

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

II. Surface Immunoglobulin Isotopes on Unprimed B Cells in the
Spleen*

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In the preceding paper (1) we examined the ability of cells bearing IgM, IgD, and IgG to restore the adoptive secondary response of irradiated hosts to a thymus-dependent antigen, dinitrophenyl-bovine serum albumin (DNP-BSA).¹ In the present report, we examined the ability of IgM-, IgD-, and IgG-bearing cells from the spleens of unprimed mice to restore the adoptive primary response to DNP-BSA. Studies of the adoptive primary antibody response of rats to horse spleen ferritin have shown that IgM-bearing cells in the spleens of unprimed donors are able to restore both the IgM and IgG antibody response (2). The role of IgD- and IgG-bearing cells was not examined in the latter work. Recent studies in mice suggest that IgM- but not IgG-bearing cells from the spleens of unprimed animals restore the adoptive direct plaque-forming response of irradiated hosts to the trinitrophenyl hapten.² However, the role of IgD-bearing cells in the adoptive direct or indirect plaque-forming response was not examined.

The experiments reported herein differ from those described above, in that the role of cells bearing all three major surface immunoglobulin isotypes in both the adoptive primary IgM and IgG responses was investigated. The experimental results are similar to those reported for the adoptive secondary response (1). IgM- and IgD-bearing cells give rise to the adoptive IgM and IgG responses, but IgG-bearing cells give rise only to the IgG response. The latter cells suppress the IgM response of the former. The roles of the IgG- and IgD-bearing cells in unprimed mice are discussed.

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; FACS, fluorescence activated cell sorter; 2ME, 2-mercaptoethanol; RAMIg, rabbit anti-mouse Ig antiserum; RA δ , rabbit anti-mouse δ -chain antiserum; RA γ , rabbit anti-mouse γ -chain antiserum; RA μ , rabbit anti-mouse μ -chain antiserum.

² Yuan, D., E. S. Vitetta, and J. Kettman. Cell surface Ig. XX. Antibody responsiveness of subpopulations of B lymphocytes bearing different isotypes. Manuscript submitted for publication.

Materials and Methods

Antisera. Preparation and specificity of rabbit anti-mouse Ig (RAMIg), IgM (RA μ), IgD (RA δ), and IgG (RA γ) antisera are described in the preceding report (1).

Immunofluorescent Staining for Surface Immunoglobulin. A two-stage procedure was used to stain surface Ig of mouse spleen cells (1). The IgG fraction of RAMIg, RA μ , and RA δ , or the F(ab')₂ fragment of RA γ were used as first-stage reagents. A fluorescein-conjugated IgG fraction of goat anti-rabbit IgG antiserum (Melyo Laboratories, Springfield, Va.) was used as the second-stage reagent.

Sorting and Analysis of Cells with the Fluorescence Activated Cell Sorter (FACS). Spleen cells stained for surface Ig were analyzed and sorted on the FACS using thresholds which correspond to positively staining cells as judged by fluorescence microscopy (1).

Animals. (BALB/c \times C57BL/Ka)F₁ female mice, 2–3 mo of age were used in all experiments. Animals were bred in the Department of Radiology, Stanford University School of Medicine, Stanford, Calif.

Immunization Procedures. Irradiated recipients of transferred spleen and lymph node cells were challenged with a single subcutaneous injection (0.2 ml) of an emulsion of equal volumes of DNP₁₈-BSA in saline and complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Each animal received a total of 0.5 mg protein.

Cell Suspensions. Suspensions of spleen and lymph node cells were prepared as described previously (1).

In Vitro Killing with Anti-Thy1.2 Antiserum. Spleen cells were depleted of lymphocytes by a two-stage complement-dependent in vitro cytotoxicity procedure using AKR anti-C3H thymocyte (anti-Thy1.2) antiserum (1).

Passage of Lymph Node and Spleen Cells Over Nylon Wool Columns. A purified population of T lymphocytes was obtained by passing lymph node and spleen cells over Leuko-Pak filters (Fenwall Laboratories, Inc., Morton Grove, Ill.) equilibrated with 20% fetal calf serum in minimal essential medium (3). The effluent contained greater than 95% T cells as judged by killing with anti-Thy1.2 antiserum.

X Irradiation of Mice. Mice received 750 R whole body X irradiation from a 250 kv (15 A) source as before (1).

Antibody Titrations. Serum antibodies to DNP were measured by a modification of the Farr assay (4). Determinations are expressed as the log₁₀ of the titer of serum which bound 33% of ³H-DNP-lysine (New England Nuclear Corp., Boston, Mass.). Antibody to BSA was measured by a tanned red blood cell hemagglutination technique in microtiter plates (Cooke Laboratory Products Div., Dynatech Laboratories, Inc., Alexandria, Va.) (5). 2-Mercaptoethanol (2ME)-resistant antibody was determined by incubation of serum with an equal volume of 0.1 M 2ME in saline for 30 min at room temperature before serial dilution. The titer of 2ME-sensitive antibody was calculated as follows: log₂ titer 2ME-sensitive antibody = log₂ titer total antibody – log₂ titer 2ME-resistant antibody.

Results

Analysis of the Percentage of Spleen Cells Bearing Different Ig Isotypes Using the FACS. The percentage of spleen cells staining brightly for surface IgM, IgD, IgG, and total Ig was similar in normal (unprimed) mice and in mice primed subcutaneously with antigen in adjuvant. The mean percentage of positive cells was determined in 13 experiments using a combination of primed and unprimed mice as cell donors. Cells staining positively with RAMIg, RA μ , RA δ , and RA γ accounted for a mean of 50.3, 35.3, 43.5, and 12.5%, respectively of total spleen cells as reported previously (1).

Adoptive Primary Response Restored by Anti-Thy1.2-Treated Spleen Cells. Mice were irradiated and given a constant excess number (5×10^6) of nylon wool-purified lymph node and spleen cells (helper T cells) intravenously from normal (unprimed) donors 4–8 h later. Graded numbers of anti-Thy1.2-treated spleen cells (B cells) from normal donors were injected simultaneously.

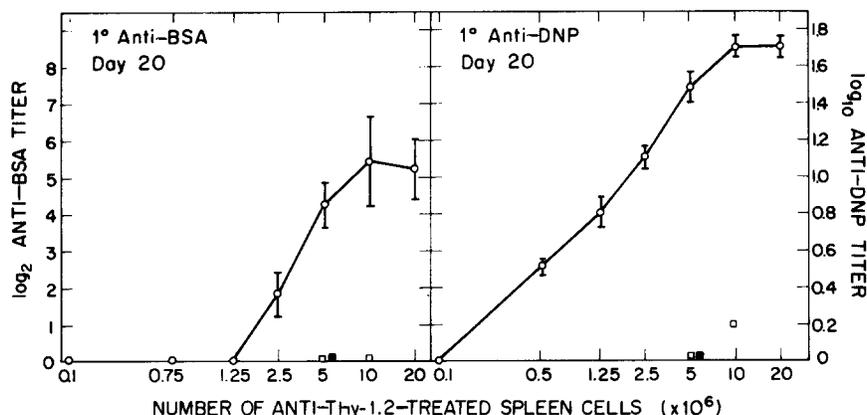


FIG. 1. Adoptive primary antibody response restored by anti-Thy1.2-treated spleen cells from unprimed mice at day 20. Irradiated hosts were injected with 5×10^6 nylon wool T cells from unprimed mice and with graded numbers of anti-Thy1.2-treated spleen cells from unprimed donors. The hosts were injected subcutaneously with BSA-DNP in complete Freund's adjuvant and the antibody response to DNP and BSA were measured. (O—O), unprimed T cells and unprimed B cells. (■—■), T cells alone. (□—□), B cells alone. Each point shows the mean response of a group of 12–16 mice. Brackets show the standard error.

Adoptive recipients were injected subcutaneously with 0.5 mg DNA-BSA in complete Freund's adjuvant within 24 h after cell transfer.

Fig. 1 shows the dose-response curve for the adoptive primary anti-DNP and anti-BSA response vs. cell dose at day 20 after antigenic challenge. A linear portion of the curve was observed between 1 and 10×10^6 cells transferred. Similar responses were restored by 10 or 20×10^6 anti-Thy1.2-treated cells. 10×10^6 of the latter cells restored a minimal response in the absence of T cells, and T cells alone produced no detectable response.

Adoptive Primary Anti-BSA Response Restored by IgM-, IgD-, or IgG-Bearing Cells. The experimental scheme used to test the ability of unprimed spleen cells to restore an adoptive primary antibody response is the same as that described in our previous paper (1). Spleen cells from normal donors were stained with RAMIg, RA μ , RA δ , or RA γ , and sorted on the FACS into bright and dull cell fractions. Thresholds were set for bright cells corresponding to positively staining cells as judged by fluorescence microscopy. The next brightest 10% of cells were discarded, and the remaining cells were included in the dull cell fraction. The bright or dull cells were injected together with unprimed T cells into irradiated hosts which were subsequently challenged with DNP-BSA in complete Freund's adjuvant. Total and 2ME-resistant antibodies to BSA were measured in the serum for 25 days. The total anti-DNP antibody response was also determined.

The restorative activity of bright and dull cells were measured at two cell doses. The high cell dose corresponded to the number of bright cells contained within 4×10^6 unfractionated spleen cells, and the low cell dose corresponded to the number contained within 0.8×10^6 unfractionated cells. The high cell dose was calculated to be on the linear portion of the dose-response curve described in the previous section. Control groups of animals were injected with 4, 0.8, or 0.04

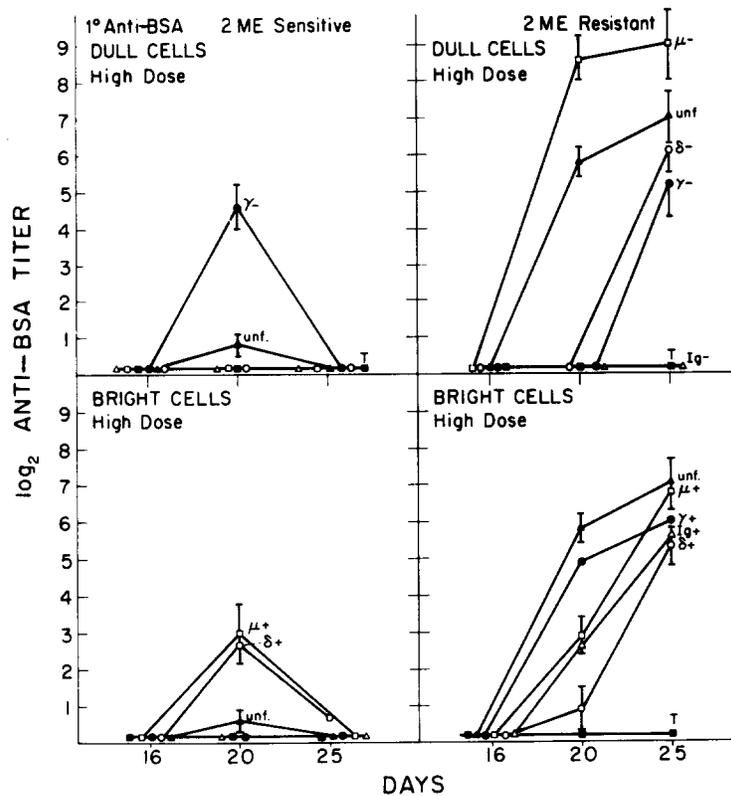


FIG. 2. Adoptive primary anti-BSA response restored by unprimed cells sorted according to isotype of surface immunoglobulin. Mice were injected with 5×10^6 purified T cells from unprimed mice and with a dose of bright or dull cells contained within 4×10^6 unfractionated, unprimed spleen cells. Controls received 4×10^6 unfractionated cells plus T cells, or T cells alone. The 2ME-sensitive antibody response was calculated by subtracting the \log_2 titer 2ME-resistant antibody from the \log_2 titer total antibody response. Each point shows the mean response of a group of mice, and brackets show the standard errors. Groups given unfractionated cells plus T cells, or T cells alone contained 12-16 mice. Groups given sorted cells contained four mice. Two sorting experiments were carried out for each Ig isotype. Data in this and subsequent figures show results of one of the two experiments. (\blacktriangle - \blacktriangle), unfractionated cells. (\circ - \circ), cells stained with RA δ . (\bullet - \bullet), cells stained with RA γ . (\square - \square), cells stained with RAMIg. (Δ - Δ), cells stained with RAMIg. (\blacksquare - \blacksquare), T cells alone.

$\times 10^6$ unfractionated spleen cells and unprimed T cells, or with unprimed T cells alone. Two sets of experiments were carried out. In each set, cells were stained and sorted for surface IgM, IgD, IgG, or total Ig and transferred to groups of four to six recipients at the high (4×10^6) and low (0.8×10^6) cell doses. The results of only one set of experiments are reported here. The second set gave similar results to the first unless otherwise noted.

Figs. 2 and 3 show the results of the adoptive primary anti-BSA response. The response restored by unfractionated cells showed a prolonged latent period such that easily detectable antibody was first observed at about day 20. IgG (2ME resistant) antibody was already predominant at this time (Table I). Cells staining brightly for total Ig restored a response similar to that of an equivalent

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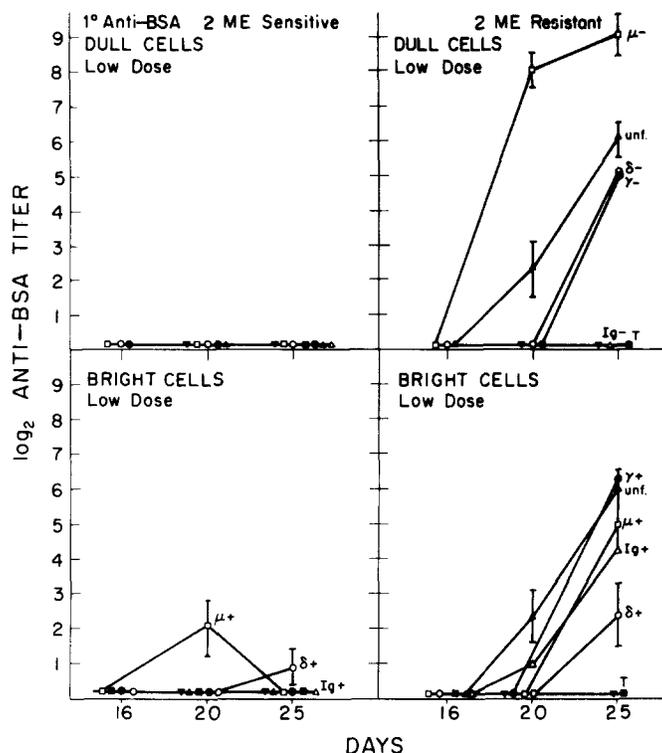


FIG. 3. Adoptive primary anti-BSA response restored by unprimed cells sorted according to isotype of surface immunoglobulin. Mice were injected with 5×10^6 purified T cells from unprimed mice and with a dose of bright or dull cells contained within 0.8×10^6 unfractionated, unprimed spleen cells. Controls received 0.8 or 0.04×10^6 unfractionated cells plus T cells, or T cells alone. The 2ME-sensitive antibody response was calculated by subtracting the \log_2 titer 2ME-resistant antibody from the \log_2 titer total antibody response. Each point shows the mean response of a group of mice, and brackets show the standard errors. Groups given unfractionated cells plus T cells, or T cells alone contained 12-16 mice. Groups given sorted cells contained four mice. (\blacktriangle - \blacktriangle) 0.8×10^6 unfractionated cells. (\blacktriangledown - \blacktriangledown), 0.04×10^6 unfractionated cells. (\circ - \circ), cells stained with RA δ . (\bullet - \bullet), cells stained with RA γ . (\square - \square), cells stained with RA μ . (\triangle - \triangle), cells stained with RAMIg. (\blacksquare - \blacksquare), T cells alone.

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

Immunoglobulin isotype on donor cells	Mean total antibody response (\log_2 titer)*	Percentage of total antibody which is 2ME resistant
IgM	5.9	13
IgD	3.9	13
IgG	5.4	100
Unfractionated	5.7	75

* Response restored by the number of bright cells contained within 4×10^6 unfractionated cells.

number of unfractionated cells. Dull cells produced no detectable response through day 25.

Cells staining brightly for surface IgM or IgD restored an anti-BSA response which was predominantly IgM (2ME sensitive) at day 20 (Table I). However, on day 25 the antibody response was all IgG (2ME resistant) (Figs. 2 and 3). The second set of experiments differed from the first in that the IgD-bearing cells restored only IgG antibody at days 20 and 25. Cells staining brightly for surface IgG restored only 2ME-resistant antibody on both days. The magnitude of the day 25 response was similar for IgM-, IgD-, and IgG-bearing cells. Dull cells contaminating the cells staining brightly for IgM or IgD could not account for the adoptive IgG response, since a dose of unfractionated cells equal to that of the contaminating cells (0.04×10^6) did not restore a detectable response.

Depletion of IgG-bearing cells (dull cells) resulted in a marked increase in the IgM response and elimination of the IgG response at day 20 at the high cell dose (Fig. 2). Elimination of the day 20 IgG response was also observed at the low cell dose, but a concomitant increase of the IgM response was not observed (Fig. 3). However, the increased IgM and decreased IgG responses were noted at both high and low cell doses in the second set of experiments. The dull cells restored an IgG response which was only slightly below that of the unfractionated cells at day 25 in the first experiment (Figs. 2 and 3). In the second experiment, the IgG response was still markedly reduced at day 25.

Depletion of IgM-bearing cells increased the IgG response at days 20 and 25 (Figs. 2 and 3). This effect was quite dramatic at the low cell dose (~60-fold increase) at day 20 (Fig. 3). On the other hand, depletion of IgD-bearing cells eliminated the IgG response on day 20, but did not affect the day 25 response as compared to that of the unfractionated cells.

These results show that IgM- and IgD-bearing cells can give rise to the adoptive primary IgM and IgG response to BSA. IgG-bearing cells give rise only to the adoptive IgG response. In addition, the IgG-bearing cells or their products suppress the adoptive IgM response. A reciprocal relationship was also observed, in that IgM-bearing cells appear to suppress the full expression of the adoptive IgG response.

Restoration of the Adoptive Primary Anti-DNP Response by IgM-, IgD-, and IgG-Bearing Cells. Fig. 4 shows that the adoptive primary total anti-DNP response restored by 4×10^6 unfractionated cells rose more rapidly than the anti-BSA response and showed a plateau at day 16. Cells staining brightly for surface IgM and IgD restored a vigorous response which was similar to that restored by an equivalent number of unfractionated cells. IgG-bearing cells restored a slow rising response which lagged that of the IgM- and IgD-bearing cells by approximately 9 days. Depletion of IgM-, IgD-, or IgG-bearing cells did not alter the total anti-DNP response as compared to unfractionated cells at both high and low cell doses.

Discussion

In these experiments, we investigated the capacity of IgM-, IgD-, and IgG-bearing spleen cells, purified by use of the FACS, to restore the adoptive primary response of irradiated (BALB/c \times C57BL/Ka)F₁ mice to DNP-BSA. The

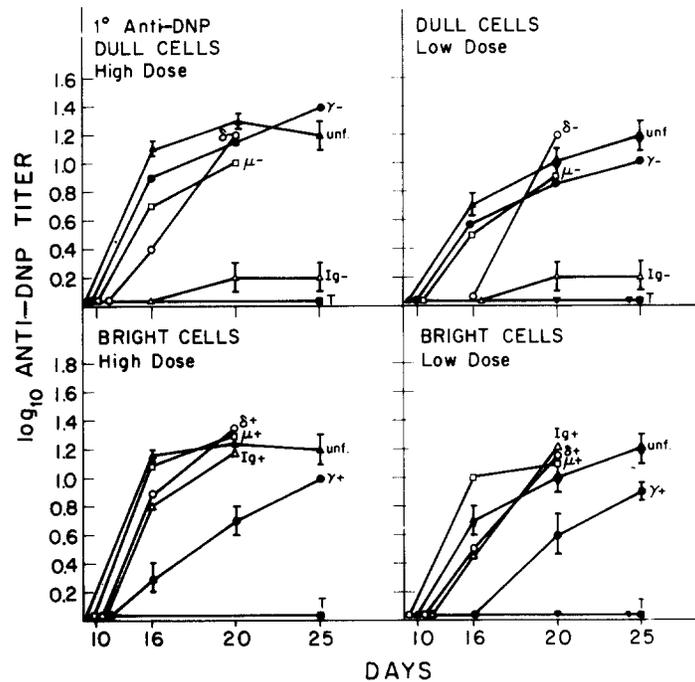


FIG. 4. Adoptive primary anti-DNP response restored by unprimed cells sorted according to isotype of surface immunoglobulin. Mice were injected with 5×10^6 purified T cells and with unfractionated or fractionated unprimed spleen cells. Recipients were given a dose of dull or bright cells contained within either 4×10^6 (high dose) or 0.8×10^6 (low dose) unfractionated cells. Controls received either 4, 0.8, or 0.04×10^6 unfractionated spleen cells plus T cells, or T cells alone. (\blacktriangle — \blacktriangle), 4×10^6 unfractionated cells. (\blacklozenge — \blacklozenge), 0.8×10^6 unfractionated cells. (\blacktriangledown — \blacktriangledown), 0.04×10^6 unfractionated cells. (\circ — \circ), cells stained with RA δ . (\bullet — \bullet), cells stained with RA γ . (\square — \square), cells stained with RA μ . (\triangle — \triangle), cells stained with RAMIg. (\blacksquare — \blacksquare), T cells alone. Each point shows the mean response of a group of mice and brackets show the standard errors. Groups given unfractionated cells or T cells alone contained 12–16 mice. Groups given sorted cells contained four mice.

experimental protocol differed from that in the preceding paper (1), since both T and B cells were obtained from unprimed instead of primed donors. In addition, adoptive recipients were challenged subcutaneously with DNP-BSA in complete Freund's adjuvant instead of in saline. Adjuvant was used because neither normal unirradiated mice nor irradiated adoptive recipients of unprimed cells made a detectable serum antibody response to the DNP or BSA determinants (I. Zan-Bar, and S. Strober, unpublished observations) during an interval of 25 days after immunization with DNA-BSA in saline.

The kinetics of the adoptive primary response lagged that of the adoptive secondary response by about 14 days in the case of BSA, and by about 10 days in the case of DNP. The magnitude of the adoptive primary response was at least 10-fold lower than that of the secondary response in both cases when equal numbers of cells were transferred. It is, therefore, clear that the primed B cells differed from unprimed B cells in three ways; (a) greater rapidity of the adoptive secondary response, (b) greater magnitude of the adoptive secondary

response, and (c) ability of primed cells to respond to antigen in aqueous solution.

Unprimed spleen cells staining brightly for surface IgM and IgD restored the adoptive primary IgM and IgG response to BSA. In contrast, IgG-bearing cells restored only the adoptive IgG response. This pattern of reconstitution is similar to that found with primed cells in the preceding paper (1). The adoptive IgG response was dependent upon the presence of IgD-bearing cells, since the removal of the latter cells resulted in a substantial reduction of this response. However, removal of the IgM-bearing cells significantly increased the adoptive IgG response. This suggests that there is a subpopulation of IgD-bearing cells which express little or no IgM. These cells may express both IgD and IgG on the cell surface.

Unprimed IgM-bearing B cells which gave rise to both the adoptive IgM and IgG responses may contain subpopulations of IgM alone as well as IgM- plus IgD-bearing cells. Evidence for the presence of the former cells in the large cell fraction of the mouse spleen separated by 1 *g* velocity sedimentation has been reported (6). It is of interest that a subpopulation of unprimed B cells which is not capable of giving rise to IgG-secreting cells, and which does not bear detectable Ia antigens on the cell surface are confined to this fraction.³ It is interesting to speculate that virgin B cells bearing only IgM give rise to only IgM-secreting cells, and that B cells bearing both IgM and IgD give rise to IgM- and IgG-secreting cells.

Removal of IgG-bearing cells augmented the adoptive IgM response and decreased the adoptive IgG response. The augmentation of the IgM response was also noted in our studies of the adoptive secondary response (1). This suggests that IgG-bearing cells in both primed and unprimed mice suppress the IgM response derived from IgM- and IgD-bearing cells.

IgG-bearing cells are active in both the adoptive primary anti-BSA and anti-DNP responses. These cells could have arisen through an antigen-independent process as proposed by Cooper and his colleagues (7), or by an antigen-dependent process from less mature B cells. In the latter case, antigenic exposure would have been accomplished through environmental contacts with cross-reacting antigens. This could give rise to IgG-bearing memory cells to the environmental antigens which have low affinity receptors for BSA-DNP. On the other hand, these cells may represent intermediate B cells which have gained surface IgG but not those functional parameters characteristic of mature memory B cells. B cells which are intermediate along the pathway of maturation between virgin and memory B cells in the rat have been described previously (8). These intermediate cells do not recirculate from the blood to the lymph, but their rate of turnover is slow (8). It would, therefore, be of considerable interest to investigate the migratory pattern and turnover rate of the IgG-bearing cells reported here.

Although the present experiments do not elucidate the role of surface IgD, the experiments with unprimed B cells suggest that IgD may play a role in allowing

³ Press, J. L., S. Strober, and N. R. Klinman. Characterization of B cell populations by velocity sedimentation, surface Ia antigens, and immune function. *Eur. J. Immunol.* In press.

for switching from IgM to IgG antibody synthesis. The IgD-bearing cells differed from the IgM- or IgG-bearing cells in that they restored the adoptive IgM response, and made a substantial contribution to the IgG response as judged by depletion experiments. These findings are consistent with the role of IgD discussed in a previous model of B-cell differentiation (9). It is clear from the present experiments and those discussed in the preceding paper (1) that cells bearing surface IgD are active in both the adoptive primary and secondary antibody responses.

Fig. 9 of the preceding paper (1) summarizes our experimental results with both primed and unprimed B cells. It should be noted that the relative contribution of IgM-, IgD-, and IgG-bearing cells to the adoptive primary and secondary IgG response differed. In addition, there appears to be a reciprocal feedback mechanism of the IgM- and IgG-bearing cells on the adoptive primary IgG and IgM antibody responses, respectively. This indicates that subpopulations of B cells bearing different isotypes modulate the immune response of one another so that a "coordinated" or "balanced" response is achieved.

Summary

We investigated the ability of IgM-, IgD-, and IgG-bearing cells from the spleens of unprimed (BALB/c \times C57BL/Ka)F₁ mice to restore the adoptive primary anti-BSA and anti-DNP antibody responses. Purified populations of isotype-specific cells were prepared by immunofluorescent staining 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 complete Freund's adjuvant. Unfractionated spleen cells as well as IgM- and IgD-bearing cells restored the adoptive primary IgM and IgG antibody response. IgG-bearing cells restored a vigorous adoptive response which was all IgG (2-mercaptoethanol resistant). Depletion of IgG-bearing cells markedly increased the adoptive IgM response, and depletion of IgM-bearing cells markedly increased the IgG response. However, depletion of IgD-bearing cells resulted in a considerable reduction in the IgG response. The latter finding indicates that there is a subpopulation of IgD-bearing cells which express little or no surface IgM and which make a considerable contribution to the adoptive primary IgG response.

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