ENRICHMENT OF ANTIGEN-SPECIFIC B LYMPHOCYTES
BY THE DIRECT REMOVAL OF B CELLS
NOT BEARING SPECIFICITY FOR THE ANTIGEN*

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Antigen-specific B cells (ASC)\(^1\) can be isolated from other lymphoid cells by their ability to specifically bind antigen. ASC were shown to bind to antigen-coated glass bead columns by the inability of the passed cells to respond to that antigen (1, 2). Since attempts to elute the cells with excess antigen were unsuccessful (2), other means of obtaining the ASC were developed. Three different approaches have been used: (a) adsorption to an insoluble antigen matrix and eluting the cells by either mechanical disruption (3) or dissolution of the matrix (4–7), (b) adsorption to an anti-hapten affinity absorbent and eluting with excess hapten (8, 9), and (c) separation electronically with the fluorescence activated cell sorter (10, 11).

In the present study, ASC were obtained by another approach which does not directly remove ASC from other lymphoid cells as outlined above, but rather indirectly provides ASC through removal of the nonantigen-specific B cells. Advantage was taken of the observation that B cells, upon binding large amounts of antigen, eventually lose their surface immunoglobulin (Ig), becoming relatively "nude" (12). It was postulated that such nude ASC could be separated from the remaining B cells bearing surface Ig by rosetting with erythrocytes (RBC) conjugated with anti-mouse Ig (13). In fact, treating spleen cells with specific antigen followed by removal of the Ig-bearing cells resulted in retention of B cells specific for that antigen, while over 90% of the other B cells were removed. Since lymphoid cells other than the B cells remained with the ASC, such antigen-specific B-cell-enriched spleen cell populations responded to

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\(^1\) Abbreviations used in this paper: anti-Ig-RBC, anti-mouse immunoglobulin-coated erythrocytes; APC, antibody precursor cells; ASC, antigen-specific B cells; BRBC, burro erythrocytes; BSS-S, balanced salt solution with serum; FCS, fetal calf serum; KLH, keyhole limpet hemocyanin; NIP, 4-hydroxy-3-iodo-5-nitro-phenylacetyl; PBS, phosphate-buffered saline; PBS-S, phosphate-buffered saline with serum; PFC, plaque-forming cells; RBC-Sol, solubilized erythrocytes; TGG, turkey gamma globulin; TNP, trinitrophenyl.
the antigen without the addition of other cells. This indirect procedure of enriching ASC is described and compared to cell separation procedures which remove ASC directly.

Materials and Methods

Animals and Immunization. Male A/J mice (The Jackson Laboratory, Bar Harbor, Maine) were immunized at 7–12 wk of age intraperitoneally (i.p.) with 100 μg alum-precipitated turkey gamma globulin (TGG) in complete Freund's adjuvant. Or, mice were immunized either i.p. with 0.2 ml of a 10% suspension of burro RBC (BRBC) or intravenously with 0.2 ml of a 0.01% suspension of sheep RBC (SRBC) to prime T cells (14). TGG- and BRBC-primed animals were used after 5 or more wk; SRBC-primed animals were used after 6–10 days.

Antigens. TGG was isolated from serum of turkeys (Burley Farms, San Diego, Calif.) by sodium sulfate fractionation (15), followed by chromatography on Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscatway, N. J.) equilibrated with borate buffer containing 30 μg/ml gentamicin (Schering Corp., Port Reading, N. J.). Protein collected in the 150,000 mol wt area was alum precipitated (16) for use as the antigen. For in vitro experiments, after column separation TGG was adsorbed at 4°C with A/J RBC, thymus, and spleen cells until free of mouse hemagglutinating activity, sterilized by filtration (0.22 μm Millipore detergent-free filter; Millipore Corp., Bedford, Mass.), and stored at −20°C at a concentration of 2–3 mg/ml.

BRBC (burro no. 1; Burley Farms) or SRBC (sheep no. 446; Colorado Serum Co., Denver, Colo.) were used either intact, after washing three times in phosphate-buffered saline (PBS) (0.15 M NaCl and 0.001 M sodium phosphate buffer, pH 7.2), or were solubilized. Soluble RBC (RBC-Sol) antigens were prepared by lysing 0.2 ml of a washed RBC pellet with 15 ml of sterile, distilled water. After pipetting up and down several times, the membranes were pelleted by centrifuging at 2,000 g for 10 min, the supernate aspirated, and the membranes resuspended in distilled water. After centrifuging again, the pellet was resuspended in 1 ml of sterile PBS with 5% heat-inactivated fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N. Y.) (PBS-S) and sonicated in an ice-water bath with a Model W140 sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) giving three 30 s pulses with the probe powered at 20 W. The sonicate was centrifuged at 2,000 g for 10 min and the upper 3/4 removed for use. RBC-Sol were freshly prepared for each experiment.

Spleen Cell Cultures. A modified Mishell-Dutton culture system was employed for the generation of antibody-producing cells (17). Single cell spleen preparations were established in microtiter plates (no. 3040; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). To each well was added 5 × 10⁵ spleen cells in 0.3 ml minimum essential medium for suspension culture (Microbiological Associates, Bethesda, Md.) containing 7.5% FCS (lot no. A846523; GIBCO), sodium pyruvate (1 mM), nonessential amino acids (1 mM), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (30 μg/ml), and Hepes (5 mM) (Calbiochem, La Jolla, Calif.) and mercaptoethanol (4 × 10⁻⁵ M). The cultures were not fed or rocked during the incubation period. Spleen cells were cultured either without antigen or with intact RBC (3 × 10⁶ per well) or with TGG added to the cultures indirectly by prebinding the TGG (18, 19). This was done by incubating 5 × 10⁶ spleen cells with 100 μg TGG in 1 ml for 30–60 min at 4°C and washing three times. After 5–6 days of culture, cells from triplicate wells were pooled and assayed by a modification of the hemolytic plaque technique (20). TGG was coupled to indicator BRBC by incubating a 10% suspension of BRBC with a dilution of turkey antiserum to BRBC optimal for plaque-forming cell (PFC) detection. After 30 min at 37°C, the TGG-coupled BRBC were washed four times in saline. The turkey antiserum was depleted of IgM by prior chromatography on Sephadex G-200. In this experimental system PFC to BRBC were always negligible in the response to TGG and direct PFC to TGG were not generated (21). The indirect PFC were developed using rabbit anti-mouse immunoglobulin facilitating serum. Results are expressed as the mean PFC per 10⁷ original cells of duplicate pools with the standard error.

Antiserum to Mouse-Ig and its Coupling to RBC. F(ab’)₂ fragments of goat Ig anti-mouse F(ab’)₂ were prepared by pepsin digestion of purified antibodies eluted from an F(ab’)₂ mouse IgG-Sepharose 4B immunoadsorbent column (22). The antiserum precipitated all classes of mouse Ig bearing kappa light chains as demonstrated by Ouchterlony analysis.
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1 mg of F(ab')2 fragments in 0.5 ml PBS was added to 0.1 ml gluteraldehyde-fixed (23), packed SRBC in 5 ml of 0.1 M sodium-acetate buffer, pH 5.0, and the mixture rotated overnight at room temperature. The cells were then washed six times with PBS and stored at 4°C in PBS with 0.1% sodium azide. The reagent is stable for several months when prepared steriley.

Removal of Ig-bearing Cells. Ig-bearing cells were removed by a modification of the method of Parish et al. (13). Spleen cells were freed of RBC and dead cells by centrifugation through Ficoll-Isoopaque (24). The interface cells were washed twice in PBS-S and cultured in medium with 7.5% FCS for 1.5 h at 37°C at 2 x 10^7 cells/ml to attempt to elute nonspecifically bound Ig. The cells were then washed again, cooled to 4°C, and 1 x 10^9 anti-mouse Ig-coated RBC (anti-Ig-RBC) added to 5 x 10^7 lymphocytes in 1 ml balanced salt solution with 5% FCS (BSS-S) (15). This mixture was then pelleted at 140 g for 3 min at 4°C (tissue culture tube no. 3033, Falcon Plastics, Div. of BioQuest), left on ice for 30 min, and then gently resuspended by pipetting. Lymphoid cells which formed rosettes with anti-Ig-RBC were then separated from nonrosetted lymphoid cells by centrifugation at 2,000 g at 20°C for 8 min on Ficoll-Isoopaque (13). The rosettes pelleted to the bottom of the gradient, while lymphoid cells which did not rosette (non-Ig bearing) remained at the interface of medium and gradient. The interface cells were aspirated, washed two times with PBS-S, and cultured.

Retention of ASC. The procedure was similar to the removal of Ig-bearing cells, except that after removal of the dead cells and RBC by centrifugation through Ficoll-Isoopaque, the washed interface cells were incubated in BSS-S for 30-60 min at 4°C with the desired antigen. TGG (100 μg) or soluble RBC antigen (0.8 ml) (see Materials and Methods, Antigens) was added to 1 ml of 5 x 10^7 cells. The cells were then washed three times with PBS-S, resuspended in medium with 5% FCS, incubated 1.5 h at 37°C, washed, and rosetted with anti-Ig-RBC as outlined above for nonantigen-treated cells. The interface spleen cells were washed two times and cultured.

Results

Enrichment of ASC by the Removal of Other Ig-Bearing Cells. Ig-bearing cells are easily removed from mouse spleen cells by rosetting with RBC conjugated with antiserum to mouse Ig and sedimenting the rosetted cells through a Ficoll-Isoopaque gradient (13). The rosettes pellet, leaving non-Ig-bearing cells at the interface. Since it was known that B cells can be induced to lose their surface Ig by incubation with antigen (12), it was postulated that prior incubation of spleen cells with antigen before B-cell depletion could lead to an enrichment of B cells specific for the antigen used in incubation upon subsequent removal of the other B cells. Since only nonantigen-specific B cells would be removed and since B cells represent about 50% of all spleen cells, the PFC response per 10^7 spleen cells should double if all of the ASC were retained. While this is a small enrichment relative to total spleen cell number, if all the B cells were removed except the few ASC (estimated as less than 1% of the B cells), then enrichment of ASC relative to B-cell content should be considerable.

To determine if ASC could be obtained in this manner, specific antigen was incubated with spleen cells before the Ig-bearing cells were removed, as shown schematically in Fig. 1. Spleen cells were either from mice primed earlier with TGG or BRBC to elicit a secondary, indirect antibody response upon in vitro culture with antigen, or spleen cells were from mice given a low dose of SRBC 6-10 days prior, resulting in an enhanced primary, direct antibody response upon culture with SRBC. TGG was added at 100 μg/ml of 5 x 10^7 spleen cells; soluble

Falkoff and Kettman (14) have shown that a low, intravenous dose of SRBC which is subimmunogenic for B cells, as evidenced by a lack of antibody synthesis, nevertheless primes T cells resulting in an enhanced in vitro direct antibody response to SRBC.
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Mouse Spleen Cells

Add Soluble Antigen

4°C x 30 min; wash 3x

37°C x 1.5 h; wash 1x

Spleen Cells [5 x 10^7] + Anti-mouse Ig-RBC [1 x 10^9]

140k x 3 min

4°C x 30 min

Layer onto Ficoll-Isoopaque

2000g x 8 min

Remove and Wash Interface Cells

FIG. 1. Method to obtain antigen-specific B cells by removal of the other B cells. Encircled symbols represent T cells (T), macrophages (M), B cells (□), ASC (○), and anti-Ig-RBC (●).

For additional explanation, see text.

Sonicates of RBC antigen were added at the equivalent of 10^10 intact RBC/ml of 5 x 10^7 spleen cells. After 30–60 min of incubation at 4°C to saturate the Ig receptors of ASC specific for the antigen, excess antigen was removed by multiple washes. The spleen cells were then incubated 1.5 h at 37°C to permit Ig-bearing cells with bound antigen to lose their Ig receptors. This length of time was shown to be sufficient for antigen-induced loss of surface Ig (12). Also, this time period probably allowed nonspecifically bound antigen and cytophilic antibody to dissociate. After being washed, the spleen cells were incubated with anti-Ig-RBC, sedimented on Ficoll-Isoopaque and the interface cells recovered. The Fc end of the anti-mouse Ig was removed to avoid its binding to cells with Fc receptors.

The effect on the antibody response of spleen cells incubated with and without specific antigen followed by B-cell removal is shown in Tables I and II. Spleen cells primed to TGG in response to TGG generated 6,201 PFC per 10^7 spleen cells put into culture (Table I, line 2), but when depleted of B cells, they did not respond to TGG (Table I, line 3). However, if they were incubated with TGG before B-cell depletion, the PFC response was not lost upon B-cell removal, but rather it was increased to 16,614 PFC per 10^7 spleen cells put into culture, indicating retention of the TGG-specific B cells. This data is typical of six other experiments done with TGG-primed spleen cells. Next, it was attempted to retain ASC specific for SRBC antigen. As expected, spleen cells depleted of B cells did not generate PFC to SRBC upon culture (Table II, line 6). However,
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Table I
Antibody Response of TGG-Primed Spleen Cells Incubated with and without TGG before B-Cell Depletion

| Treatment* | Before B depletion | B depletion | After B depletion | PFC ± SE |
|------------|--------------------|-------------|------------------|---------|
| None -     | None               | TGG         | <39              |         |
| TGG -      | None               | TGG         | 6,201 ± 662      |         |
| None +     | TGG                | None        | 234 ± 83         |         |
| TGG +      | None               | TGG         | 16,614 ± 2,483   |         |

* Spleen cells primed to TGG were incubated with TGG either before or after B-cell depletion, as indicated. Incubation of spleen cells with TGG as described in the text made further addition of TGG unnecessary upon culture. After 6 days indirect PFC to TGG per 10^7 initial spleen cells placed into culture were determined.

Table II
Antibody Response of SRBC-Primed Spleen Cells Incubated with and without Soluble SRBC before B-Cell Depletion

| Treatment* | Before B depletion | B depletion | SRBC in Culture | PFC ± SE |
|------------|--------------------|-------------|-----------------|---------|
| None -     | None               | +           | 158 ± 28        |         |
| None -     | None               | +           | 4,393 ± 1,168   |         |
| SRBC-Sol   | -                  | +           | 2,558 ± 361     |         |
| SRBC-Sol   | -                  | +           | 4,643 ± 1,085   |         |
| None +     | -                  | TGG         | <19             |         |
| None +     | -                  | TGG         | 316 ± 296       |         |
| SRBC-Sol   | +                  | TGG         | 3,837 ± 688     |         |
| SRBC-Sol   | +                  | TGG         | 546 ± 50        |         |

* Spleen cells primed with a low dose of SRBC (2 × 10^5) in order to prime only T cells (14) were incubated with or without solubilized SRBC antigen followed by B-cell depletion, as indicated. Direct PFC per 10^7 initial spleen cells in culture were assayed after 5 days.

Prior incubation with soluble SRBC antigen followed by B-cell removal resulted in the generation of 3,837 PFC (Table II, line 7), indicating retention of B cells specific for SRBC antigen.

Tables I and II also show that preincubation with either TGG or soluble SRBC was sufficient to generate PFC without further addition of antigen. For some reason, intact SRBC were on occasion inhibitory to soluble SRBC-treated spleen cells which had been depleted of B cells (Table II, line 8). Most likely this was not due to a lack of macrophages to process the antigen, since by nonspecific esterase stain macrophages were not removed upon B-cell depletion.

Determination of the Enrichment and Purity of ASC Relative to B-Cell Content. Enrichment of ASC relative to other B cells in the spleen cell preparation was determined by rerosetting the spleen cell preparations with anti-IgRBC and quantitating the number of rosettes to determine B-cell content. Rosetting was done just before culture and after 20 h incubation of the spleen...
quantitation of B-cell content in TGG-primed spleen cell cultures enriched and nonenriched for TGG-specific B cells

| Exp. no. | Treatment* | No. of B cells/100 lymphoid cells | PFC/10^6 cells ± SE | PFC/10^9 B cells | APC/10^9 B cells† | Fold enrichment |
|----------|-------------|----------------------------------|---------------------|------------------|------------------|-----------------|
| 1        | None        | None                             | 63 ± 1              | 61 ± 12          |      –            |      –          |
|          | TGG         | None                             | 62 ± 1              | 5,612 ± 432      | 90               | 1.4            |
|          | None        | TGG                              | 4.2 ± 0.2           | 244 ± 71         |      –            |      –          |
|          | TGG         | None                             | 4.1 ± 0.3           | 23,729 ± 4,090   | 5,790            | 90.4           |
| 2        | TGG         | None                             | 48 ± 2              | 6,215 ± 310      | 130              | 2.1            |
|          | TGG + (2x)  | None                             | 1.5 ± 0.03$        | 25,816 ± 3,203   | 17,211           | 268.7          |

* Spleen cells primed to TGG were depleted of B cells either with or without prior incubation with TGG to retain TGG-specific B cells. At 0 and 20 h of culture the number of cells which rosetted with anti-Ig-RBC was determined. After 6 days indirect PFC per 10^7 original cells were measured.
† The number of antibody precursor cells was extrapolated from kinetic curves of the appearance of PFC to TGG from which it was estimated that beginning on day 3, PFC to TGG doubled every 8 h with seven doublings.
§ B-cell number after the first B depletion was 3.5 ± 0.5%.

cells to permit regeneration of surface Ig receptors, although the number of B cells at either time was essentially the same. The first experiment in Table III shows that spleen cells primed to TGG and enriched for TGG-specific B cells contained about 4% B cells as compared to 62% before the B cells were removed, indicating a 94% reduction in B-cell content. Despite this high degree of B-cell depletion, the response to TGG was increased from 5,612 PFC to 23,729 PFC per 10^7 cells. Thus, non-B-depleted cultures in response to TGG contained 1.4 antibody precursor cells (APC) per 10^6 B cells, while B-depleted, TGG-specific B-cell cultures contained 5,790 PFC per 10^5 B cells. This represented a 64-fold enrichment of ASC relative to B-cell content. In addition, it was found that if the B cells were depleted twice, rather than once, B-cell number could be further reduced. As shown in the second experiment in Table III, TGG-specific B-cell-enriched cultures contained 3.5% B cells when B depleted once and 1.5% B cells after a second B-cell depletion. Upon relating PFC to 10^5 B cells in enriched and nonenriched cultures, the purification obtained was 132-fold.

The purity of TGG-specific B cells relative to other B cells at the beginning of the culture period was estimated from the PFC response. Extrapolation of the PFC response back seven doublings led to an approximation of the number of APC, since prior kinetic curves of the generation of PFC to TGG indicated that beginning on day 3, PFC to TGG doubled every 8 h with seven doublings (21). Table III shows that TGG-primed spleen cell cultures contained 1.4 antibody precursor cells (APC) in 10^5 B cells, while cultures enriched for TGG-specific B cells contained 90 APC in 10^6 B cells. Thus, about 1 in 1,000 B cells enriched for ASC were capable of generating PFC to TGG or a purity of 0.1%.

Recovery of ASC. Next, the recovery of TGG-specific B cells was determined to assess the efficiency of the purification method. It was expected, as stated
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Recovery of TGG-Specific B Cells from the Ficoll-Isopaque Gradient

Table IV

| Treatment* | Before B depletion | B depletion | Cell recovery | Actual PFC/10⁷ cells | Expected PFC/10⁷ cells† | Recovery of ASC§ |
|------------|--------------------|-------------|---------------|-----------------------|--------------------------|------------------|
| TGG        | –                  | 100         | 5,612 ± 432   | –                     | –                        | –                |
| TGG        | +                  | 20          | 23,729 ± 4,090| 28,068                | 85                       |                  |

* Spleen cells primed to TGG were treated with TGG followed by depletion of the B cells as indicated. After 6 days indirect PFC per 10⁷ original cells were measured.
† Expected PFC/10⁷ cells = (PFC/10⁷ cellsASC-enriched) × [% cell recoveryASC-enriched]/(ASC-recoveryASC-enriched).
§ % Recovery of ASC = [(Actual PFC/10⁷ cellsASC-enriched)/(Expected PFC/10⁷ cellsASC-enriched)] × 100.

Earlier, that if all the ASC were retained and the other B cells removed, the PFC response per 10⁷ spleen cells should double. However, this is only correct if after gradient separation 50% of the cells were recovered at the interface. Table IV shows that the recovery was 20%, which indicates more than a loss of B cells. A 20% cell recovery was typical of most experiments. As a consequence, if all the ASC were retained at the interface, the PFC response should be increased fivefold, since the ASC are in 1/5 the number of cells as compared to the original cell population. In fact, Table IV shows that the PFC response was increased from 5,612 PFC to 23,729 PFC, or almost fivefold. Since 28,068 PFC were expected if all the ASC were retained, 85% of the TGG-specific B cells were recovered. Six other experiments with TGG averaged 82% recovery (54–94% range). The recovery of RBC-specific B cells averaged 19% for four experiments. For example, Table II which shows an increase in PFC to SRBC from 2,558 PFC to 3,837 PFC after B-cell removal represents a 30% recovery of SRBC-specific B cells. Thus, preincubation with specific antigen proved especially effective in preventing the depletion of TGG-specific B cells upon B-cell removal, but was less effective with SRBC-specific B cells.

Requirement of Specific Antigen for Retention of ASC. Spleen cells were incubated with either the antigen used in priming or with an unrelated antigen to determine if specific antigen was required to retain specific B cells. Table V shows that treatment of SRBC-primed spleen cells with TGG did not prevent the removal of precursors of SRBC PFC upon B-cell depletion. On the contrary, the response to SRBC, 5,800 PFC, was reduced to 519 PFC, or greater than 90%. Similarly, the second experiment in Table V shows that incubation of BRBC-primed spleen cells with TGG followed by B-cell depletion did not prevent removal of the precursors to BRBC antibody-producing cells. However, if the spleen cells were first incubated with solubilized BRBC antigen, the PFC response was significantly retained, giving 3,689 PFC before B-cell removal and 2,949 PFC after B-cell removal.

Retention of ASC in Spleen Cells Primed to Two Different Antigens. While having shown that specific antigen was required to retain ASC, it was still possible that significant numbers of B cells capable of responding to other antigens were retained with the ASC. For this reason, spleen cells primed to two
Separation of TGG-Specific B Cells and SRBC-Specific B Cells from Spleen Cells Primed to Both TGG and SRBC

| Treatment* | Before B depletion | B depletion | After B depletion | SRBC in culture | PFC ± SE |
|------------|--------------------|-------------|------------------|-----------------|---------|
|            |                    |             |                  | vs. TGG         | vs. SRBC|
| None       | None               | None        | -                | 266 ± 224       | 82 ± 0  |
| None       | None               | None        | +                | 512 ± 348       | 1,306 ± 489 |
| TGG        | -                  | None        | -                | 14,443 ± 6,284  | -       |
| None       | +                  | TGG         | -                | 82 ± 0          | <41     |
| None       | +                  | TGG         | +                | <41             | <41     |
| TGG        | +                  | None        | -                | 67,650 ± 2,050  | <41     |
| TGG        | +                  | None        | +                | 69,700 ± 4,100  | <41     |
| SRBC-Sol   | +                  | TGG         | -                | 1,653 ± 143     | 959 ± 102 |
| SRBC-Sol   | +                  | TGG         | +                | 1,411 ± 466     | 1,187 ± 371 |

* Spleen cells primed to both TGG and a low dose of SRBC to prime T cells only (14) were incubated either with or without TGG or soluble SRBC antigen followed by removal of the B cells as indicated. After 6 days in culture, indirect PFC to TGG and direct PFC to SRBC per 10^7 initial spleen cells were assayed.

Discussion

ASC were separated from B cells with specificities for other antigens by modification of a simple procedure used to remove B cells from other lymphoid cells (13). This was accomplished by incubating mouse spleen cells with specific antigen before the B cells were removed by rosetting with anti-Ig-RBC. ASC were not removed, presumably because prior incubation with antigen caused the loss of their surface Ig (12). However, it is possible that not all of the Ig was removed, but that antigen "hid" the Ig receptor.

ASC for TGG were enriched about 100-fold relative to B-cell content, which is comparable to other purification procedures. For example, ASC purified by the fluorescence activated cell sorter were enriched 20- to 500-fold depending on the antigen (11), ASC separated on an antigen-gelatin matrix were enriched 100- to 900-fold (6, 7, 25), and ASC eluted from an anti-hapten affinity absorbent were enriched 55-fold (8) and 600-fold (9).

Purity of the B-depleted, TGG-specific B cells relative to B-cell content was estimated at about 0.1%, which is far lower than other methods estimating a 15-55% purity (7-9, 11, 26). However, this difference may be partially explained by the fact that the latter figures are routinely based on antigen-binding studies, while TGG-specific B-cell content was based on APC content, estimated indirectly from the number of PFC, as has been done by others (27-29). In our
### Table VI

**Antibody Response of RBC-Primed Spleen Cells Incubated with and without TGG before B-Cell Depletion**

| Cell source | Treatment | RBC in culture | PFC ± SE |
|-------------|-----------|----------------|----------|
|             | Before B depletion | B depletion | SRBC vs. SRBC | BRBC vs. BRBC |
| SRBC-primed spleen* | None | – | – | 338 ± 62 |
|                  | None | – | + | 4,238 ± 938 |
|                  | TGG  | – | + | 5,800 ± 281 |
|                  | TGG  | + | + | 519 ± 281 |
| BRBC-primed spleen‡ | None | – | – | 144 ± 21 |
|                  | None | – | + | 7,137 ± 1,629 |
|                  | TGG  | + | – | 102 ± 21 |
|                  | TGG  | + | + | 451 ± 62 |
|                  | BRBC-Sol | – | – | 3,669 ± 868 |
|                  | BRBC-Sol | + | – | 2,949 ± 711 |

* Spleen cells from mice primed with a low dose of SRBC to prime T cells only (14) were incubated with TGG followed by B-cell depletion as indicated. After 5 days of culture direct PFC per 10^7 spleen cells put into culture were determined.

‡ Spleen cells from mice primed to BRBC 4 mo prior were incubated either with TGG or soluble BRBC antigen followed by B-cell depletion as indicated. After 6 days of culture, indirect PFC per 10^7 spleen cells put into culture were determined. The few direct PFC were subtracted.

Experiments, TGG-primed spleen cells were estimated to contain about one APC in 100,000 B cells or 0.001% ASC, which means that a 100-fold enrichment leads only to 0.1% purity. However, it is not unusual for antigen-binding studies to show that 0.1–2% of unpurified spleen cell preparations bind an antigen, such as dinitrophenyl (DNP) (30, 31), 4-hydroxy-3-iodo-5-nitro-phenylacetyl (NIP) conjugate (26), human gamma globulin (11, 32), and keyhole limpet hemocyanin (KLH) (8, 11) which means that a 100-fold enrichment of ASC would lead to a 10–100% purity in terms of antigen-binding cells. Thus, despite equal enrichment factors, since the initial number of ASC to TGG is small, the resultant purity is less than if the initial number of ASC were higher.

Antigen-binding cells were not measured in this study since their number appears to inaccurately reflect the number of cells which can respond to antigen. For example, it seems unlikely that 1%, or even 0.1% of the spleen, or 1 in 500 B cells is specific for one antigen if B cells are unipotential. Estimates for the number of B cells capable of responding to an antigen have ranged from 1 in 10,000 to 1 in 1,000,000 (29). In fact, it has been shown that with certain antigens as high as 90% of the cells which bind antigen are not capable of producing antibody to that antigen (33, 34). Also, Nossal and co-workers showed that NIP-specific B cells could be obtained at greater than 30% purity in terms of antigen-binding cells (26), while purity was 2% as assessed by cloning analysis (25). Thus, antigen-binding cell studies may overestimate the number of "functional" ASC, such that in actuality the purity could be considerably less than claimed.

On the other hand, the number of ASC determined indirectly from PFC
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Generation may be underestimated for two reasons. First, the number of PFC does not take into account TGG-specific B cells which proliferated into memory cells, instead of into antibody-producing cells, and the plaque assay itself may not detect antibody-producing cells beyond a certain antibody affinity. Thus, since estimations of absolute purity determined either by antigen binding or functionally by PFC generation may be subject to considerable error, it seems that the only valid means of evaluating various purification methods is in terms of the fold-purification, which does not depend on knowing the absolute purity of ASC, but rather the relative ASC content before and after purification.

In the present study, B cells specific for TGG were recovered by more than 80%, while the recovery of RBC-specific B cells was much less. This was most likely due to the antigen and not the method, since the RBC-Sol antigen may not have contained all the RBC antigens in high enough concentration to saturate the Ig receptors. That ASC specific to RBC were retained at all was surprising since the RBC-Sol preparation was so crude. Experiments are in progress with more purified antigens, such as trinitrophenyl (TNP)-KLH, to determine if B cells specific for other antigens can be recovered as efficiently as those specific for TGG. In addition, the initial number of B cells specific for TNP may be higher than for TGG, leading to a higher purity of ASC upon separation.

The procedure is highly antigen specific in that B cells primed to one antigen can be separated from B cells primed to another antigen. While other purification procedures have shown that ASC can be obtained and that they respond to antigen, the response of the purified ASC to other antigens was usually not measured (9, 11). However, Nossal and Pike (25) have shown by generation of PFC that B cells enriched for NIP antigen do not contain cells capable of responding to DNP antigen. It is important to measure the response to two different antigens, since only a few nonantigen-specific B cells could lead to a significant contamination of the very small number of functional ASC obtained.

Advantages of the indirect purification procedure are the simplicity of obtaining the ASC and their ability to respond to antigen without addition of other cells. ASC can be obtained now as routinely as B cells have been removed in the past, and since the ASC are obtained indirectly by removal of the other B cells, sufficient numbers of T cells and macrophages remain to permit a response to antigen. This contrasts with direct purification procedures which obtain ASC so depleted of others that in order to get an antibody response, filler cells must be added (25, 35). Or, if the ASC are specific for a T-dependent antigen, helper T cells must be added (9, 11). Thus, this latter population is pure largely in the sense that nonantigen-specific B cells have been removed, which is precisely what the indirect procedure accomplishes, but more rapidly. However, if one desires to study the effects of various T-cell subpopulations on the ASC, T cells in the ASC can be removed by anti-T-cell serum and complement and T cells added. Thus, this indirect selection procedure of ASC should be as applicable as methods currently employing the direct purification of ASC.

Summary

Antigen-specific B cells (ASC) were purified from other B cells by prior incubation with specific antigen followed by rosetting with erythrocytes conju-
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Gated with anti-mouse Ig and sedimenting on Ficoll-Isopaque. This procedure allowed the removal of most of the B cells, while those specific for the antigen used in incubation were retained. Relative to the B-cell content, ASC were enriched 64- to 132-fold. The method is highly specific in that B cells primed to two different antigens, turkey gamma globulin and sheep erythrocytes, could be separated from each other. The advantages of this indirect purification procedure over purification procedures which obtain ASC directly are the simplicity of obtaining the ASC and the ability of the ASC of respond to antigen without the addition of other cells.

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