Studies using both human and murine lymphoid cells have established that the predominant immunoglobulin isotypes on the cell surface of B lymphocytes are IgM and IgD (1–6). Moreover, the majority of B cells express both isotypes simultaneously (2, 3, 7–9). A minority of B cells bear surface IgG (10, 11).

Recently, several investigators have studied the immune function of primed (memory) B cells bearing the different isotypes. Mason (12) found that IgG- but not IgM-bearing cells in the thoracic duct lymph of rats carried immunological memory for the adoptive indirect plaque-forming response to the dinitrophenyl (DNP) hapten. Similar findings were reported by Okumura et al. (13) and Yuan et al. (1) for murine splenic memory B cells to DNP and sheep red blood cells, respectively. Other investigators have suggested that some memory B cells bear surface IgM. Strober (14) showed that small memory cells found in rat thoracic duct lymph express surface IgG, but that large memory cells present shortly after priming express surface IgM. Abney et al. (15) demonstrated that passaged memory B-cell clones which give rise to IgG2a antibody bear IgM receptors on their cell surface. Yuan et al. (1) have recently reported that cells giving rise to a secondary IgM response may bear little IgM as compared to cells giving rise to the primary IgM response.

The goal of the present studies was to prepare populations of cells bearing specific isotypes by means of the fluorescence activated cell sorter (FACS), and to test the immune function of these populations by adoptively transferring them to irradiated syngeneic mice. We determined the ability of these cells to restore the adoptive primary and secondary serum antibody response of irradiated mice.

--

* Supported by NIH grants AI-70018, 11851, 12789, and 10293, and by American Cancer Society grant IM-63.

‡ Postdoctoral Fellow of the Arthritis Foundation.

§ Investigator, Howard Hughes Medical Institute.

1 Yuan, D., E. S. Vitetta, and J. Kettman. 1976. Cell surface Ig. XX. Antibody responsiveness of subpopulations of B lymphocytes bearing different isotypes. Manuscript submitted for publication.

2 Abbreviations used in this paper: BSA, bovine serum albumin; FACS, fluorescence activated cell sorter; FCS, fetal calf serum; 2ME, 2-mercaptoethanol; NP40, Nonidet P40; PAGE, polyacrylamide gel; PBS, phosphate-buffered saline; RAMIg, rabbit anti-mouse Ig serum; RAδ, rabbit anti-mouse δ-chain antiserum; RAγ, rabbit anti-mouse γ-chain antiserum; RAμ, rabbit anti-mouse μ-chain antiserum; SDS, sodium dodecyl sulfate; TMG, Tris buffer containing MgCl₂.
ated recipients. Recipients of primed B cells were immunized such that unprimed B cells did not contribute to the adoptive response. The experimental protocols described herein had several advantages as compared to previous investigations: (a) all three major surface isotypes were studied; (b) cells were purified by both a positive and negative selection procedure; (c) both the adoptive IgM and IgG antibody responses were measured serially during an interval of 2-4 wk; and (d) the immune function of both unprimed and primed cells were assayed for their response to the same antigen.

The first of the two papers describes the preparation and specificity of the anti-immunoglobulin reagents, B-cell isolation procedures, and experimental results of the transfer of primed B cells. The second paper reports the results of the transfer of unprimed B cells.

Materials and Methods

Antisera

Rabbit Anti-Mouse Ig (RAMIg) (16). This antiserum contained antibodies against, μ, γ, α, κ, and λ-chains.

Rabbit Anti-Mouse-μ (RAμ) (16). This serum was prepared against μ-chains isolated from MOPC-104E and was monospecific for both serum and cell surface IgM.

Rabbit Anti-Mouse-γ (RAγ) (14). This serum was prepared against γ-chains for serum IgG and was monospecific for IgG.

Rabbit Anti-Mouse-δ (RAδ). The anti-δ serum was prepared and assayed by a modification of the technique described by Abney et al. (17).

Preparation of Plasma Membranes. Lots of 250 frozen spleens prepared from adult, outbred mice were purchased from Pel-Freez Bio-Animals, Inc., Rogers, Ark. The spleens had been frozen in 250 ml of phosphate-buffered saline (PBS), pH 7.3, 3-5 days before their arrival in Texas. Within 1-3 wk after their removal from the mice, the spleens were thawed and rinsed in PBS. Batches of spleens were suspended in 40 ml 0.1 M Tris buffer, pH 7.4, containing 0.001 M MgCl₂ (TMG buffer) and samples were homogenized at 4°C with 50-100 strokes of a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.). The homogenate was centrifuged at 12,000 g for 15 min and the pellets were resuspended in a small volume of TMG buffer by homogenization with a loose-fitting Dounce homogenizer. The homogenate was adjusted to a final concentration of 55% sucrose in 50 ml for each 100 spleens. Samples were then pipetted into centrifuge tubes and overlayed with 45, 35, and 25% sucrose in TMG buffer. Samples were centrifuged for 20 h at 27,000 rpm in the SW27 rotor of the Spinco ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

The material with the lowest density, containing the majority of the plasma membrane, was removed with a Pasteur pipet, diluted with TMG buffer, and centrifuged at 100,000 g for 45 min in the 65 rotor of the Spinco ultracentrifuge. The pellet was washed once and recentrifuged. The yields varied from 0.3 to 0.7 g of plasma membrane per 100 spleens.

Preparation of Membrane Ig for Immunization. The membrane pellet was lysed in 2-10 ml of 0.5% Nonidet P40 (NP40) (Gallard Schlesinger) containing 10 μl diisopropylfluorophosphate as an inhibitor of proteolysis. Samples were dialyzed for 16 h at 4°C against PBS and then centrifuged at 10,000 g for 30 min at 4°C.

The extract was mixed with Sepharose-anti-μ and incubated for 15 min at 37°C. Preliminary experiments had established the volume of Sepharose-anti-μ necessary to bind >70% of the membrane IgM. This was done by mixing a small aliquot amount of lysate from 131I-splenocytes with a known volume of the membrane extract, absorbing the material to Sepharose anti-μ, centrifuging, and precipitating the supernate with RAMIg. The material which did not adhere to the anti-μ immunoabsorbant was further incubated for 15 min at 4°C with a previously determined volume of Sepharose-anti-Ig in order to bind the remaining Ig. The Sepharose-anti-Ig was centrifuged, washed four times with PBS, and then injected subcutaneously into two rabbits. The initial injection consisted of the Sepharose-anti-Ig-bound material from the plasma membranes of 500 spleens. Rabbits were boosted at 2- to 4-wk intervals with the membrane IgD from 250 to 500 spleens and were bled 1-2 wk after each boost. The serum used for these experiments was a pool from both rabbits after injections of the membrane Ig from 1,500 to 2,000 spleens.
Absorption of the Antiserum. The pooled serum was diluted with an equal volume of PBS and absorbed with a predetermined volume of Sepharose-euglobulin for 30 min at 4°C. The Sepharose was removed by centrifugation, washed once in PBS, and the wash pooled with the supernate. The supernate was further absorbed with 1 mg/ml mouse acetone liver powder (Cappel Laboratories, Downingtown, Pa.). The liver powder had been suspended in PBS and washed until the OD280 of the supernate was less than 0.1 before its addition to the serum. In the studies described here, absorption with thymocytes was found to be unnecessary, although subsequent pools of the serum required absorption with packed thymocytes.

Assay of the Antiserum. The anti-δ serum was used to bind purified Ig's or surface Ig from radioiodinated spleen cells using Staphylococcus aureus to bind the immune complexes. The pool used in these studies was found to be 20-30% as strong as our anti-Ig and was therefore used at a fivefold higher concentration than our anti-Ig in the biochemical assays.

Radioiodination of Splenocytes

5 x 10^7 splenocytes from A/J mice were radioiodinated (18), lysed, and the lysates immunoprecipitated as described previously (16). Briefly, the aliquots of the lysate were incubated with the rabbit anti-mouse Ig, μ-, δ-, or γ-sera described above and the complexes bound to S. aureus (19, 20). The bacterial pellets were washed and the radioactivity eluted in 1% sodium dodecyl sulfate (SDS) containing 8 M urea and 0.5 M 2-mercaptoethanol (2ME). Aliquots of the eluate were mixed with H-μ and L chains from the HP-76 myeloma and electrophoresed for 16 h at 4 mA/gel on 7.5% SDS-polyacrylamide gels (SDS-PAGE). Gels were fractionated, the double label counted, and the marker proteins aligned in comparing different gels.

Preparation of IgG from Antisera

The antisera were brought to 50% saturation with (NH₄)₂SO₄ and stirred for 4 h at 4°C. The precipitates were centrifuged, dissolved in distilled water, and desalted by dialysis against water. The IgG fraction was prepared on DEAE-Sephadex A50 using 0.05 M phosphate buffer, pH 7.8. The IgG was desalted and lyophilized.

Preparation of F(ab')₂ Fragments of IgG (21)

IgG was dissolved in 0.1 M sodium acetate, pH 4.3, at 10 mg/ml and was digested for 16 h at 37°C with 2% wt/wt pepsin. The digest was neutralized with NaOH, dialyzed against PBS, and applied to a Sephadex G150 column equilibrated with PBS. The major peak was desalted, lyophilized, and assessed for the absence of intact IgG by SDS-PAGE. Gels were stained with 0.25% Coomassie blue.

Preparation of Euglobulin

500 ml of normal mouse serum (Pel-Freez Bio-Animals, Inc.) was brought to 50% saturation with (NH₄)₂SO₄ and stirred for 4 h at 4°C. The sample was centrifuged and the precipitate dissolved in a minimal volume of water. The dissolved material was dialyzed for 24-48 h against two to three changes of water. The dialyzed material was centrifuged and the resultant pellet termed "euglobulin."

Conjugation of Euglobulins and Ig to Sepharose

Proteins were dissolved in 0.1 M NaHCO₃, pH 9, at 5–10 mg/ml. Samples were conjugated to cyanogen bromide-activated Sepharose 4B according to published procedures (22). After the conjugation, the gel was washed in 1 M ethanolamine in 0.02 M Tris, pH 8, and occasionally in saline containing 0.05 M glycine-HCl, pH 2.8. Samples were stored at 4°C in PBS. 70-95% of the protein was conjugated in all experiments as determined by the OD₂₈₀ of the unbound material. The binding capacity of each immunoabsorbant was determined using either lysates of radioiodinated cells or radioiodinated myeloma proteins.

Immunofluorescent Staining for Surface Immunoglobulin

Immunofluorescent staining of spleen cells for surface Ig was performed using a two-stage procedure. Spleen cells (20 x 10⁶ cells/ml) suspended in tissue culture medium 199 and 5% fetal calf serum (FCS) were incubated for 30 min at 4°C with the IgG fraction of RAMIg, RAμ, RAδ, or with the F(ab')₂ fragment of RAγ. The cell suspension was subsequently layered over FCS and centrifuged for 10 min at 150 g. The cell pellet was resuspended in similar culture medium with the
IgG fraction of a fluorescein-conjugated goat anti-rabbit IgG antiserum (Meloy Laboratories, Springfield, Va.) thoroughly absorbed with mouse thymocytes. Cells were washed again and used for sorting or analysis.

In order to determine the appropriate concentration of the first-stage rabbit anti-mouse Ig reagents, spleen cells were incubated in over a 40-fold range of concentrations (4 mg/ml-0.1 mg/ml). The percentage of positively stained cells was determined on the FACS at several concentrations. A concentration vs. percentage of positive cells curve was constructed, and a plateau was observed for each reagent. Thereafter, cells were stained at a reagent concentration which was on the plateau portion of the curve (0.1 mg/ml, RAMIg; 1 mg/ml, RAμ; 2 mg/ml, RAγ; 2 mg/ml, RAδ). The concentration of the second-stage reagent was similarly determined to be on the plateau portion of a dilution curve.

Sorting and Analysis of Cells with FACS

Stained spleen cells were analyzed for the percentage of bright fluorescent cells, and sorted on the same basis using the FACS (23). Thresholds were set so that bright cells corresponded to positive cells as judged by fluorescence microscopy. For purposes of sorting, the next brightest 10% of cells were discarded, and the remaining cells fell into the dull cell fraction. Details of the sorting procedure have been described elsewhere (24). Approximately 40-50% of spleen cells were recovered in the combined bright and dull cell fractions. Bright cells were contaminated with up to 5% of dull cells, and dull cells were contaminated with up to 3% of bright cells as judged by repeat analysis of sorted cells. Approximately 5 × 10⁷ cells were sorted in each experiment at a rate varying between 3 and 5 × 10⁵ cells/s.

Fluorescence Microscopy

Stained cells were smeared onto a glass slide, dried in air, and fixed in methanol for at least 1 h. Thereafter, slides were dried, a drop of glycerol and PBS (1:1) was placed over the cells, and a glass cover slip applied. Cells were examined with a Zeiss microscope at 400× using a vertical illuminator (Carl Zeiss Inc., New York). The microscope was equipped with a combination of barrier filters for the selective visualization of either fluorescein or rhodamine stains. The total number of cells in each field was determined by phase-contrast illumination.

Animals

(BALB/c × C57BL/Ka)F₁ mice obtained from the colony of Dr. R. Kallman, Department of Radiology, Stanford University School of Medicine, Stanford, Calif., were used in all experiments. Only female mice, 2- to 3-mo old, were selected for investigation.

Immunization Procedures

Donors of carrier-primed cells were immunized with a subcutaneous injection (0.2 ml) of an emulsion of equal volumes of bovine serum albumin (BSA) (Calbiochem, Los Angeles, Calif.) in saline and complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Each animal received a total dose of 0.5 mg protein.

Donors of hapten (DNP)-primed cells were immunized with DNP₁₈-BSA in complete Freund's adjuvant as described above. BSA was dinitrophenylated with dinitrophenyl benzene sulfonate as reported previously (25). Adoptive recipients were challenged intraperitoneally with 200 μg DNP-BSA in saline 1 day after cell transfer.

Preparation of Cell Suspensions

Cell suspensions were prepared by mincing lymph nodes and spleens with a scissor, and gently pressing the fragments through a nylon mesh. Cells were harvested by centrifugation at 150g, and resuspended in minimum essential medium without sodium bicarbonate [minimal essential medium (MEM)] (Grand Island Biological Co., Grand Island, N. Y.) before intravenous injection into the lateral tail vein of irradiated recipients.

In Vitro Killing with Anti-Thy1.2 Antiserum

T cells were depleted from spleen cell suspensions by a two-stage in vitro cytotoxicity procedure. The cells were first incubated with anti-Thy1.2 (AKR anti-C3H) antiserum at a dilution of 1:10 for 45 min at 37°C. Cells were collected by centrifugation and resuspended in guinea pig complement (Grand Island Biological Co.) at a dilution of 1:5 for 30 min at 37°C. The remaining cells were spun
down, washed once, and resuspended in MEM before injection into adoptive hosts. Approximately 50–60% of spleen cells were killed by this procedure.

Passage of Lymph Node and Spleen Cells Over Nylon Wool Columns

T lymphocytes from the spleen and lymph nodes were purified by passage over a nylon wool column as described by Julius et al. (26). Cell suspensions were applied to LP-1 Leuko-Pak filters (Fenwall Laboratories, Inc., Morton Grove, Ill.) equilibrated with 10% FCS in MEM and maintained at 37°C. The percentage of T cells in the effluent was greater than 95% as judged by killing with anti-Thy1.2 antiseraum. The percentage of Ig-bearing cells (B cells) was less than 5% as judged by immunofluorescent staining with RAMIg.

X Irradiation of Mice

Mice were placed in Lucite containers and given 650 R whole body X irradiation from a single 250 kV (15 A) source. The dose rate was 54 R/min (0.25 mm Cu plus 0.55 mm Al filtration) with a 52 cm source axis distance. Cells were transferred to adoptive hosts 4–6 h after irradiation.

Antibody Titrations

Blood samples were collected from the retro-orbital vein. Serum was separated by centrifugation and stored at −20°C. Antibodies to DNP were measured by a modification of the Farr assay (27). Serial fivefold dilutions of serum were made in 20% normal rabbit serum in saline. 3H-DNP-lysine (New England Nuclear Corp., Boston, Mass.) at a concentration of 2.5 × 10−8 M was added to each tube. Globulins were precipitated by the addition of ammonium sulfate, and aliquots of the supernates were counted in a Beckman Model LS 3133P scintillation counter. Antibody determinations are expressed at the log10 of the titer of antiserum which bound 33% of 3H-DNP-lysine.

Antibodies to BSA were measured by a tanned red blood cell hemagglutination procedure (28). Serial twofold dilutions of serum were made in microtiter plates (Cooke Laboratory Products Div., Dynatech Laboratories, Inc., Alexandria, Va.) in 1% normal rabbit serum. The total and 2-ME-resistant antibody titer of each serum sample was measured simultaneously. The latter titer was determined by incubation of serum with an equal volume of 0.1 M 2-ME in saline for 30 min at room temperature before serial dilution. Antibody determinations are expressed as the log2 of the reciprocal of the final dilution of serum showing a smooth carpet of agglutinated cells. The titer of 2-ME-sensitive antibody = log2 titer total antibody − log2 titer 2-ME-resistant antibody.

Incubation of representative serum samples with 2-ME did not alter the titer of anti-DNP antibodies measured by the Farr assay. Therefore, only total anti-DNP antibody titers were recorded.

Results

Monospecificity of the Anti-8-Serum. As shown in Table I, the fully absorbed anti-8 serum did not bind IgG, IgM, or IgA, even though these Iggs could be effectively bound by sera specific for the appropriate H or L chains. When this absorbed serum was tested on lysates of radioiodinated splenocytes, only 8 and L chain could be precipitated (Fig. 1). However, despite its monospecificity, the absorbed serum was only 10–30% as strong as the anti-Ig serum and was therefore used at a fivefold higher concentration.

As shown in Fig. 2, prior depletion of the lysate with anti-8 (control) (panel A) failed to deplete either IgM or IgD from the supernate, as assessed by subsequent precipitation with anti-Ig. In contrast, pretreatment of the lysates with anti-8 (panel B), anti-8 (panel C), or anti-8 plus anti-8 (panel D) resulted in significant depletion or loss of the respective isotypes from the supernate. Prior precipitation of the lysate with anti-Ig (Fig. 3, panel B) but not anti-8 (Fig. 2, panel A) resulted in the depletion of radioactive molecules which were reactive with anti-8.

Taken together, these results suggest that the absorbed anti-8 serum recog-
### Table I

**Immunoprecipitation of Radioiodinated Myeloma Proteins With Monospecific Antisera**

| Rabbit antisera | Myeloma proteins | cpm immunoprecipitated |
|-----------------|------------------|------------------------|
|                 | IgG (~,κ)        | IgM (~,λ)              | IgA (~,κ)              |
| Anti-μ           | 3,120            | 45,785                 | 2,690                  |
| Anti-γ           | 56,110           | 1,798                  | 3,120                  |
| Anti-κγ          | 61,205           | 47,306                 | 56,580                 |
| Anti-MEM.κγ      | 58,314           | 41,207                 | 53,666                 |
| Anti-δ§          | 2,068            | 2,890                  | 2,785                  |

* Myeloma proteins (70,000 cpm/μg) were dissolved in PBS, centrifuged, and treated with saturating amounts of rabbit antisera. Complexes were bound to S. aureus, washed, eluted, and analyzed by SDS-PAGE.

† Unabsorbed anti-δ.

§ Anti-δ after absorption with Sepharose-euglobulin.

---

**Fig. 1.** Cell surface Ig precipitated from lysates of radioiodinated A/J splenocytes by unabsorbed (upper panel) or absorbed anti-δ serum. Each precipitate was dissolved, reduced, mixed with 3H-μ and L chains and electrophoresed for 16 h at 4 mA/gel. The 3H-IgM marker (HP-76 myeloma protein) has a heavy chain which occasionally migrates 0.5–1 tube ahead of normal μ-chain. In plotting the gel patterns, the marker proteins were aligned.

izes a surface Ig which is not IgM, IgA, or IgG. This Ig has a heavy chain of 65–68,000 daltons and by analogy with the human is murine IgD. The specificity of our antiserum is thus similar to that described by Abney et al. (15).

**Double Staining of Spleen Cells for Surface IgM and IgD.** In order to further test the specificity of the anti-IgD reagent, a series of experiments were
Fig. 2. Cell surface Ig recovered from the lysates of radioiodinated A/J splenocytes after removal of molecules reactive with (A) anti-\(\phi_X\), (B) anti-\(\delta\), (C) anti-\(\mu\), and (D) anti-\(\mu\) plus anti-\(\delta\). The supernate of each precipitate (A-D above) was treated with RAMIg and S. aureus. Material was eluted from the S. aureus and electrophoresed as described in Fig. 1.

**Fig. 3.** Cell surface IgD recovered from the lysates of radioiodinated A/J splenocytes after removal of molecules reactive with (A) anti-\(\phi_X\) and (B) anti-Ig. The supernate of each precipitate was treated with anti-\(\delta\) and S. aureus. The material eluted from the S. aureus was electrophoresed as described in Fig. 1.

designed to determine whether the reagent (a) stains predominantly those cells which also stain for IgM, (b) stains surface determinants which move to one pole of the cell (capping) independently of the surface IgM, and (c) stains surface determinants which can also be capped by the RAMIg reagent.

Double staining of spleen cells for surface IgM and IgD was carried out using a modified two-stage procedure. Cells were first stained for surface IgD as described in the Materials and Methods section except that a rhodamine-conjugated goat anti-rabbit IgG (kindly supplied by Doctors L. A. Herzenberg and M. Loken, Department of Genetics, Stanford University School of Medicine) was
Table II

Immunofluorescent Staining of Spleen Cells for Surface IgM and IgD Determined by Fluorescence Microscopy

| Two-stage staining procedure | Stage number | Staining pattern of IgM-bearing cells (fluorescein) | Staining pattern of IgD-bearing cells (rhodamine) |
|-----------------------------|--------------|---------------------------------------------------|--------------------------------------------------|
| Anti-IgD, 4°C and azide     | 1            | Diffuse*                                          | Diffuse‡                                         |
| Anti-IgM, 4°C and azide     | 2            |                                                   |                                                  |
| Anti-IgM, 37°C              | 1            | Capped                                            | Diffuse                                         |
| Anti-IgD, 37°C              | 2            |                                                   |                                                  |
| Anti-IgM, 4°C and azide     | 1            | Diffuse                                           | Capped                                          |
| Anti-IgD, 4°C and azide     | 2            |                                                   |                                                  |
| Anti-Ig, 37°C               | 1            | Capped                                            | –                                                |
| Anti-IgM, 4°C and azide     | 2            |                                                   |                                                  |
| Anti-Ig, 37°C               | 1            | –                                                 | Capped                                          |
| Anti-IgD, 4°C and azide     | 2            |                                                   |                                                  |

27% of total cells were doubles (IgM plus IgD).

* 29% of total cells stained positively for IgM.

‡ 31% of total cells stained positively for IgD.

used as the counterstain, and sodium azide was added to the tissue culture medium (0.1%). Under the latter conditions there is minimal capping of surface determinants. Cells were subsequently stained for surface IgM under similar conditions with a fluorescein-conjugated IgG fraction of rabbit anti-mouse \( \mu \)-chain antiserum (kindly supplied by Doctors L. A. Herzenberg and M. Loken).

Table II shows that, in a representative experiment, 29% of cells stained with fluorescein and 31% with rhodamine as judged by fluorescence microscopy. Double-staining cells accounted for 27% of the total cells counted. Less than 5% of cells showed cap formation.

The above experiment was repeated except that staining for surface IgD was performed at 37°C without azide (capping conditions). The majority of these cells showed staining with rhodamine at one pole (capping), but diffuse staining with fluorescein (Table II). In a similar experiment, cells were first stained with the fluorescein-conjugated anti-\( \mu \)-chain reagent at 37°C without azide (capping conditions), and then stained for surface IgD at 4°C with azide. The majority of these cells showed fluorescein caps, but diffuse staining with rhodamine (Table II). These findings show that surface determinants identified by the anti-\( \mu \) and anti-\( \delta \) chain reagents cap independently. In the last group of experiments, cells were first stained with RAMIg as described in the Materials and Methods section except that the temperature was maintained at 37°C (capping conditions). The cells were subsequently stained for surface IgM or IgD at 4°C with azide. In both cases, the fluorescein and rhodamine stains were localized to one pole of the cell (Table II). This shows that the anti-\( \delta \) chain reagent identifies a surface determinant which is an immunoglobulin or co-caps with immunoglobulin.

Analysis of the Percentage of Spleen Cells Bearing Different Ig Isotypes Using
**Table III**

Percentage of Mouse Spleen Cells Staining Brightly with Rabbit Anti-Mouse Total Ig or Heavy Chain Antiserum

| Exp. | Anti-Ig | Anti-delta | Anti-mu | Anti-gamma* |
|------|---------|------------|---------|-------------|
| 1    | 49      | 36         | 37      | 7           |
| 2    | 50      | 41         | 40      | 12          |
| Mean ± SD | 50.3 ± 9.2 | 43.1 ± 10.8 | 35.3 ± 5.83 | 12.5 ± 5.6 |

* F(ab')₂ fragment.
† Results of 13 experiments using groups of primed and unprimed mice. There were no differences observed between the two groups.

the FACS. Spleen cells were stained for surface Ig with RAMIg, RAμ, RAδ, or RAγ and counterstained with fluorescein-conjugated goat anti-rabbit IgG as described in the Materials and Methods section for the remainder of the experiments presented herein. Table III shows the mean percentage of cells staining brightly for total Ig (50.3%), μ-chain (35.3%), δ-chain (43.1%), and γ-chain (12.5%) as judged by analysis on the FACS. The mean percentage of cells staining positively for δ-chain exceeds that for μ-chain by about 8% and indicates that a minority of δ-bearing cells may have little or no μ-chain on the surface. However, these cells may have other surface isotypes such as IgG in addition to IgD. It is of interest that the sum of IgM- and IgG-positive cells approximates the percentage of cells positive for total Ig. The percentage of γ-staining cells was not spuriously elevated due to binding via the Fc receptor of the rabbit reagent, since F(ab')₂ fragments were used. Incubation of spleen cells for 1 h at 37°C before staining with RAγ did not alter the percentage of positively stained cells. This suggests that the IgG-bearing cells did not passively absorb IgG in vivo. However, macrophages were not removed from the cell suspensions and could contribute to the observed IgG-bearing cells. Background staining using the second-stage reagent alone was uniformly less than 1%.

Adoptive Transfer of Selected Cell Populations. In order to test the ability of purified IgM-, IgD-, or IgG-bearing cells to carry immunological memory, a syngeneic cell transfer system was designed using (BALB/C × C57/Ka)F₁ recipients given 650 R whole-body X irradiation. A dose-response curve was constructed by transferring graded numbers of anti-Thyl.2-treated spleen cells from donors immunized to DNP-BSA 8–12 wk earlier. An excess of nylon wool-purified lymph node and spleen cells from BSA-primed donors (helper T cells) was given simultaneously, and the adoptive hosts were challenged intraperitoneally with 200 μg of DNP-BSA in saline within 24 h after the cell transfer. Serum antibodies to both DNP and BSA were measured for 9 days thereafter.

Dependence of the Responses on T Cells. The antibody response to both DNP and BSA was found to be linear in the range of 0.1–5 × 10⁶ anti-Thyl.2-treated cells transferred, and was dependent upon the presence of primed T cells (Fig. 4). Cells from unprimed mice did not restore the adoptive response in the range tested (up to 20 × 10⁶ cells). The adoptive secondary response was higher and more rapid than the adoptive primary response at any B-cell dose as discussed in the subsequent paper (29). It is, therefore, likely that only B memory cells or their immediate precursors are active in this cell transfer system.
In subsequent experiments spleen cells were stained for surface μ, γ, δ-chains, or total Ig and sorted on the FACS. The dull and bright cells were injected with an excess of primed T cells into irradiated hosts (Fig. 5). Two doses of sorted cells equal to the number of bright or dull cells contained in either 2 x 10⁶ or 0.5 x 10⁶ unfractionated spleen cells were transferred. The higher dose was calculated to lie on the linear portion of the B-cell dose-response curve.

**FUNCTION OF Ig-BEARING CELLS.** Cells staining brightly with RAMIg restored an anti-BSA response similar to that of unfractionated cells at days 7 and 9 (Figs. 6 and 7). The dull cells made no detectable response at day 9. This indicates that the thresholds used for sorting are biologically meaningful, since they separate functional B cells from other cell types in the spleen. Both unfractionated and Ig-bearing spleen cells restored an anti-BSA antibody response which was all IgG (2-ME resistant) at days 7 and 9 (Figs. 6 and 7). Although 0.5 x 10⁶ anti-Thy1.2-treated spleen cells restored a minimal response at day 7 (Fig. 4), 0.5 x 10⁶ unfractionated cells produced a considerable response (Fig. 7). This could be due to damage of residual B cells by the killing procedure.

**FUNCTION OF CELLS BEARING IgM OR IgD.** Cells bearing surface IgM or IgD restored an anti-BSA response which was predominantly IgM at day 7 and IgG at day 9 (Figs. 6 and 7, Table IV). Dull cells contaminating the bright IgM- or IgD-bearing cells could not account for the IgG response on day 9 at the low dose, since a dose of unfractionated cells equal to the number of contaminating dull cells (0.02 x 10⁶) did not restore a detectable response (Fig. 7). Results of the adoptive secondary anti-DNP response were similar to the BSA response (Fig. 8).
FUNCTION OF CELLS BEARING IgG. The response stored by cells staining brightly for γ-chain was all IgG at both 7 and 9 days (Figs. 6 and 7). In addition, removal of bright γ-cells resulted in a dramatic increase in the IgM response, and a marked decrease in the IgG response on day 7 as compared to unfractionated cells. However, the IgG response at day 9 was similar to that of unfractionated cells. Removal of μ- or δ-bearing cells did not substantially alter the IgG
Fig. 6. Adoptive secondary anti-BSA response restored by DNP-BSA-primed cells sorted according to isotype of surface immunoglobulin. Mice were injected with $5 \times 10^6$ BSA-primed T cells and with a dose of bright or dull cells contained within $2 \times 10^6$ unfractionated BSA-DNP-primed spleen cells. Controls received $2 \times 10^6$ unfractionated cells plus T cells, or T cells alone. (A---A), unfractionated cells. (O---O), cells stained with RAγ. (●---●), cells stained with RAμ. (△---△), cells stained with RAMIg. (■---■), T cells alone. Each point represents the mean response of a group of mice and brackets show the standard error. There were 4 mice in each group given dull or bright cells, and 6-12 mice in each control group. Log$_2$ titer 2-ME-sensitive antibody = log$_2$ titer total antibody - log$_2$ titer 2-ME-resistant antibody.

Discussion

In this study, we tested the ability of IgM-, and IgD-, and IgG-bearing cells from the mouse spleen to carry immunological memory to the hapten-protein conjugate, DNP-BSA. The preparation and specificity of the rabbit anti-mouse IgG, IgM, and total Ig (RAγ, RAμ, and RAMIg) reagents have been reported in detail previously (16). Preparation of the rabbit anti-IgD reagent was similar to that reported by Abney et al. (15) and the serum was judged to be monospecific by several criteria. (a) The serum precipitated only the $^{125}$I-labeled IgD-like molecule extracted from the spleen cell surface after treatment with NP40 as judged by analysis on SDS-PAGE. (b) The antiserum does not cross-react with
Fig. 7. Adoptive secondary anti-BSA response restored by DNP-BSA-primed cells sorted according to isotype of surface immunoglobulin. Mice were injected with $5 \times 10^6$ BSA-primed T cells and with a dose of bright or dull cells contained within $0.5 \times 10^6$ unfractionated BSA-DNP-primed spleen cells. Controls received $0.5 \times 10^6$ unfractionated cells, $0.02 \times 10^6$ unfractionated cells plus T cells, or T cells alone. ($\Delta--\Delta$), $0.5 \times 10^6$ unfractionated cells. ($\nabla--\nabla$), $0.02 \times 10^6$ unfractionated cells. (○—○), cells stained with RA8. (●—●), cells stained with RAy. (□—□), cells stained with RAμ. (△—△), cells stained with RAMIg. (●—●), T cells alone. Each point represents the mean response of a group of mice and brackets show the standard error. There were 4 mice in each group given dull or bright cells, and 6–12 mice in each control group.

**Table IV**

| Immunoglobulin isotype on donor cells | Mean total antibody response* (log, titer) | Percentage of total antibody which is 2-ME resistant |
|--------------------------------------|------------------------------------------|--------------------------------------------------|
| IgM                                  | 9.3                                      | 2                                                |
| IgD                                  | 6.9                                      | 8                                                |
| IgG                                  | 8.7                                      | 100                                               |
| Unfractionated                       | 8.7                                      | 100                                               |

* Response restored by the number of bright cells contained within $2 \times 10^6$ unfractionated cells.
FIG. 8. Adoptive secondary anti-DNP response restored by DNP-BSA-primed cells sorted according to isotype of surface immunoglobulin. Mice were injected with $5 \times 10^6$ BSA-primed T cells and with unfractionated or fractionated BSA-DNP-primed spleen cells. Recipients were given a dose of dull or bright cells contained within either $2 \times 10^6$ (high dose) or $0.5 \times 10^6$ (low dose) unfractionated cells. Controls received either $2$, $0.5$, or $0.02 \times 10^6$ unfractionated cells plus T cells, or T cells alone. (A--A), $2 \times 10^6$ unfractionated cells. (C--C), $0.5 \times 10^6$ unfractionated cells; (V--V), $0.02$ unfractionated cells. (O--O), cells stained with RA8. (C--C), cells stained with RA9. (O--O), cells stained with RA9. (A--A), cells stained with RAM1g. (B--B), T cells alone. Each point represents the mean response of a group of mice and brackets show the standard error. There were 4 mice in each group given dull or bright cells, and 6-12 mice in each control group.

serum IgG, IgA, or IgM as judged by radioimmunoprecipitation. (c) 90% of (BALB/c x C57/Ka)F1 splenocytes stained with anti-IgD, also stained with anti-IgM. The surface determinants stained by the former reagent move to one pole of the cell (cap) independently from the surface IgM and visa versa. The determinants stained by the anti-IgD reagent are capped with the RAM1g reagent.

It was found that 35.3% of spleen cells stained with RA9, 43.1% with RA8, 12.5% with RA9, and 50.3% with RAM1g. Thus, the majority of the cells appeared to have IgM and IgD; a minority expressed either IgD or IgG alone, or both IgD and IgG together. However, double staining for IgG and IgD was not
performed. The percentage of IgA-bearing cells was not determined in the present study.

Cells expressing different Ig isotypes were prepared from the spleens of animals primed 8–12 wk earlier with DNP-BSA in adjuvant by means of the FACS. The "bright" or "dull" cells selected with each antiserum were adoptively transferred into syngeneic, irradiated mice, and the animals were given an excess of BSA-primed helper T cells. Within 1 day after transfer the animals were challenged with DNP-BSA. Serum antibody from the immunized mice was assayed by both the Farr technique and by passive hemagglutination. In the latter assay, 2-ME was used to distinguish IgM from IgG antibody.

These experimental results demonstrated that memory B cells or their immediate precursors bear surface IgM, IgD, or IgG. The IgM- and IgD-bearing cells carry immunological memory for both IgM and IgG responses. The IgG-bearing cells carry memory for only the IgG response. There are several possible interpretations for this data which depend on a number of different assumptions. If we assume that transferred cells can differentiate (and presumably acquire new isotypes) in the environment of the adoptively transferred host, then the precise surface characteristics of the memory cell at the time of triggering by antigen will be difficult to establish by the cell transfer technique. If differentiation does occur in the transferred host, then we could argue that less mature cells (perhaps IgM-only cells) would give rise to a wider spectrum of responses (IgM and IgG) than would the more mature cells (IgG only). On the other hand, if we assume that cell populations which are selected in the cell sorter represent stable, nondifferentiating cells, then memory cells can exist as multiple populations which express a variety of isotypes. Perhaps the exact nature of the isotype on a cell will depend on the route and nature of the immunization and the interval between immunization and adoptive transfer.

Recent findings in a number of systems have already suggested that cells giving rise to IgG memory can have either IgM or IgG on their surface (12–15, 30, 31) while cells giving rise to IgM memory usually bear predominantly IgM early after immunization, but decreased amounts of IgM as the interval between priming and adoptive transfer increases. In situations where IgM-bearing cells give rise to IgG memory there may be continual stimulation of virgin cells in the host such that recently activated memory cells express IgM. On the other hand, immunization with antigen in the absence of adjuvants or where the interval between priming and adoptive transfer of primed cells is long, may result in terminal differentiation of only IgG-bearing memory cells. Thus, the duration and nature of the antigenic stimulus, the efficiency of recruitment of cells into a lymphoid organ, and the dynamic and continuous differentiation of B lymphocytes in the host all play a role in determining the isotype on the memory cell.

IgG-bearing cells or their products suppress the ability of the IgD- or IgM-bearing cells to restore the IgM anti-BSA memory response, since depletion of IgG-bearing cells markedly enhanced this response. Evidence for the suppression of the IgM antibody response by passive infusion of IgG antibody has been reported previously (32). However, it is also possible that B-cell differentiation can be regulated by cell-cell interaction between subpopulations of B cells or perhaps by complex interactions between suppressor T cells and subsets of B cells. Regardless of the mechanism of suppression, this could account for the
minimal IgM memory response after secondary challenge with T-dependent antigens.

Our data may be summarized as shown in Fig. 9. Memory cells giving rise to IgM antibody lack IgG, but express IgM, IgD, or both. In contrast cells bearing IgM, IgD, or IgG can give rise to IgG memory. The latter cells suppress the IgM response of the former. We did not determine whether switching of surface immunoglobulin isotype can occur after B cells have acquired memory function.

Summary

We investigated the ability of IgM-, IgD-, and IgG-bearing cells from the spleens of (BALB/c × C57BL/Ka)F1 mice primed to dinitrophenyl-bovine serum albumin (DNP-BSA) to restore the adoptive secondary anti-BSA and anti-DNP antibody responses. A rabbit anti-mouse IgD antiserum was prepared and the specificity documented by radioimmunoprecipitation, and cell surface staining. Purified populations of IgM-, IgD-, and IgG-bearing cells were prepared by immunofluorescent staining with isotype-specific reagents, and sorting on the fluorescence activated cell sorter. Bright or dull cells were transferred to irradiated syngeneic recipients which were challenged with DNP-BSA in saline. Unfractionated spleen cells restored an adoptive secondary serum antibody response which was all IgG (2-mercaptoethanol resistant). Purified IgM- or IgD-bearing cells restored both the secondary IgM and IgG antibody response. IgG-bearing cells restored only the IgG response. In addition, the IgG-bearing cells appear to suppress the adoptive secondary IgM response, since depletion of IgG-bearing cells from transferred spleen cells results in a marked increase in the adoptive IgM response.

We thank Mr. F. Assisi, Ms. M. Bagby, Mr. Y. Chinn, Mr. S. Diase, Ms. C. Doss, Mr. M. Knapp, Mr. S. Lin, Ms. G. Sloane, and Ms. J. Terry for expert technical assistance. We are indebted to Dr.
IMMUNOGLOBULIN ISOTYPES ON MEMORY B CELLS

L. A. Herzenberg for his advice concerning the use of the FACS and gift of reagents, and to Doctors J. W. Uhr, I. L. Weissman, and Ms. D. Yuan for helpful discussions and comments concerning this work.

Received for publication 7 February 1977.

References

1. Van Boxel, J., W. E. Paul, W. D. Terry, and I. Green. 1972. Communications. IgD-bearing human lymphocytes. J. Immunol. 109:648.

2. Knapp, W., R. L. Bolhuis, J. Radl, and W. Hijmans. 1973. Independent movement of IgD and IgM molecules on the surface of individual lymphocytes. J. Immunol. 111:1295.

3. Rowe, D. S., K. Hug, L. Forni, and B. Pernis. 1973. Immunoglobulin D as a lymphocyte receptor. J. Exp. Med. 138:965.

4. Fu, S. M., R. J. Winchester, and H. G. Kunkel. 1974. Occurrence of surface IgM, IgD, and free light chains of human lymphocytes. J. Exp. Med. 139:651.

5. Abney, E., and R. M. E. Parkhouse. 1974. Candidate for immunoglobulin D present on murine B lymphocytes. Nature (Lond.). 252:600.

6. Vitetta, E. S., U. Melcher, M. McWilliams, J. Phillips-Quagliata, M. Lamm, and J. W. Uhr. 1975. Cell surface Ig. XI. The appearance of an IgD-like molecule on murine lymphoid cells during ontogeny. J. Exp. Med. 141:206.

7. Vitetta, E. S., and J. W. Uhr. 1976. Cell surface Ig. XV. The presence of IgM and IgD-like molecules on the same cell in murine lymphoid tissue. Eur. J. Immunol. 6:140.

8. Cooper, M. D., J. F. Kearney, A. R. Lawton, E. R. Abney, R. M. E. Parkhouse, J. L. Peud'homme, and M. Seligmann. 1976. Generation of immunoglobulin class diversity in B cells: a discussion with emphasis on IgD development. Ann. Immunol. (Paris) 127:573.

9. Goding, James 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.

10. McWilliams, M., M. E. Lamm, and J. M. Phillips-Quagliata. 1974. Surface and intracellular markers of mouse mesenteric and peripheral lymph node and Peyer's patch cells. J. Immunol. 113:1326.

11. Winchester, R. J., S. M. Fu, T. Hoffman, and H. G. Kunkel. 1975. IgG on lymphocyte surfaces; technical problems and the significance of a third cell population. J. Immunol. 114:1210.

12. 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.

13. 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.

14. Strober, S. 1976. Maturation of B lymphocytes in rats. III. Two subpopulations of memory B cells in the thoracic duct lymph differ by size, turnover rate, and surface immunoglobulin. J. Immunol. 117:1288.

15. Abney, E. R., K. D. Keefer, R. M. E. Parkhouse, and H. N. A. Wilcox. 1976. IgM receptor memory cells of IgG antibody forming clones. Eur. J. Immunol. 6:443.

16. Vitetta, E. S., J. Forman, and J. R. Kettman. 1976. Cell surface Ig. XVIII. Functional differences of B lymphocytes bearing different cell surface immunoglobulin isotypes. J. Exp. Med. 143:1055.

17. Abney, E., I. R. Hunter, and R. M. E. Parkhouse. 1976. Preparation and characterization of an antiserum to the mouse candidate for immunoglobulin D. Nature (Lond.). 259:403.
18. Vitetta, E. S., S. Baur, and J. W. Uhr. 1971. Cell surface Ig. II. Isolation and characterization of Ig from mouse splenic lymphocytes. *J. Exp. Med.* 134:242.

19. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent. Parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* 115:1617.

20. Vitetta, E. S., M. D. Poulik, J. Klein, and J. W. Uhr. 1976. Beta 2-microglobulin is selectively associated with H-2 and TL on murine lymphoid cells. *J. Exp. Med.* 144:179.

21. Nisonoff, A., F. C. Wissler, L. N. Lipmann, and D. L. Woernley. 1960. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. *Arch. Biochem. Biophys.* 89:230.

22. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. *J. Biol. Chem.* 245:3059.

23. Bonner, W. A., H. R. Hulett, R. G. Sweet, and L. A. Herzenberg. 1972. Fluorescence activated cell sorter. *Rev. Sci. Instrum.* 43:404.

24. 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.

25. Eisen, H. N., M. E. Carsten, and S. Belman. 1954. Studies of hypersensitivity to low molecular weight substances. III. The 2,4-dinitrophenyl group as a determinant in the precipitin reaction. *J. Immunol.* 73:296.

26. 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.

27. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I*-BSA and antibody. *J. Infect. Dis.* 103:239.

28. Stavitsky, A. B. 1954. Micro methods for the study of protein and antibodies. I. Procedure and general apparatus of haemagglutination and haemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. *J. Immunol.* 72:360.

29. 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 spleen. *J. Exp. Med.* 145:1206.

30. Pierce, C. W., S. M. Solliday, and R. Asofisky. 1972. Immune responses in vitro. IV. Suppression of primary γM, γG, and γA plaque-forming cell responses in mouse spleen cell cultures by class-specific antibody to mouse immunoglobulins. *J. Exp. Med.* 135:675.

31. Kishimoto, T., and K. Ishizaka. 1972. Regulation of antibody response in vitro. IV. Heavy chain antigenic determinants on hapten-specific memory cells. *J. Immunol.* 109:1163.

32. Uhr, J. W., and G. Möller. 1968. Regulatory effect of antibody on the immune response. *Adv. Immunol.* 8:81.