THE RELATIONSHIP BETWEEN SURFACE IMMUNOGLOBULIN ISOTYPE AND IMMUNE FUNCTION OF MURINE B LYMPHOCYTES

III. Expression of a Single Predominant Isotype on Primed and Unprimed B Cells*

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There are now several lines of evidence which indicate that B cells bearing different surface immunoglobulin isotypes are functionally distinct subsets of cells (1-10). Thus, negative (5) and positive (3, 4, 8, 9) selection studies as well as studies utilizing anti-Ig¹ reagents to block immune responses in vitro (1, 2, 6, 7, 10) suggest that cells bearing IgM or IgD give rise to an IgM antibody response followed by an IgG response. In contrast, cells bearing IgG give rise only to an IgG response (3-6, 8, 9) and suppress the IgM response of the cells bearing IgM and IgD (8, 9).

Because the majority of splenic B cells in the mouse bear more than one surface Ig isotype (11-14), a further delineation of B-cell subsets requires the use of additional techniques to determine the function of cells bearing one as opposed to multiple isotypes. For example, it has been hypothesized previously (15) that cells bearing both IgM and IgD give rise to an IgM response, and that cells bearing only IgD give rise only to an IgG response.

The goal of the present study was to determine whether B cells in the mouse spleen, which restore the adoptive antibody response to bovine serum albumin (BSA), contain subpopulations that express a single predominant Ig isotype. The experimental results reported here show that subpopulations of primed and unprimed B cells bearing predominantly IgM, IgD, or IgG (μ p, δ p, or γ p cells) are found in the spleen but in lower quantities than those subpopulations that

* Supported by National Institutes of Health grants AI 10293, 11851, and 12789.

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter; Ig, immunoglobulin; FCS, fetal calf serum; 2-ME, 2-mercaptoethanol; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline; RAMIg, rabbit anti-mouse Ig serum; RA δ , rabbit anti-mouse δ -chain antiserum; RA γ , rabbit anti-mouse γ -chain antiserum; RA μ , rabbit anti-mouse μ -chain antiserum, μ p, γ p, δ p cells, cells bearing predominantly surface IgM, IgG, or IgD, respectively.

express a combination of isotypes. The IgD-predominant cells differ from cells bearing combinations of IgM and IgD ($\mu^+\delta^+$) in that the former cells are found only in primed donors, and give rise only to the adoptive IgG antibody response. The latter cells are found in both unprimed and primed donors, and give rise to the adoptive IgM and IgG responses. IgG-predominant cells, also present in unprimed and primed animals, give rise to an adoptive IgG response, and (unlike the $\delta\mu$ cell) suppress the adoptive IgM response of IgM and IgD bearing cells. The relationships of the several B-cell subpopulations are discussed in the context of antigen-dependent and independent B-lymphocyte maturation.

Materials and Methods

Experimental Design. To prepare cells bearing a single Ig isotype, spleen cells were stained with a combination of antisera against two of the three major surface isotypes (e.g., anti- μ and anti- δ), and the positive cells were depleted by sorting on the fluorescence-activated cell sorter (FACS). The remaining B cells that express the surface isotype not stained for were used as the source of isotype-predominant (e.g., $\gamma\mu$) cells.

In cell transfer studies of the adoptive secondary response, spleen cells from (BALB/c $\times$ C57BL/Ka)F₁ mice immunized to DNP-BSA (Calbiochem, San Diego, Calif.) 8–12 wk earlier were sorted on the FACS and injected into syngeneic irradiated (650 rads) recipients. An excess of nylon wool-purified cells from donors immunized to BSA 8–12 wk earlier (helper T cells) were given to recipients, and the adoptive hosts were challenged within 24 h with DNP-BSA in saline. Serum antibodies to both BSA and DNP were measured for 2 wk thereafter. Control recipients received either 2×10^6 or 0.4×10^6 unfractionated DNP-BSA-primed spleen cells, and experimental recipients received a dose of bright (positively stained) or dull (negatively stained) cells from the FACS contained within 2×10^6 or 0.4×10^6 unfractionated cells. These cell doses were previously determined to be on the linear portion of a dose-response curve constructed by transferring graded numbers of anti-Thy 1.2-treated DNP-BSA-primed spleen cells to irradiated recipients given an excess of BSA-primed T cells (8). The anti-DNP and anti-BSA responses are both dependent on the presence of carrier-primed T cells and primed B cells (8).

In cell transfer studies of the adoptive primary response, spleen cells from unprimed mice were stained with various combinations of rabbit anti-mouse Ig isotype antisera, sorted on the FACS, and transferred to syngeneic irradiated (750 rads) recipients. Adoptive hosts were given an excess of nylon wool-purified T cells (5×10^6 cells) from unprimed donors, and challenged subcutaneously within 24 h with DNP-BSA in complete Freund's adjuvant. Serum antibodies to both BSA and DNP were measured for 4 wk thereafter. Control recipients received either 4 or 0.8×10^6 unfractionated cells, and experimental recipients received a dose of bright or dull cells contained within either 4 or 0.8×10^6 unfractionated cells. Our previous studies (9) show that the adoptive primary responses are completely dependent upon the transfer of the nylon wool T cells, and that the spleen cell doses chosen are on the linear portion of a dose-response curve constructed by transferring graded numbers of unprimed spleen cells treated in vitro with anti-Thy 1.2 antisera to irradiated recipients given an excess of T cells. Results of the adoptive primary and secondary anti-BSA response from representative experiments are reported here. Results of the anti-DNP response will be the subject of a separate communication.

Antisera

Rabbit Anti-Mouse- μ (RA μ ; 16). This serum was prepared against μ -chains isolated from MOPC-104E and was monospecific for both serum and cell surface IgM. The IgG fraction of this serum was purified on DEAE-Sephadex A50 (Pharmacia Fine Chemicals, Piscataway, N. J.) as described previously (8), and used for staining of cell surface IgM as outlined below.

Rabbit Anti-Mouse- δ (RA δ). This serum was made against a purified fraction of spleen cell surface Ig by a modification of the technique of Abney et al. (12) as described by Zan-Bar et al. (8). Details of the preparation of the membrane extract, immunizations, and absorption procedures, and assays for specificity have been published elsewhere (8). The batch of anti- δ used in these studies was extensively absorbed with thymocytes before use. In brief, the specificity of the antiserum used in the present report was documented as follows: (a) precipitation of only the " δ "

and "L" chain peaks of lysates of radioiodinated splenocytes as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8); (b) inability to bind radioiodinated IgM, IgG, and IgA myeloma proteins (8); (c) binding to the surface of spleen cells that bear IgM with redistribution (capping) of surface determinants independently of surface IgM (8); (d) binding to spleen cell surface determinants that co-cap with surface Ig identified with rabbit anti-mouse Ig antiserum (8); (e) failure to stain thymocytes (8); (f) failure to stain spleen cells previously stripped with either anti-Ig (M. Knapp, F. Assisi, and S. Strober, unpublished observations) or allotypic (17) anti- δ (F. Ligler, E. S. Vitetta, and J. Goding, unpublished observations).

Rabbit Anti-Mouse γ (RA γ ; 5). This serum was made against γ -chains (prepared from reduced and alkylated serum IgG) and was monospecific for IgG. The F(ab')₂ fragment of the IgG fraction of the serum was prepared as described previously (8), and used in all cell surface staining procedures in order to minimize binding via the Fc receptor.

Rabbit Anti-Mouse Ig (RAMIg; 16). This antiserum contains antibodies against μ -, γ -, α -, κ -, and λ -chains and is a pool of rabbit sera prepared against MOPC-104E (μ, λ), TEPC-15 (α, κ), and serum IgG.

Immunofluorescent Staining for Surface Ig. Spleen cells were stained for surface Ig with rabbit anti-mouse Ig isotype antisera, and counter-stained with the IgG-fraction of fluoresceinated goat anti-rabbit-IgG antiserum (Meloy Laboratories Inc., Springfield, Va.). The latter antiserum was thoroughly absorbed with thymocytes before use. Spleen cells were suspended in tissue culture medium 199 with 5% fetal calf serum (FCS) at a concentration of 20×10^6 cells/ml during all incubations. In some experiments cells were stained for three surface isotypes (IgM, IgD, and IgG) by either sequential incubations with RA μ , RA δ , and RA γ at 4°C for 30 min each, or by a single incubation with a combination of all three antisera. Similar procedures were used to stain cells for surface isotypes (IgM and IgD; IgM and IgG; or IgD and IgG) by incubating cells with RA μ and RA δ , RA μ and RA γ , or RA δ and RA γ , respectively. After incubation with rabbit antisera, the cell suspension was layered on FCS and centrifuged for 10 min at 150 *g*. The cell pellet was resuspended in culture medium with fluoresceinated goat anti-rabbit IgG antiserum for 30 min at 4°C, and washed before sorting or analysis.

All rabbit antisera were used for staining at a dilution determined to give the optimum percentage of positive cells over a 40-fold range of concentrations tested (4-0.1 mg/ml). RA γ was used at 1 mg/ml, RA δ at 2 mg/ml, and RA μ at 0.5 mg/ml. The optimum concentration of the goat antiserum was similarly chosen.

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 (18). 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 constituted the dull cell fraction. Details of the sorting procedure have been described elsewhere (19). 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 5×10^7 cells were sorted in each experiment at a rate varying between 3 and 5×10^3 cells/s.

In the cell transfer experiments, spleen cells were stained in culture medium that contained either a single anti-isotype antiserum, a combination of two anti-isotype antisera, or a combination of all three anti-isotype antisera (anti- μ + anti- γ + anti- δ). Cells were counterstained with a fluorescein-conjugated goat anti-rabbit Ig antiserum and sorted on the FACS. Experiments using all three antisera (Figs. 1 and 7) showed that the dull cells (IgM⁻, IgD⁻, IgG⁻) were unable to restore a detectable primary or secondary response to BSA, and the bright cells (IgM⁺ + IgD⁺ + IgG⁺) restored a response similar to that of unfractionated cells. It was, therefore, concluded that any restorative activity found in the dull cells after staining with a combination of two antisera is due to residual B cells which bear predominantly the isotype not stained for. For example, B cells contained within the dull cell fraction after staining with anti- μ and anti- δ antisera are those cells that express predominantly IgG (γ p). The isotype-predominant cells may bear small quantities of other isotypes that are below thresholds of detection by immunofluorescence microscopy. It is for this reason that the term "isotype predominant" is used.

The adoptive responses of these isotype-predominant cells (contained in the dull cell fraction)

were compared to those bright cells obtained after staining for the same isotype with a single antiserum. The latter cells (isotype-positive cells) contain both the isotype-predominant cells, and cells that express that isotype in combination with others.

Animals. (BALB/c $\times$ 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.

Irradiation of Mice. Mice were placed in Lucite containers and given either 650 rads (secondary response) or 750 rads (primary response) whole body X-irradiation from a single 250 kV (15 Å) source. The dose rate was 54 rads/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.

Immunization Procedures. Donors of carrier-primed cells were immunized with a subcutaneous injection (0.2 ml) of an emulsion of equal volumes of BSA (Calbiochem) 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 DNP-benzene sulfonate as reported previously (20).

In studies of the adoptive secondary response, irradiated recipients were challenged intraperitoneally with 200 μ g DNP-BSA in saline 1 day after cell transfer. Recipients used in the adoptive primary response were challenged subcutaneously with DNP-BSA in Freund's adjuvant as noted above.

Preparation of Cell Suspensions. Cell suspensions were prepared by mincing lymph nodes and spleens with scissors, and gently pressing the fragments through a nylon mesh. Cells were harvested by centrifugation at 150 g, and resuspended in Eagle's Minimum Essential Medium without sodium bicarbonate (MEM, Grand Island Biological Co., Grand Island, N. Y.) before intravenous injection into the lateral tail vein of irradiated recipients.

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. (21). Cell suspensions were applied to LP-1 Leuko-Pak filters (Fenwall Electronics, Framingham, Mass.) equilibrated with 5% FCS in MEM and maintained at 37°C. The percentage of T cells in the effluent was >95% as judged by killing with anti-Thy 1.2 antiserum. The percentage of Ig-bearing cells (B cells) was <5% as judged by immunofluorescent staining with RAMIg.

Collection of Serum Samples. Blood samples were collected from the retro-orbital vein. Serum from each sample was separated by centrifugation and stored at –20°C.

Antibody Titrations. Antibodies to BSA were measured by a tanned red blood cell hemagglutination procedure (22). 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-mercaptoethanol (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.

Results

Analysis of the Percentage of Spleen Cells Stained with Anti-Isotype Antisera Using the FACS. Spleen cells were stained with combinations of anti-isotype antisera, and sorted on the FACS to determine whether cells bearing a single predominant Ig isotype could be identified. Results of representative experiments are shown in Table I. In experiment 1 (Table I), cells were stained with RA μ , RA δ , and RA γ and sorted on the FACS to deplete the (μ^+ + δ^+ + γ^+) cells. The residual dull cells were restained with all four antisera (RAMIg, RA μ , RA δ , and RA γ). The percentage of Ig⁺, μ^+ , δ^+ , or γ^+ cells was <2% above the background level. The results of experiment 2 (Table I) show that depletion

TABLE I
Percentage of Spleen Cells Staining Positively for Surface Ig Isotype

Exp	Cells depleted by sorting on FACS	Positive cells before depletion (unfractionated cells)	Positive cells after depletion (dull cells)*	Positive cells corrected for background staining
		%	%	%
1	$\mu^+ + \delta^+ + \gamma^+$	Total Ig - 58.3	2.53	0.96
		μ^+ - 32.5	2.55	0.98
		δ^+ - 50.5	3.40	1.83
		γ^+ - 8.2	3.10	1.53
		BKG‡ - 0.5	1.57	-
2	$\delta^+ + \gamma^+$	Total Ig - 48.2	7.1	5.4
		μ^+ - 32.6	6.7	5.0
		δ^+ - 26.1	2.6	0.9
		γ^+ - 11.2	2.0	0.3
		BKG‡ - 0.5	1.7	-
3	$\mu^+ + \gamma^+$	Total Ig - 63	11.9	10.2
		μ^+ - 51	2.3	0.6
		δ^+ - 28	8.6	6.9
		γ^+ - 9.5	1.9	0.2
		BKG‡ - 1.5	1.7	-
4	$\mu^+ + \delta^+$	Total Ig - 60.9	4.1	2.4
		μ^+ - 31.0	1.2	0
		δ^+ - 28.2	1.3	0
		γ^+ - 14.9	2.8	1.1
		BKG‡ - 1.2	1.7	-

* Dull cells accounted for 42, 60, 43, and 40% of unfractionated cells in experiments 1-4, respectively.

‡ Background staining with second stage alone.

of ($\delta^+ + \gamma^+$) cells leaves a residual population of 6.7% μ^+ cells (background-1.7%) which accounts for almost all of the Ig-positive cells (7.1%). The percentage of μ^+ cells is disproportionately higher than that of the δ^+ (2.6%) and γ^+ (2.0%) cells. This indicates that there is a subpopulation of spleen cells that are μ^+ , but that do not bear quantities of surface IgD or IgG above the threshold set on the FACS and corresponding to detection by the naked eye. We have defined these cells as μ -predominant (μ p) cells.

Experiment 3 (Table I) shows that depletion of ($\mu^+ + \gamma^+$) cells leaves a residual population that contains a disproportionately high percentage (8.6%) of δ^+ cells (background-1.7%) as compared to μ^+ (2.3%) or γ^+ (1.9%) cells. The percentage of μ^+ and γ^+ cells was <1% above background. Thus, a small population of δ p as well as μ p cells is found in the adult spleen.

The results of experiment 4 suggest that there is a subpopulation of splenic γ p cells because the percentage of γ^+ cells (2.8%) after depletion of ($\mu^+ + \delta^+$) cells is above that of the μ^+ (1.2%), δ^+ cells (1.3%), and background (1.7%). However, the size of this subpopulation is variable (1-4%) and falls within the limits of error (1-2%) of our analysis of stained cells in most experiments.

FIG. 1. Adoptive secondary anti-BSA response restored by ($\mu^+ + \delta^+ + \gamma^+$) spleen cells. Cells were stained with a combination of RA μ , RA δ , and RA γ , and separated on the FACS. Irradiated mice received a dose of bright ($\mu^+ + \delta^+ + \gamma^+$) or dull (μ^- , δ^- , γ^-) cells contained within 2 or 0.4×10^6 unfractionated cells. All recipients were given 5×10^6 BSA-primed T cells. Controls received 2 or 0.4×10^6 unfractionated (unf) spleen cells plus T cells or T cells alone. Δ , 0.02×10^6 ($\mu^+ + \delta^+ + \gamma^+$) cells (dose of bright cells contaminating dulls); $\square$, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error. $\log_2$ titer 2-ME-sensitive antibody (IgM) = $\log_2$ titer total antibody - $\log_2$ titer 2-ME-resistant antibody (IgG).

Nevertheless, functional studies discussed below show that γ p cells can be readily detected by an independent assay system.

The variability of the percentage of μ^+ and δ^+ cells in the adult spleen before fractionation (Table I) has been observed previously (8, 9). This variation occurs between different groups of mice, but repeat staining and analysis of the spleen cells from a single group performed on the same day give percentages that are within the limits of error noted above.

The actual percentage of isotype-predominant cells in the whole spleen is lower than that shown in the dull cell fractions in Table I, because the dull cells represent only a portion of the whole spleen cell population. Accurate calculation of the percentage of isotype-predominant cells in the spleen is difficult, because there is a gap between the thresholds of the bright and dull cells due to our efforts to minimize contamination of the sorted cells. However, if one assumes that the dull cell population is homogeneous and contains all cells not stained positively, then the maximum net percentage of δ p, μ p, and γ p cells in the whole spleen is 3-4, 2-3, and 0.5-1%, respectively. As approximately 55% of splenic lymphocytes are B cells (8, 9), 11-16% of these B cells bear only one surface Ig isotype.

Secondary Anti-BSA Response Restored by Splenic B Cells. Fig. 1 shows the anti-BSA response restored by spleen cells stained for all three surface isotypes

FIG. 2. Adoptive secondary anti-BSA response restored by ($\delta^+ + \gamma^+$) spleen cells. Cells were stained with a combination of RA δ and RA γ , and separated on the FACS. Irradiated mice received a dose of bright ($\delta^+ + \gamma^+$) or dull (δ^-, γ^-) cells contained in 2 or 0.4×10^6 unfractionated cells. All recipients were given 5×10^6 BSA-primed T cells. Controls received 2 or 0.4×10^6 unfractionated cells plus T cells or T cells alone. Δ , 0.04×10^6 ($\delta^+ + \gamma^+$) cells (dose of bright cells contaminating dulls); $\square$, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error.

(μ , γ , δ) and sorted on the FACS. The kinetics and magnitude of the response produced by the ($\mu^+ + \delta^+ + \gamma^+$) cells were similar to those of the unfractionated cells. Anti-BSA antibodies in both cases were all IgG (2-ME resistant) at both days 7 and 9. Spleen cells depleted of all three isotypes produced no detectable anti-BSA response by day 9. These results indicate that staining cells with a combination of all three anti-isotype antisera depletes all functional B cells as efficiently as staining with a polyvalent anti-Ig (8).

Secondary Anti-BSA Response Restored by μp Cells. Fig. 2 shows the anti-BSA response restored by spleen cells stained for both surface " δ " and " γ ," and sorted on the FACS. The ($\delta^+ + \gamma^+$) cells restored an IgG response that was similar to that of the unfractionated cells. On the other hand, the (δ^-, γ^-) cells (μp cells) restored both an IgM and IgG response. The IgM antibody (2-ME sensitive) was predominant on day 7 and accounted for approximately 90% of the measured antibody (Table II). All antibody was IgG by day 9. Although a substantial response was produced by both ($\delta^+ + \gamma^+$) and unfractionated cells at the low cell dose (0.4×10^6 cells), no response was observed with a similar dose of (δ^-, γ^-) cells. These results indicate that the μp cells given in sufficient numbers can generate adoptive IgM and IgG responses.

Secondary Anti-BSA Response Restored by δp Cells. Fig. 3 shows the anti-BSA response restored by ($\mu^+ + \gamma^+$) and (μ^-, γ^-) (δp) spleen cells. Antibodies produced by the ($\mu^+ + \gamma^+$) and unfractionated cells were all IgG, and showed

TABLE II
Relative Quantity of 2-ME-Sensitive and 2-ME-Resistant Anti-BSA
Antibody on Day 7 of the Adoptive Secondary Response

Surface Ig isotype of donor cells*	Mean total antibody response ($\log_2$ titer)†	Percentage of 2-ME-resistant (IgG) antibody
		%
μ p	6.6	10
δ p	6.5	95
γ p	9.3	100
μ^+	9.3	2
δ^+	6.9	8
γ^+	8.7	100
$(\mu^+ + \delta^+)$	4.0	13
$(\mu^+ + \gamma^+)$	4.9	100
$(\mu^+ + \delta^+ + \gamma^+)$	4.6	100
$(\delta^+ + \gamma^+)$	8.0	100

* μ^+ , δ^+ , γ^+ denotes cells staining positively for given isotype. μ p, δ p, γ p denotes cells bearing predominantly single isotype.

† Adoptive antibody response restored by high dose of cells.

FIG. 3. Adoptive secondary anti-BSA response restored by $(\mu^+ + \gamma^+)$ spleen cells. Cells were stained with a combination of RA μ and RA γ , and separated on the FACS. Irradiated mice received a dose of bright $(\mu^+ + \gamma^+)$ or dull (μ^-, γ^-) cells contained in 2 or 0.4×10^6 unfractionated cells. All recipients were given 5×10^6 BSA-primed T cells. Controls received 2 or 0.4×10^6 unfractionated cells plus T cells or T cells alone. Δ , 0.04×10^6 $(\mu^+ + \gamma^+)$ cells (dose of bright cells contaminating dulls); $\square$, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error.

FIG. 4. Adoptive secondary anti-BSA response restored by ($\mu^+ + \delta^+$) spleen cells. Irradiated mice received a dose of bright ($\mu^+ + \delta^+$) or dull (μ^-, δ^-) cells contained within 2 or 0.4×10^6 unfractionated cells. All recipients were given 5×10^6 BSA-primed T cells. Δ , 0.03×10^6 ($\mu^+ + \delta^+$) cells (dose of bright cells contaminating dulls); $\square$, T cells alone. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

similar titers on days 7 and 9 for both cell doses. Although the response restored by (μ^-, δ^-) (δp) cells was similar to that of the ($\mu^+ + \gamma^+$) cells at the high cell dose, δp cells made no response at the low cell dose. These results indicate that δp cells generate only an IgG response.

Secondary Anti-BSA Response Restored by γp Cells. The anti-BSA response restored by ($\mu^+ + \delta^+$) cells (Fig. 4) differed from that of the ($\mu^+ + \gamma^+$), ($\gamma^+ + \delta^+$), and ($\mu^+ + \delta^+ + \gamma^+$) cells in that the former cells produced a predominantly IgM response at day 7 (Table II), and the latter cells produced only IgG antibody. The (μ^-, δ^-) cells (γp) produced only IgG antibody with kinetics similar to that of unfractionated cells at the high cell dose. However, the γp cells (as well as δp and μp cells) produced no detectable response at the low cell dose. These results indicate that γp cells generate an IgG response, and suppress the IgM response of μ^+ and δ^+ cells.

Comparison of the Adoptive Secondary Anti-BSA Response Restored by Isotype-Positive and Isotype-Predominant Cells. Figs. 5 and 6 compare the adoptive IgM and IgG anti-BSA responses restored by μ^+, δ^+ , and γ^+ cells with those restored by μp , δp , and γp cells, respectively, at high and low cell doses. The isotype-positive cells ($\mu^+, \delta^+, \gamma^+$) were purified by staining with a single anti-isotype antiserum and sorting on the FACS. Fig. 5 shows that the kinetics and magnitude of the responses produced by μp and μ^+ , and γp and γ^+ cells, respectively, are similar. In contrast, the response produced by the δ^+ cells

FIG. 5. Comparison of the adoptive secondary anti-BSA responses restored by isotype-positive and isotype-predominant cells (high cell dose). Isotype-positive cells are in the bright cell fraction obtained after staining with a single anti-isotype antiserum. Isotype-predominant cells are in the dull cell fraction obtained after staining with two anti-isotype antisera. Irradiated recipients received a dose of μ^+ , δ^+ , γ^+ , μp , δp , or γp cells contained within 2×10^6 unfractionated cells. All recipients were given 5×10^6 BSA-primed T cells. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

FIG. 6. Comparison of the adoptive secondary anti-BSA responses restored by isotype-positive and isotype-predominant cells (low cell dose). Irradiated recipients received a dose of μ^+ , δ^+ , γ^+ , μp , δp , or γp cells contained within 0.4×10^6 unfractionated cells. All recipients were given 5×10^6 BSA-primed T cells. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

FIG. 7. Adoptive primary anti-BSA response restored by ($\mu^+ + \delta^+ + \gamma^+$) spleen cells. Cells were stained with a combination of RA μ , RA δ , RA γ and separated on the FACS. Irradiated mice received a dose of bright ($\mu^+ + \delta^+ + \gamma^+$) or dull (μ^- , δ^- , γ^-) cells contained within 4 or 0.8×10^6 unfractionated cells. All recipients were given 5×10^6 unprimed T cells. Controls received 4 or 0.8×10^6 unfractionated (unf) spleen cells plus T cells or T cells alone. Δ , 0.08×10^6 ($\mu^+ + \delta^+ + \gamma^+$) cells (dose of bright cells contaminating dulls); $\square$, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error. $\log_2$ titer 2-ME-sensitive antibody (IgM) = $\log_2$ titer total antibody - $\log_2$ titer 2-ME-resistant antibody (IgG).

differed from that of the $\delta\mu$ cells in that the former is predominantly IgM at day 7 (Table II) and the latter is all IgG. In addition, the 7-day IgG response of the $\delta\mu$ cells is considerably greater than that of the δ^+ population. This suggests that subpopulations within the set of δ^+ cells may modulate the immune responses of one another.

Fig. 6 shows that the isotype-predominant cells were unable to restore the anti-BSA response at the low cell dose, but the same dose of isotype-positive cells restored a considerable response. A comparison of Figs. 5 and 6 shows that about five times as many isotype-predominant cells are required to give a response similar to that restored by a given number of isotype-positive cells. This indicates that the large majority of memory cells that contribute to the adoptive secondary anti-BSA response bear more than one surface Ig isotype.

Primary Anti-BSA Response Restored by Splenic B Cells. Fig. 7 shows the adoptive primary anti-BSA response restored by spleen cells stained for all three surface isotypes, and sorted on the FACS. The kinetics and magnitude of the response restored by the ($\mu^+ + \delta^+ + \gamma^+$) cells were similar to that of equivalent doses of unfractionated cells. The response was mainly IgG at days 20 and 25. The (μ^- , δ^- , γ^-) cells produced no detectable response at the high and

FIG. 8. Adoptive primary anti-BSA response restored by $(\gamma^+ + \delta^+)$ spleen cells. Cells were stained with a combination of RA δ and RA γ , and separated on the FACS. Irradiated mice received a dose of bright $(\gamma^+ + \delta^+)$ or dull (γ^-, δ^-) cells contained in 4 or 0.8×10^6 unfractionated cells. All recipients were given 5×10^6 unprimed T cells. Controls received 4 or 0.8×10^6 unfractionated cells plus T cells or T cells alone. Δ , 0.04×10^6 $(\gamma^+ + \delta^+)$ cells (dose of bright cells contaminating dulls); $\square$, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error.

low cell doses. These results indicate that staining cells with a combination of anti-isotype antisera depletes all functional B cells as efficiently as staining with polyvalent anti-Ig (9).

Primary Anti-BSA Response Restored by μp Cells. Fig. 8 shows that the $(\delta^+ + \gamma^+)$ spleen cells restored a vigorous anti-BSA response at both cell doses similar to that restored by the unfractionated cells. Almost all antibody at day 20 was IgG. On the other hand, the $(\delta^-\gamma^-)$ cells (μp) restored a vigorous response only at the high cell dose, and most of the antibody was IgM at days 20 and 24 (see Table III). However, all antibody was IgG at day 29. Thus, the μp cells, although present in small numbers, can give rise to both IgM and IgG responses.

Primary Anti-BSA Response Restored by γp Cells. The adoptive primary response produced by $(\mu^+ + \delta^+)$ cells was mainly IgM at day 20 and switched to IgG by day 25 at the high cell dose (Fig. 9, Table III). The γp cells restored a detectable response only at the high cell dose. The latter response was all IgG (Fig. 9). Thus, γp cells are found in the unprimed animal and can give rise to an IgG response.

Primary Anti-BSA Response Restored by δp Spleen Cells. Fig. 10 shows that $(\mu^+ + \gamma^+)$ cells restored only an IgG response similar to that of unfractionated cells at days 20 and 25 at both cell doses. It is of interest that the former response was delayed about 4 days as compared with the latter. The δp cells failed to make a detectable response at both the high and low cell doses, and, thereby differed in their restorative activity from the μp and γp cells.

TABLE III
Relative Quantity of 2-ME-Sensitive and 2-ME-Resistant Antibody to BSA
on Day 20 of the Adoptive Primary Response

Ig isotype on donor cells	Mean total anti-body response ($\log_2$ titer)*	Percentage of 2-ME-re-sistant (IgG) antibody
		%
μ P	3.0	0
δ P	—	—
γ P	2.5	100
μ^+	5.9	13
δ^+	3.9	13
γ^+	5.4	100
$(\mu^+ + \delta^+)$	7.3	8
$(\mu^+ + \gamma^+)$	5.4	100
$(\delta^+ + \gamma^+)$	6.0	100
$(\mu^+ + \delta^+ + \gamma^+)$	6.0	70

* Adoptive antibody response restored by high dose of cells.

FIG. 9. Adoptive primary anti-BSA response restored by $(\mu^+ + \delta^+)$ spleen cells. Cells were stained with a combination of RA μ and RA δ , and separated on the FACS. Irradiated mice received a dose of bright $(\mu^+ + \delta^+)$ or dull (μ^-, δ^-) cells contained in 4 or 0.8×10^6 unfractionated cells. All recipients were given 5×10^6 unprimed T cells. Controls received 4 or 0.8×10^6 unfractionated cells plus T cells or T cells alone. Δ , 0.03×10^6 $(\mu^+ + \delta^+)$ cells (dose of bright cells contaminating dulls); $\square$, T cells alone. Each point represents the mean response of a group of four to eight mice, and brackets show the standard error.

FIG. 10. Adoptive primary anti-BSA response restored by ($\mu^+ + \gamma^+$) spleen cells. Irradiated mice received a dose of bright ($\mu^+ + \gamma^+$) or dull (μ^-, γ^-) cells contained within 4 or 0.8×10^6 unfractionated cells. All recipients were given 5×10^6 unprimed T cells. Δ , 0.06×10^6 ($\mu^+ + \gamma^+$) cells (dose of bright cells contaminating dulls); $\square$, T cells alone. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

FIG. 11. Comparison of the adoptive primary anti-BSA responses restored by isotype-positive and isotype-predominant cells (high cell dose). Isotype-positive cells are in the bright cell fraction obtained after staining with a single anti-isotype antiserum. Isotype-predominant cells are in the dull cell fraction obtained after staining with two anti-isotype antisera. Irradiated recipients received a dose of μ^+ , δ^+ , γ^+ , μp , δp , or γp cells contained within 4×10^6 unfractionated cells. All recipients were given 5×10^6 unprimed T cells. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

FIG. 12. Comparison of the adoptive primary anti-BSA responses restored by isotype-positive and isotype-predominant cells (low cell dose). Irradiated recipients received a dose of μ^+ , δ^+ , γ^+ , μp , δp , or γp cells contained within 0.8×10^6 unfractionated cells. All recipients were given 5×10^6 unprimed T cells. Each point represents the mean of a group of four to eight mice, and brackets show the standard error.

Comparison of the Primary Anti-BSA Response Restored by Isotype-Positive and Isotype-Predominant Cells. Fig. 11 compares the IgM and IgG responses restored by isotype-positive and isotype-predominant cells at the high cell dose. Although the μ^+ and μp cells produced both an adoptive IgM and IgG response, the switch to IgG synthesis with the latter cells was delayed by 4 days with respect to the former. This suggests that the μp cell is a less mature B cell than the $\mu^+ \delta^+$ cell and, therefore, requires further maturation in the adoptively transferred host before giving rise to the IgG response. The γ^+ and γp cells produced similar adoptive responses which were all IgG. The most dramatic difference between isotype-positive and predominant cells was observed with the δ^+ and δp cells. The former subpopulation restored both the IgM and IgG response, but the latter produced no detectable antibody at the high cell dose. These results suggest that the IgM-IgD double bearers are the major precursors of the primary IgM response but that these cells do not undergo significant differentiation into δp cells in the absence of antigen.

Fig. 12 shows that the adoptive responses restored by isotype-positive cells were considerably reduced at the low cell dose. However, none of the isotype-predominant cells produced detectable responses at the same dose. This shows that the contribution of isotype-predominant cells to the adoptive primary response is less than that of isotype-positive cells.

Discussion

The object of these experiments was to identify B cells in the mouse spleen that bear a predominant Ig isotype and to determine the functional activity of these cells in the adoptive primary and secondary anti-BSA responses. To isolate and examine such putative isotype-predominant B cells, we first stained

spleen cells for all three major surface isotypes (μ , δ , γ) simultaneously and separated the bright (positively staining) and dull (negatively staining) cells on the FACS. The restorative activity of the bright cells was similar to unfractionated cells, but no detectable activity was observed with the dull cells. In subsequent experiments, spleen cells were stained for two of the three surface isotypes, and the restorative activity of the dull cells was examined. Adoptive responses produced by such dull cells were attributed to the presence of B cells bearing only the remaining surface isotype which was not detected by the staining procedure.

Comparisons were made between the responses restored by isotype-positive cells (bright cells isolated by the FACS after staining spleen cells for a single surface isotype) and those restored by isotype-predominant cells. The isotype-positive cells include all cells staining positively for a given isotype, and include isotype-predominant cells as well as those cells that bear that isotype in combination with others. The results show that the contribution of isotype-predominant cells to the adoptive primary and secondary responses restored by a given number of spleen cells is at least five times smaller than that of the isotype-positive cells. This suggests that the large majority of B cells bearing a given surface isotype express that isotype in combination with others. This finding is consistent with the considerably greater percentage of isotype-positive as compared with isotype-predominant cells observed in the spleen by direct analysis of stained cells on the FACS. For example, the mean percentage of δ^+ cells in spleen cells was 43% (8, 9), and the calculated percentage of δp cells in the present experiments was 3-4%. Similarly determined values for μ^+ and μp are 35 and 2-3%, respectively, and for γ^+ and γp are 13% and 0.5-1% (8, 9), respectively.

The experimental findings show that μp , δp , and γp cells are all active in restoring the adoptive secondary anti-BSA response and, therefore, carry immunological memory. The response produced by μp cells is almost all IgM at day 7, and switches to IgG antibody (2-ME resistant) by day 9. On the other hand, both the δp and γp cells restore only an IgG response with rapid kinetics so that day 7 titers are already approaching the plateau levels. The μp cells may not be typical long-lived, recirculating memory cells, but rather, recently activated virgin B cells which are their immediate precursors. Recent studies in rats have shown that IgM-bearing blast cells that carry immunological memory are found in the thoracic duct lymph for several weeks after priming with antigen in complete Freund's adjuvant (23).

The kinetics and magnitude of the secondary anti-BSA response restored by μ^+ and μp cells on the one hand, and by γ^+ and γp cells on the other, were similar at the high cell dose (equivalent of 2×10^6 cells). The IgG antibody produced by the μp cells could not be accounted for by the contaminating (γ^+ + δ^+) cells because the transfer of a dose of (γ^+ + δ^+) cells that represents the maximum number that could have contaminated the μp inoculum does not produce a detectable IgG response. In addition, the (μ^- , δ^- , γ^-) cells, which should have the same γ^+ contamination as the (γ^- , δ^-) cells, do not restore an adoptive response.

These findings strongly suggest that only B cells bearing surface IgM (alone

or in combination with other isotypes) give rise to IgM secreting progeny. This interpretation is consistent with our own previous results (8, 9), the results of Pierce et al. (1, 2), Yuan et al. (5), Coffman and Cohn (6), and Cambier et al. (10). However, our finding that μp cells give rise to IgG secreting progeny is more controversial because several laboratories have reported that only IgG-bearing cells give rise to the adoptive secondary IgG response (3, 4). Some of the differences may be reconciled by the differences in the kinetics of the IgG response restored by different B-cell subpopulations. For example, IgG-positive cells give rise to a rapid IgG response approaching the plateau on day 7. On the other hand, IgM-positive or IgM-predominant cells give rise to a 7-day response that is almost all IgM, and only produces a substantial IgG response at day 9. Measurement of the early indirect plaque-forming cell response at only one time-point would suggest that only the γ^+ cells carry immunological memory.

The response restored by the δ^+ cells differed from that of the δp cells at this dose, in that the former generated first an IgM and then an IgG response, whereas the latter generated only an IgG response. This suggests that cells bearing both IgM and IgD give rise to progeny that secrete first IgM and then IgG. In contrast, δp cells (which are μ^-) give rise to progeny that secrete only IgG. This observation is consistent with a model of B-cell differentiation suggested previously (15). Thus, as IgM-IgD double bearers are stimulated by antigen they may lose surface IgM and become δp cells. The δp cells are committed to IgG synthesis. Although the δp cells are a minor subpopulation of B cells in the spleen, these cells appear to make up the majority of B cells in the thoracic duct lymph (24).

Regulatory interactions between subpopulations of B cells were observed in experiments in which μ^+ and/or δ^+ cells were mixed with γ^+ or γp cells. Vigorous adoptive IgM responses were restored by μ^+ , δ^+ , μp cells, or a combination of μ^+ and δ^+ cells. However, no IgM response was produced by combinations of ($\mu^+ + \gamma^+$), ($\delta^+ + \gamma^+$), or ($\mu^+ + \delta^+ + \gamma^+$) cells. This shows that γ^+ cells or their products suppress the IgM response of the μ^+ and δ^+ cells. It is likely that only the γp cells within the γ^+ population are capable of suppressing the IgM response, because the combination ($\mu^+ + \delta^+$) cells gives rise to IgM antibody, and this population lacks only the γp subpopulation as compared to ($\mu^+ + \delta^+ + \gamma^+$) cells. The γp cells maintain their suppressive activity even at the low doses at which they are unable to restore a detectable IgG response. Thus, ($\mu^+ + \gamma^+ + \delta^+$) cells produce only IgG antibody, ($\mu^+ + \delta^+$) cells produce both IgM and IgG antibody, and γp cells produce no detectable antibody at the low cell dose. The findings raise the possibility that the mechanism of suppression may occur via cell-cell interaction rather than by serum IgG antibody. However, we have not excluded the possibility that low levels of serum antibody not detectable by the procedures employed are responsible for the inhibition. It is interesting to note that the δp cells do not appear to suppress the IgM response of μ^+ cells, despite the fact that δp cells give rise only to IgG antibody. The difference in the ability of the two subsets to suppress the IgM response may be due to the affinity of the antibody they produce. Thus a small amount of high affinity IgG produced by the γp cells may adequately suppress the IgM response. In contrast, δp cells may give rise to lower affinity antibody which, when present in small amounts, is not suppressive.

The results of our studies of the adoptive primary anti-BSA response were similar to those of the adoptive secondary. Unprimed μp cells restored the adoptive primary IgM and IgG responses, but unprimed γp cells restored only the IgG response. The contribution of μp and γp cells to the adoptive primary response was considerably smaller than that of the μ^+ and γ^+ cells (isotype-positive cells), because only the latter cells made a detectable response at the low cell dose (equivalent of 0.8×10^6 cells). The anti-BSA response of the μp cells differed from that of the μ^+ cells in that the switch from IgM to IgG antibody was delayed by about 4 days. These results suggest that the μp cells are a less differentiated population of cells than the majority of the μ^+ cells (which are $\mu^+ \delta^+$ doubles). The existence of μp cells has been postulated previously, and it is possible that these cells are the precursors of the $\mu^+ \delta^+$ cells. As in the adoptive secondary response, the γp cells suppressed the IgM response of the μ^+ and δ^+ cells. This is shown by a comparison of the adoptive primary anti-BSA responses restored by $(\mu^+ + \delta^+)$ cells and $(\mu^+ + \delta^+ + \gamma^+)$ cells. The latter cells include the former, and in addition contain γp cells. Whereas the $(\mu^+ + \delta^+)$ cells produce an adoptive response that is almost all IgM at day 20, the $(\mu^+ + \delta^+ + \gamma^+)$ produce a response of similar magnitude that is mainly IgG.

The role of γ^+ and γp cells in the adoptive primary response remains to be elucidated. The cells could be the product of prior exposure of the normal (BALB/c $\times$ C57BL/Ka)F₁ mice to environmental antigens that cross react with determinants expressed by BSA. On the other hand, these subpopulations may be derived from B-cell precursors via an antigen-independent maturation sequence as suggested by Cooper and his colleagues (25). In that case, the γp or γ^+ cells may be members of a separate line of B cells that mature parallel with the μp and $\mu^+ \delta^+$ cells.

The ability of the unprimed μp cells to give rise to IgG antibody extends and confirms our earlier observation that B cells that do not stain positively for surface IgG can, nevertheless, transfer an adoptive primary IgG response. Contamination of μp cells staining positively for surface IgG cannot account for the adoptive IgG response because the transfer of a dose of IgG-positive cells equivalent to the maximum number that could contaminate the μp cells does not produce a detectable adoptive primary IgG response. In addition, the $(\mu^-, \delta^-, \gamma^-)$ cells, which should have the same γ^+ contamination as the γ^-, δ^- (μp) cells, do not restore an adoptive response. The present study did not determine whether μp cells must express a small quantity of surface IgG below the levels of detection by fluorescence microscopy, or whether the cells acquire surface IgG during residence in the adoptive host.

The inability of δp cells to transfer the adoptive primary anti-BSA response contrasts with the considerable restorative activity of μp and γp cells in the primary response, and δp cells in the secondary response. This suggests that functional δp cells are present in very small numbers, if at all, among unprimed B cells, but are present in much larger quantities relative to all other B-cell subpopulations after priming. Indeed, it is possible that significant restorative activity in the δp subpopulation of a given mouse is a marker for prior exposure to the antigenic determinants under investigation. Because the relationship between the two IgG precursors (δp and γp) is not clear at present, it is difficult

to speculate on the presence of the latter but not the former in unprimed mice. It is possible that the two cells are derived as separate lineages or that the accumulation of γp cells is less antigen dependent than that of the δp cells. In addition, there may be unique regulatory mechanisms in unprimed mice that allow the accumulation of γp but not δp cells.

The placement of the isotype-predominant cells in the scheme of antigen-independent and antigen-dependent B-cell maturation remains speculative. However, a model consistent with our experimental findings would place B-cell precursors bearing only surface IgM in the neonatal spleen and adult bone marrow. These cells may give rise to the unprimed B cells in the adult spleen which include populations of μ^+ , δ^+ , and γ^+ cells. All three populations make a considerable contribution to the adoptive primary response. The μp and γp cells are minor subpopulations contained within the set of μ^+ and γ^+ cells. It is possible that the γ^+ set present in unprimed mice are the result of stimulation of μ^+ and δ^+ cells with environmental antigens that cross react with the determinants under investigation. On the other hand, these cells may be members of a lineage that develops from the young μp cells via antigen-independent maturation in parallel with the (μ^+ , δ^+) cells.

This general maturation scheme may be recapitulated after antigenic stimulation. Primed μp cells may represent recently activated virgin B cells that differentiate into (μ^+ , δ^+) and γ^+ memory cells. On the other hand, γ^+ memory cells may arise in parallel to (μ^+ , δ^+) memory cells from γ^+ virgin cells.

Summary

We determined whether primed and unprimed B cells in the spleen of (BALB/c $\times$ C57BL/Ka)F₁ mice contain subpopulations that express a predominant surface Ig isotype. Spleen cells were stained for surface isotypes and sorted on the fluorescence-activated cell sorter (FACS) in order to obtain B cells bearing predominantly IgM (μp cells), IgD (δp cells), or IgG (γp cells). Each population was assayed for its capacity to restore the adoptive primary and secondary anti-bovine serum albumin (BSA) antibody response in irradiated syngeneic recipients. In addition, the adoptive response restored by isotype-predominant cells was compared to that restored by isotype-positive cells (B cells bearing a given surface isotype alone or in combination with others). The experimental results show that μp cells restore the adoptive primary and secondary IgM and IgG responses to BSA, and γp cells restore only the primary and secondary IgG response. δp Cells restored the adoptive secondary IgG response, but failed to restore the adoptive primary response at the cell doses tested. γp Cells but not δp cells suppressed the IgM response of the μ^+ and δ^+ cells. The contribution of isotype-predominant cells to both the adoptive primary and secondary anti-BSA response was smaller than that of B cells bearing a combination of surface isotypes. Differences in the Ig isotype pattern expressed on the surface of primed and unprimed B cells are discussed.

We acknowledge the expert technical assistance of Ms. M. Bagby, Mr. Y. Chinn, Mr. S. Lin, Ms. G. Sloan, Mr. M. Knapp, Ms. C. Doss, and Ms. J. Terry. We thank Ms. M. Janisch and Ms. J. Hahn for secretarial assistance.

Received for publication 28 October 1977.

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