CELL SURFACE IMMUNOGLOBULIN

XX. Antibody Responsiveness of Subpopulations of B Lymphocytes Bearing Different Isotypes*

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The major surface immunoglobulins (Igs) on human and murine B lymphocytes are IgM and IgD (1-6). Small populations of B cells also bear IgG (7, 8) or IgA (9). A large proportion of B cells express more than one isotype on their surface (3, 4, 6, 10, 11-13). In this regard, we have recently demonstrated that neonatal murine B cells bear only IgM (14, 15); that cells later acquire IgD and that they subsequently greatly reduce or completely lose their surface IgM (15).

The present studies were aimed at establishing the role of B cells bearing different receptor isotypes in generating an immune response. In these experiments, the ability of B cells, depleted of subpopulations bearing different Ig isotypes, to transfer a primary response to the hapten, 2,4,6-trinitrophenyl (TNP),[1] and a secondary response to the carrier, sheep erythrocytes (SRBC), was evaluated.

Materials and Methods

General Experimental Plan (Fig. 1). Spleen cells from mice primed with SRBC were treated in vitro with complement (C') and either anti-μ, anti-γ, anti-μ plus anti-γ, anti-κ, anti-Ig, or medium (control) under conditions which specifically killed a maximum number of cells. Graded numbers of cells were then injected into syngeneic, lethally irradiated, carrier (SRBC) primed recipients. Animals treated in this way had radioresistant helper T cells, but no functional B cells (16) so that plaque-forming cell (PFC) responses measured should be a function of the injected B cells only. The recipient animals were challenged with heavily haptenated TNP-SRBC and 5 days later spleens were removed and assessed for a primary IgM response to TNP and secondary IgM and IgG responses to SRBC. This experimental design made it possible to assess the effect of depleting cells bearing Ig, IgM, or IgG from the same primed spleen population on both primary and secondary responses in single recipients.

Animals. (C57BL/6 × DBA/2)F₁ mice (BDF₁) (The Jackson Laboratories, Bar Harbor, Maine), 6-10 wk of age were primed by an intravenous injection of 200 μl of a 10% suspension of SRBC 2-20

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[1] *Abbreviations used in this paper:* BSS, balanced salt solution; GARIg, goat anti-rabbit IgG; HRBC, horse erythrocytes; NP40, Nonidet P40; PBS, phosphate-buffered saline; PFC, plaque-forming cell(s); RAMIg, rabbit anti-mouse Ig; RAγ, rabbit anti-mouse-γ; RAκ, rabbit anti-mouse-κ; RAμ, rabbit anti-mouse-μ; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gels; TNP, 2,4,6-trinitrophenyl.
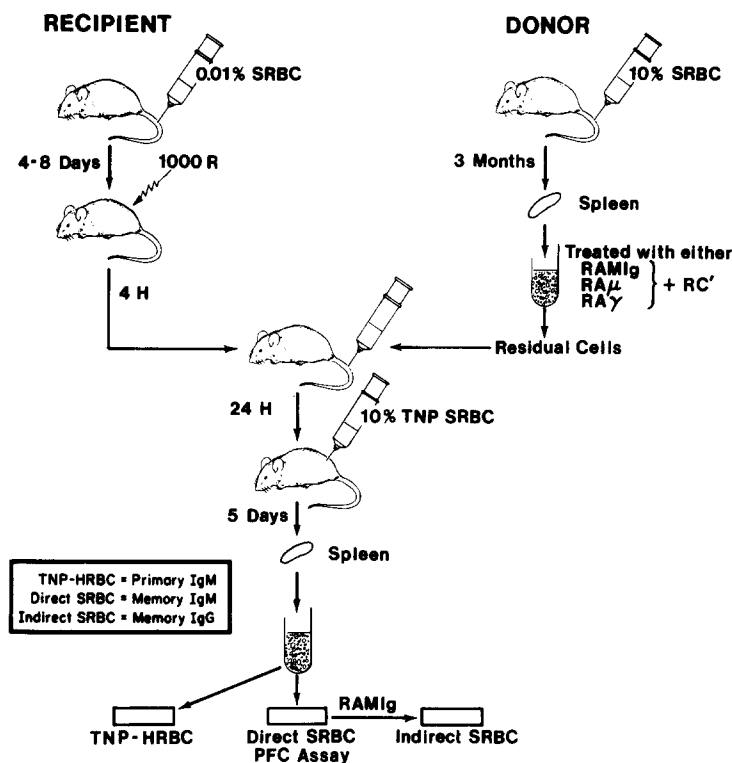
FIG. 1. Experimental protocol.

wk before the spleens were removed and used as donor cells. Syngeneic recipients were injected intravenously with 200 μl of a 0.01% suspension of SRBC 4-8 days before lethal X irradiation (1,000 R, ^{137}Cs source given 4 h before reconstitution). This low dose priming of recipient animals assured that an excess of carrier-specific, thymus-derived, radioresistant helper cells were present (17).

Antigen and PFC Assay. The recipients were challenged intraperitoneally with 200 μl of a 10% suspension of heavily substituted TNP-SRBC (18) the day after reconstitution and sacrificed 5 days later. The spleens were teased, washed once, and resuspended in 5-10 ml of balanced salt solution (BSS). The cells were assayed for direct PFC to SRBC and TNP-horse erythrocytes (HRBC) using a slide modification of the assay described by Jerne and Nordin (19). Indirect PFC were determined after developing the slides further by incubation with rabbit anti-mouse Ig (Cappel Labs, Dowingtown, Pa.), at a dilution of 1/200 with additional C' (guinea pig C', Pel-Freez Bio-Animals, Inc., Rogers, Ark.).

Antisera

RABBIT ANTI-MOUSE Ig (RAMIg) (20). RAMIg contained antibodies specific for mouse μ-, γ-, α-, κ-, and λ-chains and was a pool of sera from rabbits immunized with a variety of purified myeloma proteins.

RABBIT ANTI-MOUSE-κ (RAκ) SERUM. RAκ serum was prepared against free light chains obtained from reduced and alkylated TEPC-15 (α, κ). The serum reacted with serum Ig and kappa-containing, but not lambda-containing, myeloma proteins.

RABBIT ANTI-MOUSE-μ (RAμ) (20) SERUM. RAμ serum was prepared by immunizing a rabbit with μ-chains isolated from MOPC-104E.

RABBIT ANTI-MOUSE-γ (RAγ). Normal mouse serum (Pel-Freez Bio-Animals, Inc.) was brought

to 50% saturation with $(NH_4)_2SO_4$. The resultant precipitate was centrifuged, dissolved in distilled water, and desalted by dialysis against H_2O. The IgG fraction was prepared on DEAE-Sephadex A50 using 0.05 M phosphate buffer, pH 7.8. The IgG was desalted, lyophilized, and dissolved in 0.2 M Tris, pH 8.0, at 10 mg/ml. The γ-chains were prepared by reduction of the IgG with 20 mM dithiothreotol and alkylation with 40 mM recrystallized iodoacetamide. Preparations were dialyzed against 1 M propionic acid and the γ-chain fraction was prepared by gel filtration on a G100 Sephadex column equilibrated with 1 M propionic acid. The heavy chain fraction was pooled, concentrated, and rechromatographed. Aliquots containing 50 μg of the γ-chain were frozen and stored at $-20°C$. The purity of the fraction was determined by electrophoresing 50 μg of purified material on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) followed by staining the gel. In addition, 20 μg of the purified gamma chain was radioiodinated and 1×10^6 cpm of the labeled protein analyzed by SDS-PAGE. Greater than 95% of the radioactivity migrated as γ-chain. Rabbits were injected subcutaneously and intramuscularly at 1- to 2-mo intervals with 50 μg of γ-chain in Freund's complete adjuvant (Grand Island Biological Co., Grand Island, N. Y.).

GOAT ANTI-RABBIT IgG (GARIg) (20) ANTISERUM. GARIg antiserum contained antibodies against rabbit γ and L chain. All sera were evaluated by immunoprecipitation of both radioiodinated purified immunoglobulins and immunoglobulins in lysates of radioiodinated spleen cells.

Radioimmunoassay

PURIFIED MYELOMA PROTEIN. Purified IgM from MOPC-104E, IgA from TEPC-15, and serum IgG were enzymatically iodinated with ^{125}I (21) and dialyzed against phosphate-buffered saline (PBS), pH 7.3. Immediately before use, samples were centrifuged at 10,000 g for 30 min and acid-precipitable radioactivity determined. 100,000 cpm of each protein, representing 1–6 μg were diluted in 1.0 ml of PBS. 50 μl of rabbit anti-serum against Ig, μ, or γ was added and incubated for 15 min at 37°C. Complexes were precipitated by the addition of a slight excess of GARIg. Precipitates were centrifuged, washed twice in PBS, transferred to fresh tubes, and counted on a Beckman 300 gamma counter (Beckman Instruments, Inc., Fullerton, Calif.).

SPLEEN CELL SURFACE Ig. 5×10^7 splenocytes from adult A/J mice (The Jackson Laboratory) were radioiodinated (22), lysed in 0.5% Nonidet P40 (NP40) (Gallard-Schlesinger Chemical Mfg. Corp., Long Island, N. Y.), and lysates supplemented with fetal calf serum (GIBCO) to a final concentration of 5%. Lysates were dialyzed for 1–16 h at 4°C against PBS and centrifuged at 10,000 g for 30 min. Protein-associated radioactivity was determined in 5% trichloracetic acid (22). Equal aliquots of the lysate were treated with saturating amounts (50 μl) of either anti-μ, anti-γ, anti-Ig, or anti-κ. Complexes were absorbed to fixed Staphylococcus aureus (Cowan-I strain) (23), pelleted, washed, and the complexes eluted from the bacteria in 1% SDS, 8 M urea, 0.1 M 2-mercaptoethanol, pH 8.4, by boiling for 1–2 min. The eluate was electrophoresed for 16 h at 4 mA on 7.5% SDS-PAGE with internal markers consisting of ^3H-μ and ^3H-L chains (14). Gels were fractionated and radioactivity determined by double-label counting.

C'-Mediated Cytolysis by Specific Anti-Igs.   Donor cells were washed twice in BSS and were resuspended in Eagle's minimal essential medium (MEM) (GIBCO) supplemented with 5% fetal calf serum and nonessential amino acids. Suspensions were incubated with optimal amounts of either heat-inactivated RAMIg, RAκ, or RAγ at 4°C in the presence of 0.01 M sodium azide (final concentration) for 1 h. The cells were then pelleted and resuspended at 4×10^7/ml in medium containing 20% rabbit C' (Pel-Freez Bio-Animals, Inc.) and azide. Cells were incubated at 37°C for 35 min. Incubation of cells with anti-immunoglobulin serum caused capping even at 4°C, which protected the cells from C'-mediated cytolysis. The addition of sodium azide to the cell suspensions increased the efficiency of the specific lysis, presumably by preventing the capping. When killing with RAμ was performed, a one-step procedure was utilized where the antiserum was incubated with the cells, azide, and C' at 37°C for 35 min. This was necessary since the RAμ serum did not kill effectively in a two-step procedure. Treatment with anti-μ plus anti-γ was performed in a two-step procedure, using anti-γ plus azide followed by anti-μ, azide, and C'.

Determination of Surface Ig Isotype of Cells Remaining after C'-Mediated Cytolysis. 5×10^7 splenocytes from adult BDF_1 mice were radioiodinated (22) and washed once in PBS and once in MEM containing 5% fetal calf serum. The cells were subsequently treated with specific antisera and C' as described in the previous section. After washing once, the cells were fractionated on a 1 g fetal calf serum gradient to remove dead cells (20). The fractions from the gradient containing greater than 98% small cells with greater than 95% viability were lysed in 0.5% NP40. The lysates

TABLE I
*Immunoprecipitation of Radioiodinated Myeloma Proteins with Monospecific Antisera**

Rabbit antiserum	Myeloma proteins‡		
	IgG (γ, κ)	IgM (μ, λ)	IgA (α, κ)
	cpm immunoprecipitated		
Anti-Ig§	87,630	91,340	87,829
Anti-μ	4,023	93,662	3,764
Anti-γ	91,828	3,786	2,858
Anti-φχ (control)	3,890	4,285	3,163

* Myeloma proteins which were >95% acid precipitable were dissolved in 1.0 ml PBS and centrifuged at 10,000 g for 30 min. A saturating amount of rabbit antiserum and a slight excess of GARIg were added. The precipitates were centrifuged, washed, and counted. Aliquots of each were further assessed by SDS-PAGE.

‡ 100,000 cpm (1-6 μg) of each protein was used in the assay.

§ This antiserum contained antibodies against μ, γ, κ, and λ-chains.

were dialyzed overnight and immunoprecipitated with RAMIg as described in the preceding section.

Results

Specificity of the Antisera Used in C'-Mediated Cytolysis. The monospecificity of each of the antisera used for selective killing of B-cell subpopulations was tested by immunoprecipitation of ¹²⁵I-labeled myeloma proteins. Table I shows that under saturating conditions of antiserum, 87–93% of the total radioactivity associated with each myeloma protein was bound only by the appropriate antiserum.

To show that the antisera were also specific for normal murine splenic surface Igs, spleen cells were radioiodinated, lysed, and immunoprecipitated with each antiserum, using fixed *S. aureus* to bind the immune complexes. The PAGE patterns in Fig. 2 show that RAMIg serum precipitated both IgM and IgD from lysates of spleen cells by virtue of its activity against L chains, whereas RAμ serum precipitated only IgM from another aliquot of the same lysate. IgG was not detected on enzymatically iodinated lymphocytes in confirmation of previous reports (14, 24, 25). Presumably, it is present in quantities too low for detection by this assay. When the same sera were tested for cytotoxic activity against spleen cells, the net cytotoxicities using RAMIg, RAκ, RAμ, and RAγ were 54, 48, 42, and 0 → 15%, respectively.

Effect of Eliminating B-Cell Subpopulations from "Long-Term" Primed Donors. Cells from animals immunized 12–20 wk previously (long-term primed) with a high dose of SRBC were treated with either RAMIg, RAμ, RAγ, or an equivalent volume of culture medium in the presence of rabbit C'. The cells were centrifuged, resuspended in medium, and injected intravenously into lethally irradiated, carrier-primed, syngeneic recipients. The number of PFC in the spleen of each of the recipients was determined 5 days after antigenic challenge with heavily substituted TNP-SRBC. Since the injected cells were prepared from spleens of animals which had not been previously exposed to the hapten
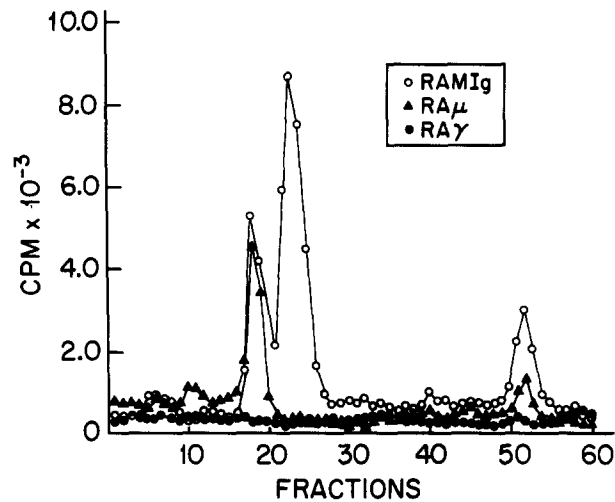
1mm

Fig. 2. SDS-PAGE analysis of cell surface Ig precipitated by RAMIg, RAμ, or RAγ. Separate aliquots of the lysate from radioiodinated spleen cells were incubated with either RAMIg, RAμ, or RAγ and complexes were absorbed to *S. aureus*. The immune complexes were then eluted from *S. aureus* in SDS-urea, reduced, and electrophoresed on 7.5% SDS-PAGE for 16 h at 4 mA/gel. Gel profiles were plotted such that ^{3}H-μ chain markers (fractions 15-20) were aligned for all three gels. Similar analysis of immunoprecipitates using RAκ revealed a pattern identical to that of the RAMIg precipitate.

TNP, the PFC response assayed against TNP-HRBC represents a primary B-cell response against TNP, whereas PFC against SRBC are derived mainly from memory B cells. No indirect PFC were observed with TNP-HRBC at the time chosen for assay.

Fig. 3 shows the results of such a depletion experiment. The direct and indirect PFC responses of animals which received spleen cells treated with RAμ and C' are presented together with the responses of two types of control animals: those which received cells treated with C' alone and those which received cells treated with RAMIg plus C'. It can be seen that the PFC response is linearly related to the number of live cells injected and that carrier-primed, irradiated animals which were not reconstituted gave less than 100 PFC/spleen, indicating complete dependence of the response on the donor cells. Treatment with RAμ in the presence of C' reduced the response to TNP by more than 99% as compared to the C' control, indicating that cells bearing IgM (with or without another isotype) are responsible for the primary (anti-TNP) response. In contrast, neither the direct nor the indirect (IgG) anti-SRBC PFC responses were reduced. Indeed, an apparent enrichment of approximately twofold for these responses was observed. This apparent enrichment is due to the manner in which the results are displayed, i.e., the response is plotted as a function of the remaining live cells injected. [If the size of the response was plotted as a function of the initial number of cells to be transferred (regardless of the number subsequently killed), the slopes of the anti-SRBC PFC in the C' control and anti-μ plus C' group would be approximately equal.] Elimination of cells bearing IgG with RAγ and C' (Fig. 4) reduced the indirect PFC response to SRBC to less than 15% of the C' control, whereas neither the primary IgM (anti-TNP) nor the second-
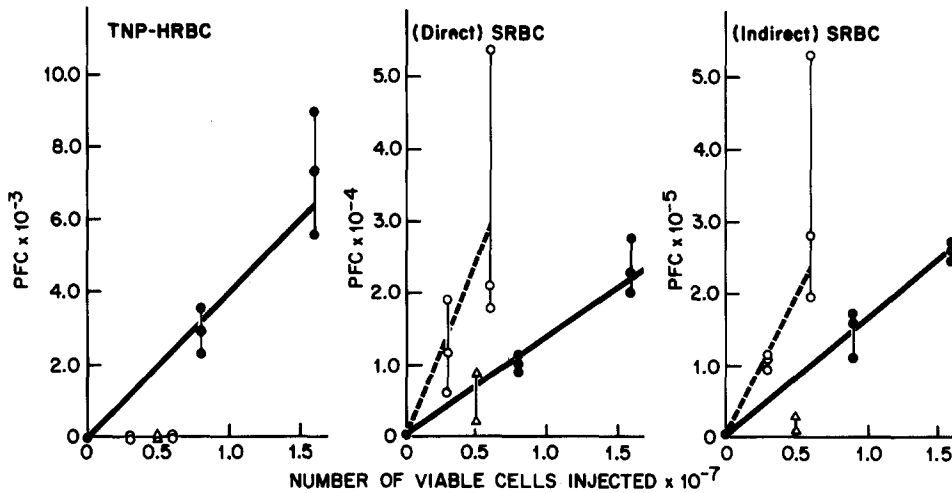
Fig. 3. PFC responses of lethally irradiated animals reconstituted with SRBC-primed spleen cells depleted of IgM-bearing cells. Recipient animals were injected with 200 μl of spleen cells previously treated with either RAMIg and C' (△), RAμ and C' (○), or C' alone (●). Reconstituted mice were challenged with TNP-SRBC on the following day. PFC responses per spleen against TNP-HRBC and SRBC assayed 5 days after challenge are shown in the vertical axis. Each point represents the response of one recipient. The number of viable cells injected represents a different percentage of the original spleen cell population since each antiserum had a different net cytotoxicity.
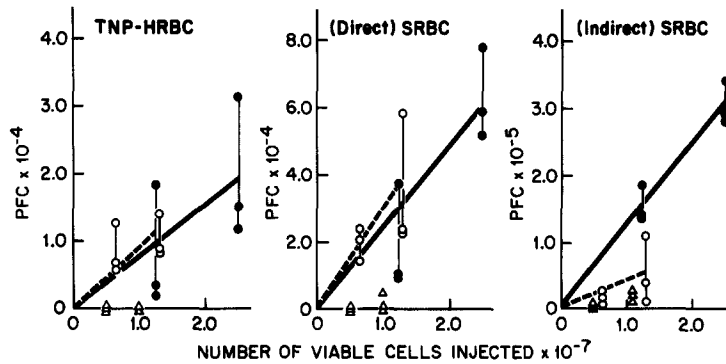

Fig. 4. PFC responses of lethally irradiated animals reconstituted with SRBC-primed spleen cells depleted of IgG-bearing cells. Recipient animals were injected with 200 μl of spleen cells treated previously with either RAMIg and C' (△), RAγ and C' (○), or C' alone (●). Reconstituted mice were challenged with TNP-SRBC on the following day. PFC responses per spleen against TNP-HRBC and SRBC assayed 5 days after challenge are shown in the vertical axis. See legend for Fig. 3 for further details.

ary IgM (anti-SRBC) response was significantly reduced. As seen in Fig. 4, the effect of killing by RAγ on the IgG response was not as complete as the effect of RAμ killing (Fig. 3) on the primary IgM response. It is possible that the number of IgG molecules on some of the memory cells is too low for RAγ-mediated killing. Alternatively, a portion of the IgG response may arise from cells bearing other isotypes. In both experiments, the responses of animals adoptively trans-

ferred with cells treated with RAMIg serum and C' were reduced by at least 87% (Figs. 3 and 4).

Although cells giving rise to the secondary IgM response could not be eliminated by either RAμ or RAγ, they could be deleted by RAMIg. Such cells therefore bear amounts of Ig which are sufficient for C'-mediated lysis using a polyvalent reagent. It is possible that such cells bear both IgM and IgG but not in sufficient density to be lysed by either anti-μ or anti-γ and C'. Therefore, a combination of RAμ plus RAγ and C' might eliminate these cells. Primed donor cells were therefore first incubated with RAγ (in azide), washed, and subsequently incubated with RAμ and C' (in azide). Table II indicates that all three responses, including the secondary IgM response can be eliminated by such a procedure. Furthermore, prior absorption of either the anti-μ or anti-γ serum with their corresponding isotype (IgM or IgG) abrogated their ability to eliminate these responses (Table II), indicating that the effect of these antisera is due to their anti-Ig specificities. The effect of treatment by RAμ plus RAγ plus C' on the deletion of the secondary response was not as complete as when RAMIg or RAκ were used (Table II). These experiments suggest that cells giving rise to the secondary IgM response bear low levels of both IgM and IgG on their surface.

The depletion experiments using each of the three specific antiserum were performed three to six times (Table II). In depletion experiments involving RAμ or RAγ, enhancement of the PFC responses of the surviving subpopulation was sometimes observed. In control experiments, where cells were treated with antisera plus heat-inactivated C' before adoptive transfer, we have also observed sporadic enhancement of the PFC response. The explanation for this finding is not clear. Table III summarizes the results of our depletion experiments and suggests that the long-term memory cells giving rise to the primary and secondary adoptive responses each bear different isotypes.

Effect of Varying the Interval between Priming and Selective Cytolysis on the Ability to Deplete Specific Subpopulations. In order to determine whether susceptibility of the various B-cell subpopulations to killing by specific anti-immunoglobulin sera and C' changes with time after immunization, we reconstituted irradiated hosts with cells from donor animals primed 2–8 wk previously.

Results shown in Fig. 5 indicate the effect of treating cells from donors primed for various periods of time with either RAμ or RAγ on their ability to respond to a challenge with TNP-SRBC. After elimination of the IgG-bearing subpopulation by RAγ plus C', the effect on each of the three responses was constant. On the other hand, elimination of IgM-bearing cells from donor spleens removed shortly after primary immunization, resulted in considerable inhibition (more than 75%) of the secondary IgM anti-SRBC PFC response, although the effect on the IgG anti-SRBC PFC response remained minimal and the inhibition of the anti-TNP primary response also remained constant.

The decreasing susceptibility to killing by RAμ and C' of the cells giving rise to the secondary IgM response suggests that the number of IgM molecules on this population of cells decreases with time after immunization.

Examination of Surface Isotypes on Cells Surviving Treatment with Antibody Plus C'. A subpopulation of cells bearing large amounts of IgD has been

TABLE II

Inhibition of PFC Responses of Cells from Long-Term Primed Animals by Treatment with Specific Rabbit Anti-Immunoglobulins and C'*

Antiserum	Exp. no.	Percent inhibition‡		
		TNP-HRBC IgM	SRBC IgM	SRBC IgG
RAMIg	1§	97.9	87.2	95.7
	2 ‖	99.3	98.7	97.7
	3	98.0	98.1	96.8
	Average	98.4	94.7	96.7
RAκ	1	94.8	96.2	96.6
	2	97.2	97.9	97.8
	Average	96.0	97.0	97.2
RAμ	1§	98.9	−28.0¶	0.1
	2	80.6	2.9	−56.8
	3	88.4	13.3	−0.8
	Average	89.3	−3.9	−19.2
RAγ	1 ‖	23.4	25.3	85.0
	2	−48.3	−43.0	46.4
	3	−49.6	19.3	50.1
	4	10.4	−9.1	79.9
	5	−19.0	15.3	84.2
	6	19.1	0.5	70.3
	Average	−10.6	1.4	69.3
RAμ + RAγ	1	96.8	86.8	85.0
	2	99.0	89.9	96.5
	3	92.7	72.2	49.2
	4	92.9	82.7	87.6
	Average	95.4	82.9	79.6
RAμ + RAγ (absorbed)**	1	−28.4	6.7	29.7

* Cells were obtained from donor mice 3–4 mo after primary immunization.

‡ Average (Av.) PFC response of cells treated – with C' only – [(Av. PFC response of same number of cells treated with antiserum + C')/(Av. PFC response of cells treated with C')] × 100.

§ Data plotted in Fig. 3.

‖ Data plotted in Fig. 4.

¶ Negative values indicate enhancement.

** RAμ serum absorbed with IgM from MOPC-104E. RAγ serum absorbed with IgG from normal mouse serum prepared as described in Materials and Methods.

observed in our previous experiments. Thus, RAμ plus C'-mediated killing of radioiodinated A/J spleen cells left a residual population in which only surface IgD was detected (13). A similar experiment using normal BDF₁ spleen cells indicates that the same subpopulation is present in this strain. As shown in Fig.

TABLE III

Surface Isotypes on Precursors of PFC

Response	% of response accounted for by cells bearing:			
	IgM*	IgG*	IgM + IgG‡	Other§
Primary IgM	<u>89</u>	0	6	3
Secondary IgM	0	1	<u>82</u>	12
Secondary IgG	0	<u>69</u>	10	17

* Average value from Table II. The negative values are expressed as 0.

‡ Average value from Table II for deletion with anti-μ plus anti-γ minus individual values obtained with anti-μ and anti-γ. All negative values are expressed as 0 in the calculations.

§ Average value from Table II for deletion with anti-Ig minus average value obtained with anti-μ plus anti-γ. This difference could reflect a difference in efficiency of killing between anti-L and anti-H chain. However, it could be due to cells bearing δ only, δ plus γ, or μ plus δ.
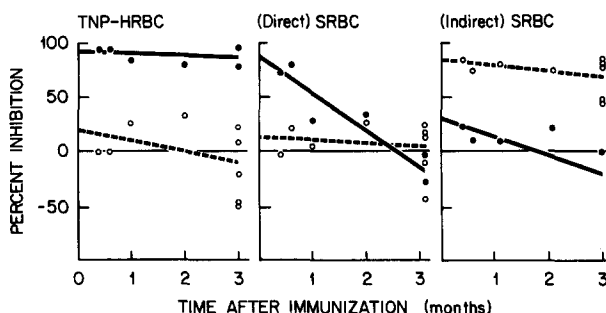

FIG. 5. The effect of time elapsed after primary immunization on the ability of RAμ and RAγ plus C' to kill subpopulations of cells giving rise to the primary, secondary IgM, and secondary IgG responses. Cells from animals primed 2-12 wk earlier with SRBC were treated with either RAμ plus C', RAγ plus C', or medium plus C', and adoptively transferred to recipients as described in Materials and Methods. The percent inhibition at each time point was calculated as described in Table II. Inhibition by RAμ plus C' (-●-). Inhibition by RAγ plus C' (-○-). The lines drawn were determined from linear regression analysis of each set of points. All slopes with one exception are not significantly different from zero as determined by an F test in an analysis of variance ($P > 0.1$ to $P > 0.25$). The slope obtained for inhibition by RAμ of the secondary IgM SRBC response (middle panel) was found to be significantly different from zero ($P < 0.01$) and has an associated correlation coefficient of -0.91.

6 A, elimination of cells bearing IgM with RAμ and C' reduced by 73% the amount of radioiodinated IgD in the lysate of the remaining viable cells, indicating that the majority of these cells bear both IgD and IgM. However, 27% of the IgD is present on cells which express too little IgM to be killed with anti-μ plus C'. Fig. 6 B indicates that elimination of cells bearing IgG with RAγ and C' does not affect the amount of either IgM or IgD in the lysate of the remaining cells indicating that, in contrast to cells bearing both IgM and IgD, there are few cells bearing both IgG and IgD or IgG and IgM. Furthermore, treatment of spleen cells with a combination of IgG and IgM in the presence of C' does not affect the amount of IgD detectable in the lysate of the remaining cells, as
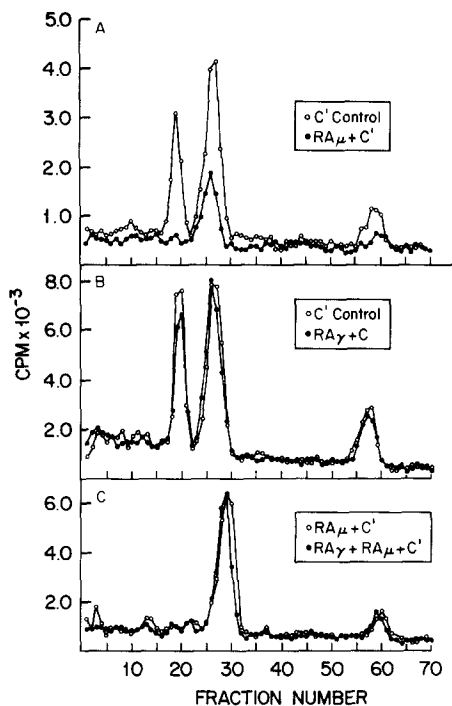
Fig. 6.   Surface Ig on radioiodinated small lymphocytes after elimination of cells bearing (A) IgM, (B) IgG, and (C) IgM plus IgG. In these experiments, labeled cells were treated with antibody plus C' or C' alone, the dead cells were eliminated on a 1 g gradient, and the Ig remaining on the small, viable cells examined after lysis, immunoprecipitation, and SDS-PAGE of the immunoprecipitate. The patterns in panel C represent cells treated with anti-μ plus C' vs. cells treated with anti-μ plus anti-γ plus C'. In comparing patterns from different gels, the L chains were aligned.

compared to cells which survive treatment with RAμ and C' (Fig. 6 C). Thus, either the IgM-IgG-bearing cells, which give rise to the secondary IgM response, bear little or no IgD on their surface or they are present in numbers too low for detection of their surface IgD by iodination.

Discussion

The present experiments provide further evidence that subpopulations of B lymphocytes bearing different surface Igs perform different functions. Thus, treatment of spleen cells from SRBC-primed mice with anti-Ig plus C' abolished the capacity of these cells to adoptively transfer an IgM- or IgG-memory response to SRBC and a primary response to TNP, indicating that the function of Ig-bearing cells only (and not Ig$^-$ progenitor cells) is being measured in the experimental system employed. When cells from mice primed 3-4 mo earlier were treated with anti-μ plus C', the adoptive primary IgM (anti-TNP) response was completely eliminated, whereas neither the secondary IgM nor IgG (anti-SRBC) responses were affected. These experiments suggest that cells giving rise to the primary IgM response bear IgM. Such results are in agreement with previous reports which demonstrated that the in vitro and in vivo primary

responses to a variety of antigens could be eliminated by treatment with anti-μ (26, 27) or could be positively selected with anti-μ using the cell sorter (28). In addition, the polyclonal IgM response to the B-cell mitogen, lipopolysaccharide, could be eliminated by anti-μ plus C' (20) or blocked by anti-μ in vitro (29, 30). To our knowledge, however, this is the first demonstration that cells giving rise to the secondary IgM response cannot be depleted by anti-μ plus C'. Such cells could, however, be eliminated by a combined treatment with RAμ and RAγ plus C'. These results suggest that the majority of cells from long-term primed animals, which give rise to a secondary IgM response, bear small amounts of both IgM and IgG on their surface. Apparently anti-serum directed against only one heavy chain isotype was not sufficient to achieve C'-dependent lysis of these cells. A correlation between the extent of C'-mediated lysis and the density of H-2 antigens on cell surfaces has been demonstrated in a number of myeloma and lymphoma cell lines (31).

It should be emphasized that a general limitation of the deletion approach is the inability to interpret definitively the failure to kill. In the present experiments, the failure probably results from a low concentration of Ig. It should also be stressed that anti-κ probably is more effective in binding surface Ig on cells bearing a particular isotype than the corresponding anti-H chain serum (32, 33). Thus, differences between the numbers of cells killed by anti-κ and an anti-H chain sera can be due either to the above reason or to a second population bearing another isotype.

In contrast to the above results, treatment of cells obtained from animals primed for relatively short periods of time with only RAμ and C' was effective in eliminating their ability to transfer a secondary IgM response. This finding suggests that shortly after priming, IgM memory cells still bear relatively large amounts of IgM. As the interval between priming and adoptive transfer increases, the concentration of surface IgM decreases.

Indeed, previous work by Pierce et al. (34), Kishimoto and Ishizaka (35), and Strober (36) using cells derived from mice primed less than 1 mo previously have shown that IgM memory is carried by IgM-bearing lymphocytes. Further, Strober has reported that IgM-bearing precursors found after short-term priming subsequently decreased in number. A less likely, but plausible, possibility is that a fraction of the memory cells present after short-term priming may be IgM-bearing plasmablasts which increase their rate of secretion and perhaps even switch to IgG after further contact with antigen. Such cells might disappear several months after priming. It was not possible to determine the exact time that IgG is acquired by the precursors of the IgM secondary response since anti-μ plus C' deleted the entire response and anti-γ plus C' alone was ineffective when the interval between priming and transfer was short.

We have also shown, by antibody-mediated killing of radioiodinated lymphocytes, that although the majority of the cells eliminated by RAμ plus C' bear IgD, the cells eliminated by a combination of RAμ and RAγ plus C' may bear no other isotype. We cannot, however, eliminate the possibility that the subpopulation bearing both IgM and IgG is too small to effect the amount of radioiodinated IgD recovered from cells surviving treatment with anti-μ plus anti-γ plus C'.

Our experiments with RAγ plus C' demonstrate that antiserum directed

solely against the IgG heavy chain markedly diminishes the secondary IgG response but not the primary or secondary IgM responses. These IgG-bearing memory cells are present in the spleen as early as 2 wk after primary immunization. These results, therefore, confirm earlier studies by Okumura et al. (37) and by Mason (38), who demonstrated that cells giving rise to memory responses bear surface IgG. A report by Abney et al. (39), however, indicates that clones of cells which elaborate IgG_1 and IgG_{2A} memory can be prohibited from binding antigen by treatment with anti-μ, but not anti-γ (or anti-δ). In studies by Pierce et al. (34) inhibition of the in vitro IgG memory response with anti-μ was also reported. It is possible, therefore, that the predominant isotype on memory IgG cells is IgG but that a portion of these cells also bear small amounts of IgM. This possibility is supported by our finding that treatment of adoptively transferred cells with C' and a combination of anti-μ plus anti-γ eliminated the IgG response more effectively than anti-γ alone.

How can the results presented here be reconciled with the failure to demonstrate IgG on murine splenocytes by enzymatic iodination (14, 24, 25)? There are several possibilities to consider: (a) Surface IgG is not readily iodinated. (b) Surface IgG is released before or after iodination. (c) The number of Ig molecules per cell and/or the number of IgG-bearing cells is small. We believe that the last explanation is the most likely—that there are a small number of IgG-bearing cells and a low density of IgG receptors per cell—because murine IgG in solution (21), in detergent-containing cell lysates (E. S. Vitetta, unpublished observations), or on plasmacytoma cells (40) is readily iodinated, suggesting that surface IgG on spleen cells should not escape effective labeling. In addition, several reports indicate that there are only 1–10% (37) IgG-bearing lymphocytes in the spleen. Therefore, the iodination method may not be sufficiently sensitive to detect the small amount of surface IgG.

In these studies, we have demonstrated that cells giving rise to a primary adoptive IgM response are easily eliminated with anti-μ plus C' and, thus, bear relatively large quantities of IgM on their surface. Precursors of the secondary IgM response bear large quantities of IgM if they are obtained from animals shortly after primary immunization. As the interval after primary immunization increases, the density of surface IgM decreases to the point, 3 or 4 mo after immunization, where the cells can no longer be eliminated by anti-μ plus C'. These long-term memory cells may also bear very low concentrations of IgG. As memory cells become precursors of the IgG response, the concentration of surface IgG increases such that the majority of IgG precursors can be eliminated by anti-γ plus C'. The present experiments were not designed to define the role of surface IgD in the adoptive responses studied because our anti-δ serum (41) is not cytotoxic. Indirectly, our results suggest that a population of IgD-bearing cells, which may also express very low amounts of IgM or IgG, contributes to the secondary IgM and IgG responses. Similar results have been obtained by positive selection, in which IgD-bearing cells contribute to the primary as well as secondary adoptive responses to dinitrophenylated bovine serum albumin (41, 42). These latter studies suggest that IgD may be present on all cells which respond to thymus-dependent antigens. In vitro studies (43) support this contention and further suggest that thymus-independent responders lack IgD. The

possibility, therefore, that IgD is a marker for a B-cell subset that can receive T-cell help deserves further exploration.

Summary

Murine spleen cells were depleted of specific B-cell subpopulations bearing different immunoglobulin isotypes by means of complement-mediated cytolysis after treatment with antisera specific for μ- and γ-chains. The functional effect of this depletion was measured by assaying both the primary and secondary plaque-forming cell responses of the residual cells after transfer to carrier-primed lethally irradiated hosts. The results suggest that cells bearing IgM are the progenitors of plaque-forming cells in the primary response and cells bearing IgG are the major progenitors of IgG plaque-forming cells in the secondary response. The quantity of IgM on progenitors of secondary IgM plaque-forming cells decreases markedly as the interval between primary immunization and antigenic challenge increases. Long-term memory cells for the secondary IgM response bear small amounts of both IgM and IgG.

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