers were bled weekly, and boosted monthly, and no drop in titer was observed over a 2<sup>1</sup>/<sub>2</sub> mo period. After 2<sup>1</sup>/<sub>2</sub> mo, the serum producers generate a large amount of antibodies that react with antibody complexes. Anti-IgD titer was assessed after absorption of the sera with a Ig-6b myeloma (C.BPC112)-Sephadex or C57BL/6 globulins-Sephadex conjugates. This procedure removes anti-Ig-6b activity from the serum in addition to any antibodies that react with complexed Ig. The anti-Ig-5b activity in absorbed sera was assessed by staining of SJL spleen cells and analysis on the FACS. This procedure permits an objective assessment of how brightly a given serum in combination with a fixed amount of fluoresceinated second step reagent will stain a fixed number of spleen cells. Serum collected over the 2<sup>1</sup>/<sub>2</sub>-mo period after the initial immunizations was used in the following studies.

The specificity of the anti-Ig-5b serum has been characterized in two ways: (a) By immunofluorescence analysis. These data have been published elsewhere (12). To the already published results we can add that our anti-Ig-5b sera do not stain any cells in SJL fetal liver nor is any activity removed from these sera by absorption with SJL fetal liver cells. These sera also do not stain any cells in the 1-day old SJL spleen whereas 5-10% of these cells can be stained with a

rabbit or goat-anti-IgM serum or with anti-Ig-6b serum. In the 4-day old SJL spleen about 5% of cells can be stained with our anti-Ig-5b serum whereas about 20% of these spleen cells are IgM bearing. In the adult spleen, most cells which have membrane IgM also stain for surface Ig-5 (IgD) (5, 7).

(b) By SDS polyacrylamide gel (SDS-PAGE) analysis of immunoprecipitates prepared from lysates of radiolabeled SJL spleen cells (S. Black, unpublished data). Briefly, anti-Ig-5b serum that did not react with IgM as shown by radioimmune analysis<sup>2</sup> or staining of 1-day old mouse spleen cells, precipitated only IgD from radiolabeled SJL spleen cell lysates. IgD was identified by the migration pattern in 10% SDS-PAGE under reducing conditions, as a molecule that had a mol wt of approximately 65,000 daltons.

*Fluorescein Conjugated Ig<sup>b</sup> anti-Ig<sup>a</sup> Allotype Serum.* This serum was raised by injecting SJL mice with complexes of *Bordetella pertussis* anti-*B. pertussis* antibodies from BALB/cN mice. Ouchterlony analysis showed reaction against Ig-1a, Ig-3a, and Ig-4a. The conjugation of this serum with fluorescein followed the protocol of Cebra and Goldstein (15). Fluoresceinated antiserum was then absorbed with SJL spleen cells until it reacted only with mouse B cells bearing Ig-1a, Ig-3a, or Ig-4a.

*Antigens and Priming.* Burro erythrocytes (BRBC) in Alsever's solution (Colorado Serum Co., Denver, Colo.) were washed three times in minimum essential medium (MEM) and  $5 \times 10^7$  were injected i.p. into experimental animals. Keyhole limpet hemocyanin (KLH) (Pacific Bio-Marine Laboratories, Inc., Venice, Calif.) and 2,4-dinitrophenyl (DNP) conjugates of KLH were prepared as previously described (16). Donors were primed with 100  $\mu$ g DNP-KLH on alum (hapten priming) or with 100  $\mu$ g KLH on alum (carrier priming). Both antigens were injected i.p. with  $2 \times 10^6$  killed *B. pertussis* (kindly supplied by the Commonwealth of Massachusetts, Department of Public Health, Boston, Mass.) Mice were used as donors a minimum of 6 wk after priming.

*Preparation of B Cells, T Cells, and Erythrocyte-Depleted Spleen Cells.* (a) T-cell killing: T cells were depleted from spleen cell suspensions by killing with anti-Thy-1 plus complement in a two-step killing protocol. Cells were incubated with congenic anti-Thy-1.2 (kindly supplied by Doctors E. A. Boyse and F. W. Shen, Sloan-Kettering Institute, New York) for 30 min at 37°C. After the first step, cells were pelleted through fetal calf serum (FCS), then resuspended and incubated with agarose-adsorbed guinea pig complement for 30 min at 37°C. After the second step, cells were resuspended and washed once before transfer. (b) T-cell-enriched populations of splenocytes were prepared by passing spleen cells through nylon wool columns (17). (c) Erythrocyte-depleted splenocytes: erythrocytes were lysed by incubating the cells for 2 min at 4°C in Gey's balanced salt solution in which the NaCl was replaced with an equimolar concentration of NH<sub>4</sub>Cl (hemolytic Gey's).

*Immunofluorescent Staining for Surface IgD.* IgD was visualized on B-cell surfaces by indirect immunofluorescence (12). Splenic lymphocytes harvested from SJL mice in MEM with 5% FCS were stained with antiserum specific for Ig-5b for 15 min at 4°C and pelleted through FCS. The cell pellet was resuspended in fluoresceinated (F\*) *b* anti-*a* allotype serum (fluorescein-labeled second step reagent) and incubated for a further 15 min at 4°C. This suspension was again pelleted through FCS and resuspended to a concentration of  $10^7$  cells/ml for separation by using the FACS-II. Concentrations of the first and second step reagents were chosen to give optimal staining. Visual analysis of stained cells on a ultraviolet Zeiss microscope, (Carl Zeiss, Inc., New York) showed that 30-40% of the population was fluorescent. No cells were stained by the second step reagent alone or after incubation with normal mouse serum.

*Sorting and Analysis with FACS-II.* FACS isolation used in these experiments allows separation of cells according to amount of bound fluorescein, amount of low-angle light scattering (size), or a selected combination of these two parameters. In these studies scatter gates or thresholds were set so that only small, live lymphocytes were analyzed or separated (18).

*Adoptive Transfer and Plaque-Forming Cell Assays.* Spleen cells (from SJL mice) in MEM were mixed with T cells as desired just before i.v. injection into BALB/c recipients, X-irradiated (650 rads) 18 h previously. Recipients were challenged at time of transfer with 10  $\mu$ g aqueous DNP-KLH or  $5 \times 10^7$  washed BRBC and killed 7 days later. Direct DNP or BRBC PFC in recipient spleens were scored by the method of Cunningham and Szenberg (19). Indirect splenic PFC were counted as the increase in DNP or BRBC PFC in chambers containing an optimal concentration

<sup>2</sup> S. J. Black, J. W. Goding, G. A. Gutman, L. A. Herzenberg, M. R. Loken, B. A. Osborne, W. van der Loo, and N. L. Warner. Manuscript in preparation.

TABLE I  
The Primary Adoptive Anti-BRBC Response

B Cells*	T Cells‡	BRBC	Anti-BRBC PFC ( $\times 10^3$ )/spleen	
			Direct	Developed
$10^7$	--	+	1.0	0.9
--	$10^7$	+	<0.1	<0.1
$10^7$	$10^7$	+	14.2	25.1
$10^7$	$10^7$	+		
	$\alpha$ -Thy-1.2 + C'	+	1.1	3.0
$10^7$	$10^7$	-	0.5	0.6

Mixtures of SJL B and T cells were injected into X-irradiated (650 rads) BALB/c mice together with  $5 \times 10^7$  BRBC. Recipient mice were killed 7 days later and spleen cells were harvested and plaqued against BRBC. The data is tabulated as anti-BRBC PFC ( $\times 10^3$ )/spleen. Direct PFC are mainly IgM-secreting PFC; developed PFC are mainly IgG-secreting PFC.

\* B cells prepared by treating unprimed SJL spleen cells with anti-Thy-1.2 and complement.

‡ Nylon wool-enriched populations.

of facilitating antiserum (rabbit anti-mouse IgG sera for IgG PFC development [10]) over the response in chambers with no facilitating antiserum. Results are expressed as PFC ( $\times 10^3$ )/spleen.

Because we have used allogeneic recipients for most of the work described here we present the data in Table I to show that the allogeneic adoptive primary anti-BRBC response has similar characteristics to syngeneic T-dependent adoptive primary responses. B cells or T cells alone generate virtually no anti-BRBC PFC in the presence of antigen. Mixtures of B and T cells, when stimulated with BRBC, give rise to substantial numbers of IgM and IgG PFC. Without antigen, few PFC were produced. T cells treated with anti-Thy-1.2 plus complement before mixing with B cells and antigen do not support the response. Thus, although SJL spleen cells were responding to BRBC in an allogeneic environment, the response was T-dependent and required antigen. The immune response obtained was threefold higher than that arising in a syngeneic transfer making this adoptive transfer system attractive for assaying the low responses usually obtained in adoptive primary studies.

Adoptive secondary anti-DNP responses in allogeneic environments also had the characteristics of a syngeneic response (see Table II). DNP-primed B cells together with carrier-primed spleen cells and DNP-KLH generated a strong IgG anti-DNP PFC response but no IgM PFC. Neither DNP-primed B cells nor carrier-primed T cells alone, or carrier-primed T cells and unprimed B cells (with antigen) generated an immune response on challenge with DNP-KLH. Further, after anti-Thy-1.2 plus complement treatment of the carrier-primed cells (Table II, line 4) DNP-primed B cells gave no adoptive anti-DNP-PFC response. Antigen given to the adoptive recipient was always required for a response in the allogeneic environment. This secondary allogeneic transfer gave rise to two to five times more PFC than were obtained in the syngeneic comparison with no changes in cell or antigen requirements (S. Black, unpublished data).

*Avidity of DNP-PFC.* Avidity of DNP-PFC was estimated by including  $\epsilon$ -DNP-lysine in the plaquing chambers at 10-fold concentration steps from  $10^{-10}$  to  $10^{-5}$  M as previously described (20). The number of cells placed into each Cunningham chamber was adjusted so that in the absence of any inhibitor, 250 PFC/chamber were counted. Six replicate chambers were counted for each concentration of inhibitor. For concentrations of  $\epsilon$ -DNP-lysine, less than  $10^{-6}$  M the standard error obtained on each set of six replicate PFC counts was  $< \pm 7\%$ .

## Results

*Staining of SJL Spleen Cells with Anti-Ig-5b Serum and Separation of IgD<sup>+</sup> and IgD<sup>-</sup> Lymphocytes.* An SJL spleen cell preparation was indirectly stained

TABLE II  
Secondary Adoptive Anti-DNP Response

DNP-Primed* B cells	KLH-Primed‡ spleen	DNP-KLH	Anti-DNP PFC ( $\times 10^3$ )/ spleen	
			Direct	Developed
$5 \times 10^6$	$10^7$	+	<0.1	102.5
$5 \times 10^6$	--	+	<0.1	11.5
--	$10^7$	+	<0.1	8.5
$5 \times 10^6$	$10^7$	+	<0.1	10.1
	$\alpha$ -Thy-1.2 + C'			
$5 \times 10^6$	$10^7$	-	<0.1	3.5

Mixtures of DNP-primed B cells and KLH-primed SJL spleen cells were injected into X-irradiated (650 rads) BALB/c mice together with  $10 \mu\text{g}$  aqueous DNP-KLH. Recipient mice were killed 7 days later and spleen cells were harvested and plaqued against DNP-BRBC. The data are tabulated as anti-DNP PFC ( $\times 10^3$ )/spleen.

\* DNP-primed splenic B cells were obtained from SJL mice primed 6 wk previously with DNP-KLH. Whole spleen cell preparations from these mice were treated with anti-Thy-1.2 plus complement.

‡ These cells were harvested from SJL mice primed 6 wk previously with KLH.

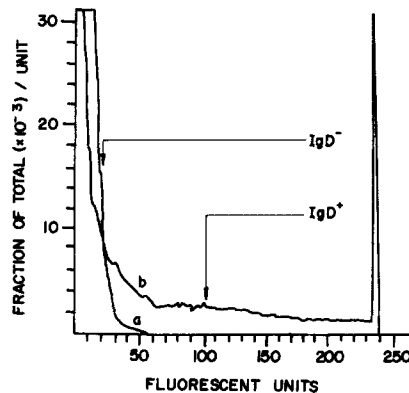


FIG. 1. FACS Analysis of SJL spleen cells stained indirectly with anti-Ig-5b serum. a) Viable small SJL spleen cells stained indirectly with SJA normal mouse serum followed by F\*b-anti- $\alpha$  allotype serum. b) Viable small SJL spleen cells stained indirectly with SJA anti-Ig-5b serum followed by F\*b-anti- $\alpha$  allotype serum. Separation gates used to isolate IgD<sup>+</sup> and IgD<sup>-</sup> lymphocytes are shown. 32% of the anti-Ig-5b stained spleen cell preparation fell between 0 and 20 fluorescence U and was taken as IgD<sup>-</sup>. 28% of the anti-Ig-5b stained spleen cell preparation fell between 100 and 240 fluorescence U and was taken as IgD<sup>+</sup>.

with specific  $\alpha$  anti-Ig-5b serum in combination with F\*b anti- $\alpha$  allotype serum. UV Zeiss microscope analysis of this cell suspension showed that about 30% of the population was fluorescent. The stained cells were analyzed on the FACS-II. Separation thresholds were set as follows (see Fig. 1): unstained (IgD<sup>-</sup>) cells were taken to be those that fell into the first one or two channels. These cells could not be distinguished from low autofluorescent cells. IgD<sup>+</sup> cells were taken as those brightly staining cells that were clearly separate from the "tail" of the IgD<sup>-</sup> population (see Fig. 1). Cells that fell between brightly fluorescent (IgD<sup>+</sup>)

TABLE III  
*Expression of IgD on the Primary Responsive B Lymphocyte*

B Cells ( $5 \times 10^6$ )	T Cells	Anti-BRBC PFC ( $\times 10^3$ )/ spleen	
		Direct	Developed
Stained unseparated spleen	$10^7$	14	25
IgD <sup>+</sup> fraction	$10^7$	13	22
IgD <sup>-</sup> fraction	$10^7$	<0.1	<0.1
--	$10^7$	<0.1	<0.1

SJL spleen cells were stained with SJA anti-Ig-5b serum and F\**b* anti-*a* allotype serum, then sorted into bright IgD<sup>+</sup> (top 28%) and dull IgD<sup>-</sup> (bottom 32%) fractions. Recipients were X-irradiated (650 rads) BALB/c mice. Washed BRBC ( $5 \times 10^7$ ) were injected along with lymphocytes. T cells are nylon wool-enriched spleen cells. The PFC response was assayed 7 days after transfer.

and dull fluorescent (IgD<sup>-</sup>) were discarded. Details of this sorting procedure have been published elsewhere (21).

More than 95% of the IgD<sup>+</sup> cells obtained in this manner were fluorescent on visual examination by using fluorescence microscopy. Direct staining of these cells with a rhodaminated-specific anti-IgM antiserum and subsequent FACS analysis showed that greater than 95% of IgD<sup>+</sup> cells also carry surface IgM. The isolated IgD<sup>-</sup> population was stained with an antiserum specific for T cells and found to contain mainly T cells.

*The Expression of IgD on the Primary Responsive Lymphocyte.* IgD<sup>+</sup> and IgD<sup>-</sup> lymphocytes were prepared as outlined above. Mixtures of stained but unseparated or separated fractions with added T cells were transferred into X-irradiated BALB/cN mice along with BRBC, and the splenic PFC response that arose 7 days later was assessed. About half of the unseparated normal spleen cells are IgD<sup>+</sup>, therefore equal numbers of IgD<sup>-</sup> and IgD<sup>+</sup> cells were transferred.

The results from the above experiment are shown in Table III. IgD<sup>+</sup> lymphocytes supplemented with T cells gave rise to a response approximately equivalent to unseparated spleen cells supplemented with T cells. IgD<sup>-</sup> cells supplemented with T cells did not generate a PFC response. This experiment is representative of four similar studies that were performed. (Similar results were also obtained on transfer of IgD<sup>+</sup> and IgD<sup>-</sup> SJL cells into SJL recipients.) Thus IgD<sup>+</sup> cells give rise to a primary response to an erythrocyte antigen. Since the IgD<sup>-</sup> lymphocyte fraction contained only about 5–10% B cells we cannot say that IgD<sup>-</sup> B cells are totally unable to participate in T-dependent primary immune responses; rather, we conclude that most, if not all, BRBC primary-reactive B cells reside in the IgD<sup>+</sup> lymphocyte pool.

*The Expression of IgD on IgG Memory Cells.* The results of adoptive transfers with primed B cells are presented in Table IV.

Both the isolated IgD<sup>+</sup> cells and the isolated IgD<sup>-</sup> cells from DNP-primed mice, when supplemented with KLH-primed spleen cells, generated substantial IgG PFC responses. Thus the IgG memory pool present 6 wk after one exposure to antigen is heterogeneous, with memory being found in both IgD<sup>+</sup> and IgD<sup>-</sup> fractions. These data are representative of results obtained in three similar

TABLE IV  
*Expression of IgD on IgG Memory Cells*

DNP-Primed B Cells* ( $5 \times 10^6$ )	DNP-KLH‡	Anti-DNP PFC ( $\times 10^3$ )/spleen
		Developed
Stained unseparated spleen	+	160
Stained unseparated spleen	-	4.7
IgD <sup>+</sup> fraction	+	50
IgD <sup>-</sup> fraction	+	44
--	+	<0.1

All recipient mice received  $10^7$  KLH-primed SJL spleen cells harvested from animals primed 6 wk previously with 100  $\mu$ g alum-precipitated KLH and *B. pertussis* organisms. SJL spleen cells were stained with SJA anti-Ig-5b serum and F\*b anti-a allotype serum, then sorted into bright IgD<sup>+</sup> (top 37%) and dull IgD<sup>-</sup> (bottom 35%) fractions. Recipients were X-irradiated (650 rads) BALB/c mice. The PFC response was assessed 7 days after transfer.

\* SJL mice were primed 6 wk previously with 100  $\mu$ g alum-precipitated DNP-KLH together with  $2 \times 10^8$  killed *B. pertussis* organisms.

‡ Where indicated (+) mice were challenged with 10  $\mu$ g aqueous DNP-KLH.

experiments using the allogeneic transfer system and one experiment in which a syngeneic transfer was performed.

In the above experiments, the proportion of IgD<sup>+</sup> memory cells comprised approximately half the memory cell population; however, in other experiments with donors taken later than 6 wk after priming, the proportion of IgD<sup>+</sup> memory cells in the memory population was consistently lower, apparently decreasing with time after antigenic exposure (S. Black, unpublished observations).

*The Avidity of PFC Generated from IgD<sup>+</sup> and IgD<sup>-</sup> Memory Cells.* As the IgG memory pool arising after one exposure to antigen proved to be heterogeneous, we investigated the avidity of the DNP-PFC populations arising from IgD<sup>+</sup> and IgD<sup>-</sup> memory cells. The experimental protocol used was identical to that described in the preceding section. The avidity of DNP-PFC was measured in the Cunningham assay by inhibition with graded concentrations of  $\epsilon$ -DNP-lysine included in the plaquing chamber. The results of this experiment are presented in Table V and are representative of two such experiments performed.

Data in Table V confirms that IgG memory is carried by both IgD<sup>+</sup> and IgD<sup>-</sup> B lymphocytes in mice primed 6 wk previously with DNP-KLH. There are differences, however, in the avidity of the PFC populations arising from each of these B-cell groups. The data in this table are presented as the percentage of the DNP-PFC response that was inhibited between the indicated concentration ranges of  $\epsilon$ -DNP-lysine. Thus, the greater the percentage of inhibition obtained with low concentrations of  $\epsilon$ -DNP-lysine present, the more PFC of high avidity were present in the population analyzed.

Stained but unseparated DNP-primed SJL spleen cells when supplemented with KLH-primed spleen cells gave rise to a PFC response of intermediate avidity. Most PFC were inhibited with between  $10^{-5}$  and  $10^{-8}$  M  $\epsilon$ -DNP-lysine present in the plaquing chamber. These account for 67% of the total PFC

TABLE V  
Avidity of DNP-PFC Generated by IgD<sup>+</sup> or IgD<sup>-</sup> Memory Cells

$\epsilon$ -DNP-lysine ( $-\log_{10}$ M) in PFC Chamber	% Inhibition of DNP-PFC response within each concentration range		
	IgD <sup>+</sup>	IgD <sup>-</sup>	Whole spleen
<5	8	3	7
5-6	28	9	22
6-7	39	24	24
7-8	12	22	21
8-9	6	22	11
9-10	4	12	9
>10	3	8	6

$5 \times 10^6$  DNP-primed cells (IgD<sup>+</sup>, IgD<sup>-</sup>, and whole spleen stained but unseparated) and  $10^7$  KLH-primed spleen cells were transferred with 10  $\mu$ g DNP-KLH into X-irradiated (650 rads) BALB/c recipients. Spleens were harvested and plaqued against DNP-BRBC on day 7 after transfer.  $\epsilon$ -DNP-lysine was incorporated into the suspending medium at the indicated concentration before placing cells into the PFC chamber. Results of the inhibition assay are expressed as percentage of the normal PFC response which is inhibited between the indicated concentration ranges of  $\epsilon$ -DNP-lysine. The developed PFC responses (without inhibitor present) obtained from each group were as follows, expressed as ( $\times 10^3$ )/spleen: IgD<sup>+</sup>, 120; IgD<sup>-</sup>, 103; unseparated, 215. Further statistical details are given in Materials and Methods.

response. The remaining PFC were inhibited over the entire range of inhibitor concentrations. IgD<sup>+</sup> B cells supplemented with carrier-primed T cells gave rise to PFC that were mainly inhibitable by concentrations of  $\epsilon$ -DNP-lysine between  $10^{-5}$  and  $10^{-7}$  M. Thus the avidity of this response was a little lower than that of the unseparated population of spleen cells. In contrast IgD<sup>-</sup> B cells in combination with carrier-primed spleen cells generated a PFC response of higher avidity than the unseparated spleen cell preparation. The majority (88%) of these PFC were inhibited with between  $10^{-6}$  and  $10^{-9}$  M  $\epsilon$ -DNP-lysine. Since increase in avidity is associated with maturation of the immune response (20), the substantially higher avidity of the IgD<sup>-</sup> component of the B-cell memory population suggests that these cells represent a later stage of memory development.

*The Expression of IgD on IgG Memory Cells after Several Exposures to Antigen.* The data presented in the preceding section suggested that IgD<sup>-</sup> IgG memory cells were more mature than IgD<sup>+</sup> IgG memory cells. To confirm this possibility, mice were exposed to more than one dose of antigen before their spleen cells were harvested, FACS-separated into IgD<sup>+</sup> and IgD<sup>-</sup> fractions, and these populations analyzed for their capacity to transfer an adoptive IgG anti-DNP-PFC response. Mice were primed with DNP-KLH 6 wk before harvesting of spleens. These animals were then split into two groups. The first set of mice was analyzed immediately for the amount of IgG memory carried in IgD<sup>-</sup> and IgD<sup>+</sup> fractions. The second group was re-exposed to 10  $\mu$ g aqueous DNP-KLH and rested for an additional 4 wk. This group was then divided. Some mice were analyzed for the amount of IgG memory carried by IgD<sup>+</sup> and IgD<sup>-</sup> B lymphocyte populations and the rest were again exposed to 10  $\mu$ g aqueous



TABLE VI  
*Expression of IgD on IgG Memory Cells after Exposure(s) to Antigen*

DNP-Primed B Cells*	No. of antigen‡ injections	Developed Anti-DNP PFC ( $\times 10^3$ )/spleen
Stained§ unseparated spleen	1	250
IgD <sup>+</sup> fraction	"	160
IgD <sup>-</sup> fraction	"	101
Stained   unseparated spleen	2	190
IgD <sup>+</sup> fraction	"	21
IgD <sup>-</sup> fraction	"	140
Stained¶ unseparated spleen	3	350
IgD <sup>+</sup> fraction	"	<0.1
IgD <sup>-</sup> fraction	"	320

\*  $5 \times 10^6$  B cells along with  $10^7$  KLH<sup>o</sup> spleen cells and  $10 \mu\text{g}$  aqueous DNP-KLH were transferred i.v. into each recipient mouse. In the absence of antigen no response was obtained.

‡ 1, primed with DNP-KLH 6 wk previously. 2, primed with DNP-KLH 10 wk previously and boosted with DNP-KLH at 6 wk later. 3, primed with DNP-KLH 14 wk previously, boosted with DNP-KLH at 6 and 10 wk after priming.

§ 43% cells stained; 28% selected as IgD<sup>+</sup>; 31% as IgD<sup>-</sup>.

|| 37% cells stained; 22% selected as IgD<sup>+</sup>; 29% as IgD<sup>-</sup>.

¶ 39% cells stained; 26% selected as IgD<sup>+</sup>; 31% as IgD<sup>-</sup>.

DNP-KLH. After another 4 wk, IgD<sup>+</sup> and IgD<sup>-</sup> cells harvested from these animals were probed for their capacity to transfer an IgG PFC response.

The results of these experiments are presented in Table VI. This experiment was performed twice with similar results. Each time the DNP-primed donor mice were exposed to antigen a change occurred in the degree of IgG memory carried by IgD<sup>+</sup> and IgD<sup>-</sup> populations of B lymphocytes. After three exposures to antigen, IgG memory became limited to the IgD<sup>-</sup> population of B lymphocytes.

These results suggest that IgD<sup>+</sup> memory cells arise early in the immune response and become converted to IgD<sup>-</sup> memory cells with repeated antigenic exposure; however, establishment of this sequence requires direct examination of isolated IgD<sup>+</sup> cells to determine their ability to give rise to IgD<sup>-</sup> memory cells. Preliminary data from such double transfer experiments is in agreement with an IgD<sup>+</sup> to IgD<sup>-</sup> switch, but definitive data on this point has not yet been obtained (S. Black, unpublished observations).

*The Expression of IgD on Primary IgM and IgG PFC.* SJL mice were injected with  $5 \times 10^7$  washed BRBC and spleen cells were harvested 5 days later. These cells were separated into IgD<sup>+</sup> and IgD<sup>-</sup> fractions on the FACS-II. Of each population of lymphocytes  $2 \times 10^5$  cells were plaqued against BRBC with or without facilitating antiserum and the distribution of direct and developed PFC noted. The results from this experiment are presented in Table VII and are representative of four such experiments conducted. Table VII

TABLE VII  
*The Expression of IgD on Primary IgM and IgG PFC*

Cells	Anti-BRBC PFC*	
	Direct PFC/(2 × 10 <sup>5</sup> )	Developed PFC/(2 × 10 <sup>5</sup> )
IgD <sup>+</sup>	80	3
IgD <sup>-</sup>	3	204

\* A day 5 anti-BRBC-PFC response after injection of 5 × 10<sup>7</sup> washed BRBC.

TABLE VIII  
*The Expression of IgD on Secondary IgG PFC*

Cells	Anti-DNP PFC*
	Developed PFC/(2 × 10 <sup>5</sup> )
IgD <sup>+</sup>	20
IgD <sup>-</sup>	320

\* A day 7 anti-DNP-PFC response generated by DNP-KLH primed SJL mice after challenge with 10 μg aqueous DNP-KLH.

shows that primary IgM anti-BRBC PFC have IgD on their membrane whereas IgG PFC do not.

*The Expression of IgD on Secondary IgG PFC.* SJL mice primed with DNP-KLH were boosted with aqueous DNP-KLH and 7 days later their spleens were harvested. These cells were separated into IgD<sup>+</sup> and IgD<sup>-</sup> fractions. Both fractions were then assayed with DNP-BRBC in the Cunningham plaque assay. Facilitating antiserum was included as no direct PFC were generated. The data from this experiment are presented in Table VIII. Most IgG PFC were IgD<sup>-</sup>. The small number of IgG PFC (4-5%) that fell in the IgD<sup>+</sup> fraction probably represents contamination of the IgD<sup>+</sup> cells with a few IgD<sup>-</sup> cells. These data are similar to results obtained in three separate experiments.

### Discussion

We have shown that IgD is present on the surface of unprimed B cells and early B memory cells but not on mature memory B cells. IgD is also present on the surface of IgM PFC but not on IgG PFC even when the IgG PFC arise as a result of a primary immune response.

The proportion of IgD<sup>+</sup> IgG memory cells is highest in recently primed animals (approximately 6 wk). If these primed animals are boosted, the proportion of IgG memory B cells found in the IgD<sup>+</sup> population of spleen cells decreases and most of these memory cells are found among IgD<sup>-</sup> B cells. These data suggest that IgD marks the early cells in the B-memory cell developmental pathway.

The lower avidity of the antibody produced by progeny of IgD<sup>+</sup> memory cells as compared with the avidity of the antibody produced by progeny of IgD<sup>-</sup> memory cells strongly supports the assertion that IgD<sup>-</sup> memory cells are the most mature. This hypothesis is further supported by the observation that all

IgG PFC in a secondary response lack surface IgD, hence when a cell enters the final stages of development to an IgG PFC it ceases to have surface IgD.

The above data confirm and extend the work of Coffman and Cohn (7) and Zan-bar et al. (8, 9) on the expression of IgD on B memory cells committed to generating an IgG secondary response. They also support the findings of Coffman and Cohn (7) that IgM-IgD lymphocytes participate in a primary immune response to a T-dependent antigen, and are consistent with previous observations on B-cell development as well (4, 5).

When the evidence gathered in these various studies is taken in concert with the evidence presented in this publication, the outlines of the B-cell maturation pathway, in which the expression of different Ig isotypes marks successive stages of maturation, can be drawn as follows: the first B cells to arise have IgM on their surface. These cells can respond to T-independent antigenic stimulus (7) but tolerance is easily induced if they are exposed to a T-dependent antigen (22). A second population of B cells later appears. These cells express surface IgM and IgD and are possibly progeny of the IgM only cells. On exposure to a T-independent antigen, at least a component of this IgM-IgD population will differentiate to IgM PFC (7-9); if a T-dependent antigen is used a component of this population will differentiate to IgM and IgG PFC. At present it is not known if the same cells in this population respond to both T-dependent and T-independent antigens.

Exposure to antigen induces formation of a memory B-cell population. The memory cell population is heterogeneous with respect to expression of surface IgD. The first memory cells appear to be largely IgD<sup>+</sup>. With time and exposure to antigen, the memory population becomes IgD<sup>-</sup>. It is likely, although definitive evidence is still lacking, that the IgD<sup>+</sup> memory cell gives rise to the IgD<sup>-</sup> memory cell.

Definitive data on the other isotypes expressed on the IgD<sup>+</sup> and IgD<sup>-</sup> memory B cells is still lacking. Most splenic memory cells carry surface IgG that indicates their class and allotype commitment (10, 11). This includes at least a significant proportion of the IgD<sup>+</sup> memory cells (7). Again, a proportion of memory cells carry surface IgM (9). It is reasonable to assume that early memory cells first express IgM and IgD, then express IgG in addition, and finally lose the IgM and IgD isotypes from their surface as the response matures (i.e., with time and exposure to antigen).

### Summary

B lymphocytes capable of generating primary IgM and IgG plaque-forming cells (PFC) responses to burro erythrocytes have surface IgD, as do primary IgM PFC. IgG memory cells arising after one injection of antigen are divided into two groups, one of which expresses surface IgD while the other has no detectable membrane IgD. PFC generated from the IgG memory cells lacking surface IgD show a higher average avidity than those arising from IgD-positive IgG memory cells, indicating that mature IgG memory cells do not have surface IgD. After more than one injection of antigen, few, if any, IgG memory cells have surface IgD. IgG PFC arising in primary or secondary immune response lack membrane-bound IgD. These data provide the outlines for a B-cell

maturation pathway in which IgD marks unprimed and early memory B cells and is lost in mature memory cells.

Studies presented here were conducted by isolating IgD<sup>+</sup> and IgD<sup>-</sup> cells with the fluorescence-activated cell sorter and functional testing of the isolated populations in adoptive transfer experiments.

We thank Ms. Sandra Scaling for providing excellent technical assistance throughout this project, Ms. Jean Anderson for assuming considerable responsibility in the preparation of this manuscript, and Ms. Lee Herzenberg for providing both critical evaluation and editorial assistance as this work was being prepared for publication. We also wish to express our appreciation to Mr. Eugene Filson for his invaluable aid with the FACS separations.

*Received for publication 25 October 1977.*

### References

1. Hood, L., J. H. Campbell, and S. C. R. Elgin. 1975. The organization, expression, and evolution of antibody genes and other multigene families. *Annu. Rev. Genet.* 9:305.
2. Rowe, D. S., K. Hug, L. Fornia, and B. Pernis. 1973. Immunoglobulin D as a lymphocyte receptor. *J. Exp. Med.* 138:965.
3. Goding, J. W., and J. E. Layton. 1976. Antigen-induced co-capping of IgM and IgD-like receptors on murine B cells. *J. Exp. Med.* 144:852.
4. Cooper, M. D., J. F. Kearney, A. R. Lawton, E. R. Abney, R. M. E. Parkhouse, J. L. Preud'homme, and M. Seligmann. 1976. Generation of Ig class diversity in B cells: a discussion with emphasis on IgD development. *Ann. Immunol. (Inst. Pasteur)* 127:573.
5. J. W. Goding. 1977. Allotypes of IgM and IgD in the mouse: a probe for lymphocyte differentiation. *Contemp. Top. Immunobiol.* 8: In press.
6. Lawton, A. R., III, and M. D. Cooper. 1974. Modification of B-lymphocyte differentiation by anti-immunoglobulins. *Contemp. Top. Immunobiol.* 3:193.
7. Coffman, R. L., and M. Cohn. 1977. The class of surface immunoglobulin on virgin and memory B lymphocytes. *J. Immunol.* 118:1806.
8. Zan-bar, I., E. S. Vitetta, and S. Strober. 1977. The relationship between surface immunoglobulin isotype and immune function of murine B lymphocytes. II. Surface immunoglobulin isotypes on unprimed B cells in the spleen. *J. Exp. Med.* 145:1206.
9. Zan-bar, I., S. Strober, and E. S. Vitetta. 1977. The relationship between surface immunoglobulin isotype and immune function of murine B lymphocytes. I. Surface Ig isotypes on primed B cells in the spleen. *J. Exp. Med.* 145:1188.
10. Okumura, K., M. H. Julius, T. Tsu, L. A. Herzenberg, and L. A. Herzenberg. 1976. Demonstration that IgG memory is carried by IgG bearing cells. *Eur. J. Immunol.* 6:467.
11. Mason, D. W. 1976. The class of surface immunoglobulin on cells carrying IgG memory in rat thoracic duct lymph: the size of the subpopulation mediating IgG memory. *J. Exp. Med.* 143:1122.
12. Herzenberg, L. A., L. A. Herzenberg, S. J. Black, M. R. Loken, K. Okumura, W. van der Loo, B. A. Osborne, D. Hewgill, J. W. Goding, G. Gutman, and N. L. Warner. 1977. Surface markers and functional relationships of cells involved in murine B cell differentiation. In *Origins of Lymphocyte Diversity. Cold Spring Harbor Symp. Quant. Biol.* XLI:33.
13. Bonner, W. A., H. R. Hulett, R. G. Sweet, and L. A. Herzenberg. 1972. Fluorescence activated cell sorting. *Rev. Sci. Instrum.* 43:404.
14. Herzenberg, L. A., and L. A. Herzenberg. 1977. Mouse immunoglobulin allotypes:

- description and specific methodology. *In Handbook of Experimental Immunology*. D. M. Weir, editor. Blackwell Scientific Publications, Oxford, England. 3rd edition. Chapter 12. In press.
15. Cebra, J. J., and G. Goldstein. 1965. Chromatographic purification of tetramethyl rhodamine-Ig conjugates and their use in the cellular localization of rabbit  $\gamma$ -globulin polypeptide chains. *J. Immunol.* 95:230.
  16. Iverson, G. M. 1977. Assay methods for antigen-mediated cell cooperation. *In Handbook of Experimental Immunology*. D. M. Weir, editor. Blackwell Scientific Publications, Oxford, England. 3rd edition. Chapter 29. In press.
  17. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus derived murine lymphocytes. *Eur. J. Immunol.* 3:645.
  18. Herzenberg, L. A., and L. A. Herzenberg. 1977. Analysis and separation using the fluorescence activated cell sorter (FACS). *In Handbook of Experimental Immunology*. D. M. Weir, editor. Blackwell Scientific Publications, Oxford, England. 3rd edition. Chapter 22. In press.
  19. Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody forming cells. *Immunology.* 14:599.
  20. Andersson, B. 1970. Studies on the regulation of avidity at the level of the single antibody-forming cell. The effect of antigen dose and time after immunization. *J. Exp. Med.* 132:77.
  21. Jones, P. P., J. J. Cebra, and L. A. Herzenberg. 1974. Restriction of gene expression of B lymphocytes and their progeny. I. Commitment to immunoglobulin allotype. *J. Exp. Med.* 139:581.
  22. Cambier, J. C., E. S. Vitetta, J. W. Uhr, and J. R. Kettman. 1977. B cell tolerance. II. Trinitrophenyl human gamma globulin-induced tolerance in adult and neonatal murine B cells responsive to thymus-dependent and independent forms of the same hapten. *J. Exp. Med.* 145:778.