

Review Article

Isolation of antigen-specific B cells

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Summary Cell separation techniques are important in immunology. Major cell populations can be separated successfully with high purity. However, isolation of cells which are specific for particular antigens is more challenging because of the relatively small numbers of antigen-specific cells, and the lack of independent markers available to determine the purity of the isolated population. In this review, the literature describing three principal techniques used to separate antigen-specific cells has been reviewed. Particular emphasis has been placed on yield and purity; the two most important parameters of any purification method. The most promising isolation methods have used immunomagnetic sorting and multiparametric flow cytometric analysis.

Key words: antigen-specific B cell, cell isolation, flow cytometry, immunomagnetic separation.

Introduction

Cell separation techniques are used widely in studies of immunity. Separation methods for isolation of particular cell populations generally use antibodies against differentially expressed cell-surface molecules as targets. Purities of 98% or better are routinely achievable in separating major cell subpopulations such as B cells and T cells.^{1–3} However, the yield of cells can be variable, and it is often necessary to choose between high purity with poor yield and good yield with a lower purity. Assessment of purity requires the use of markers that differ from those used to effect the separation, to avoid a circularity of logic.⁴ For example, if CD2 is used to isolate T cells and also to test the purity, it is possible to conclude that the preparation consists of pure T cells. However, as CD2 is also found on some non-T cells, an independent marker such as CD3 should be used to assess purity.

B cells express surface immunoglobulin (sIg) molecules (sIgM and sIgD) as antigen binding receptors.⁵ Each B cell expresses sIg with a single antigen binding specificity. Although the immune system can respond to a vast number of antigens, only a few thousand B cells express receptors that are specific for any given antigenic epitope.⁶

The binding of an antigen to a specific antigen receptor of a B cell triggers the B cell to activate and differentiate into an antibody-secreting or a memory B cell. Depending on the type of antigen involved in initiating the immune response, i.e. T-dependent or T-independent, the B cell is further stimulated by varying degrees of T cell help^{7–9} or other accessory cells.^{10,11}

The activated B cell proliferates rapidly. Genes in the B cell encoding the Ig variable region undergo somatic hypermutation followed by selection, leading to increased binding

strength between antibody and antigen. Immunoglobulin genes may also undergo further rearrangements to produce other classes of antibodies (for example, IgG, IgA, IgE) of the same antigen-specificity. The B cells that produce high affinity antibodies are selected, proliferate further and differentiate into plasma cells and memory cells.¹²

We have shown previously that Ig gene somatic hypermutation and selection occur infrequently in the human neonate¹³ and infant.¹⁴ This conclusion was reached by sequencing the hypervariable region of the Ig gene from VH₆-expressing B cells, but the antigen-specificity of the Ig produced was not studied. In order to further examine affinity maturation in antigen-driven immune responses, we set out to purify populations of B cells reactive specifically with either a protein antigen (tetanus toxoid) or with polysaccharide antigen (polyribose phosphate [PRP] of the *Haemophilus influenzae* type b bacterium). We found that excess non-biotinylated antigen inhibited binding of biotinylated antigen by some B cells, but not for all antigen binding B cells. This suggested that a proportion of B cells binding antigen did so non-specifically, in the sense that the binding was not through the specific antigen binding site, and led us to review methods used to isolate antigen-specific B cells, with an emphasis on verification of specificity.

The isolation of antigen-specific B or T cells is perhaps the most challenging application of cell separation techniques, because the frequency of cells specific for any particular antigen is usually less than 1%,^{15,16} and because it is more difficult to find a second marker to assess purity.

During the past 30 years, various methods have been developed to isolate antigen-specific B cells in order to study B lymphocyte function. Because of their relatively small numbers, most of the techniques for antigen-specific B cell isolation have used antigen priming to increase the number of specific B cells. Three principal techniques have been used to isolate antigen-specific B cells. These are:

1. capture on an antigen-coated solid matrix
2. rosetting with antigen-coated red blood cells or magnetic particles

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Table 1 Capture on an antigen-coated solid matrix

Authors	Year	ASC separation technique	Antigens	Species	Cell population	Comments
Wigzell <i>et al.</i> ¹⁷	1968	Immunoabsorbent with antigen-coated glass, plastic beads	HSA, BSA, OA	Mouse	Immunized lymph node cells	Yield 60–95%, enrichment 2.5-fold, viability 85%, non-specific binding
Truffa-Bachi <i>et al.</i> ¹⁸	1970	Immunoabsorbent with antigen-coated acrylamide beads	<i>lac</i> hapten	Mouse	Immunized spleen cells	Yield 70–90%, non-specific binding
Edelman <i>et al.</i> ¹⁹	1971	Immunoabsorbent with antigen-coated nylon fibres	Dnp ₃₈ , Tosyl ₃₀ , BSA	Mouse	Immunized spleen cells	Purity 63–88%*, viability 80–90%, non-specific binding up to 33%
Haas <i>et al.</i> ²⁰	1974	Panning with antigen-coated gelatin	DNP, FGG	Mouse	Immunized spleen cells	Purity 35–53%*, enrichment 4-fold, viability 95–100%
Pike <i>et al.</i> ²¹	1984	Panning with antigen-coated gelatin	FLU	Mouse	Unprimed spleen	Purity 11.6%, 7.5%
Liano <i>et al.</i> ²²	1987	Panning with antigen-coated gelatin	TNP	Mouse	Immunized spleen cells	Purity 8%
Steenbakkens <i>et al.</i> ²³	1993	Panning with antigen-coated plates	HIV viral antigen	Mouse	Immunized spleen cells	Yield 5%, purity 24%
De Wildt <i>et al.</i> ²⁴	1997	Panning with antigen-coated plates	U1 RNP	Human	SLE patient PBL	Purity 0.5–1.5%

* Not determined by confirmatory testing. ASC, antigen-specific cell; DNP, Dinitrophenyl; FGG, fowl IgG; FLU, fluorescein; HSA, human serum albumin; OA, ovalbumin; PBL, peripheral blood lymphocyte; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; TNP, trinitrophenyl.

3. staining with fluorescent antigen and isolation by flow cytometric cell sorting

Although these techniques have been progressively developed to achieve the best possible outcome, each has particular limitations and disadvantages.

Some important publications that have reported on isolation of antigen-specific B cells are reviewed below and are summarized in Tables 1–3. In the following discussion some of these published results are analysed in terms of the two parameters which are fundamental to assessing any purification method: yield and purity. The yield is defined as the proportion of antigen-specific cells recovered after purification from the initial mixture. Purity is the percentage of cells in the final product that binds antigen-specifically. Because of our own observations that some cells which bind antigen appear to do so non-specifically, we have particularly looked for evidence of specificity, beyond the fact that cells were purified by antigen binding.

Capture on an antigen-coated solid matrix (Table 1)

The initial attempts to isolate antigen-specific cells date back to the late 1960s. In 1968 Wigzell *et al.* isolated antigen-specific cells by passing immunized mouse lymph node cells through antigen-coated glass and plastic bead columns in order to study antigen-specific immunoglobulin receptors.¹⁷ The immunizing antigens were either human serum albumin (HSA), bovine serum albumin (BSA) or ovalbumin (OA). The columns were effective in removing antigen-specific cells. Yields of isolated antigen-specific cells were 60–95%. Haemolytic plaque assays were used to determine the specificities of isolated cell populations. However, the data presented do not allow determination of purity. Elution of the bound cells by mechanical agitation resulted in reduced cell viability and an enrichment factor of 2.5-fold, suggesting that

the isolated populations contained significant numbers of non-antigen-specific cells. Furthermore, control columns, coated with syngeneic serum only, also bound large numbers of cells.

Truffa-Bachi *et al.* used antigen-coated acrylamide beads to reduce the non-specific binding of cells when attempting to purify antigen-specific B cells.¹⁸ They described the use of antigen-coated acrylamide bead columns to isolate antigen-specific cells from spleen cells of mice immunized with phenyl- β -lactoside (*lac*) hapten. The specificity of the separated B cells was tested by detecting plaque-forming cells. Only 70–90% of the anti*lac*-specific cells bound to the antigen-coated acrylamide beads. This was thought to be due to low affinity for phenyl- β -lactoside antigen of the anti*lac* antibody expressed on the surface of the cells, or the surface antibodies being stripped away as the cells passed over the beads. In the presence of *lac*-hapten nearly all anti*lac* plaque-forming cells were recovered in the filtrate. Non-specific binding of cells to beads was overcome to only a limited extent using this method. Again, this procedure was effective for removing cells with a particular specificity, but not for recovering antigen-specific cells at high purity.

In 1971, Edelman *et al.* published a method to isolate antigen-specific cells using nylon fibres.¹⁹ In this method, Dnp₃₈-BSA, tosyl₃₀-BSA and BSA antigens were covalently coupled to transparent nylon fibres. Spleen cells collected from mice immunized with Dnp₃₈-bovine γ G immunoglobulin, tosyl₃₀-BSA and unconjugated BSA were incubated with the antigen coupled fibres. The adsorbed antigen-specific cells were removed from the fibres either mechanically, by plucking the taut fibres, or chemically, by incubating with competitive inhibitors. The viability of the cells that were removed from the fibres was 80–90%. Although antigen coupled fibres bound many more immunized spleen cells than unimmunized spleen cells, there was significant non-specific

binding, as shown by the number of cells remaining on the fibres after addition of soluble antigen to inhibit the antigen-specific binding. The antigen-specific purity of the eluted cell population was estimated to be 63–88%, in terms of cross-reactivity with a different hapten, but the data do not allow assessment of the absolute purity of the antigen-specific cells.

Haas *et al.* reported in 1974 that non-specific binding of cells to an immunoadsorbent and removal of bound cells without affecting cell viability and function were the two major obstacles in isolating antigen-specific lymphocytes using solid phase immunoadsorbents.²⁰ They described the use of antigen-coated gelatin as an immunoadsorbent for cell fractionation in an attempt to reduce these limitations. Dinitrophenyl (DNP) and fowl IgG (FGG) immunized mouse spleen cells were isolated on antigen-coated gelatin surfaces. The bound cells were recovered with relatively little loss of viability by melting the gelatin at 37°C. The specificities of the bound cell populations were 53% and 35% for DNP and FGG, respectively, in terms of cross-reactivity. Furthermore, only 4-fold enrichment of antibody-secreting cells was seen in the isolated cell population.

In 1984, Pike and Nossal tested the capacity of four fluorescein (FLU) haptenated T-independent antigens; *Escherichia coli* lipopolysaccharide (LPS), *Brucella abortus*, Ficoll and polymerized flagellin to cause activation, proliferation and differentiation of B cells into antibody-secreting cells.²¹ In this experiment, FLU-specific B cells were isolated from mouse spleen cells by incubating the mouse spleen cells in Petri dishes coated with FLU-gelatine and removing the bound cells by melting the gelatine. Fluorescein-specific B cells were cultured at limiting dilution and antibody production was assessed by a haemolytic anti-FLU plaque-forming assay. Only up to 11.6 and 7.5% of isolated B cells produced anti FLU plaques when stimulated with FLU-LPS and FLU-Ficoll, respectively. The yield and enrichment were not reported.

In 1987, Liano and Abbas used the panning technique with haptenated gelatine to isolate hapten, 2,4,6-trinitrophenyl (TNP) and FITC-specific B cells from primed mouse spleen cells in order to assess antigen presentation.²² The isolated B cells were cultured *in vitro* and antibody secretion was measured by anti TNP and FITC plaque-forming assays. If plaque formation following *in vitro* culture is considered to reflect the purity of the isolated B cells, the purity of the TNP-specific B cell population was only about 8%.

In order to generate monoclonal antibodies, Steenbakkens *et al.* in 1993 isolated HIV-specific B cells from spleen cells of mice immunized with HIV antigens by panning on antigen-coated culture plates.²³ The isolated antigen-specific B cells were fused with a myeloma cell line and cultured at limiting dilutions to produce antibody-secreting hybridomas. By measuring antibody production, the yield and purity of the isolated HIV specific B cells were assessed as 5% and 24%, respectively.

De Wildt *et al.* reported the isolation of single B cells for the production of monoclonal antibody fragments.²⁴ They isolated U1 ribonucleoprotein (U1 RNP) auto-antigen-specific B cells from peripheral blood of patients with systemic lupus erythematosus (SLE) by incubating the cells in U1 RNP coated culture wells and removing the bound cells with trypsin. The harvested cells were labelled with B cell markers and sorted as single B cells into microcultures, which were

used for proliferation assays for immunoglobulin gene cloning. When the supernatants of the B cell cultures were tested for specific antibody secretion, only 0.5–1.5% of B cell cultures were positive for anti U1 RNP specific antibodies. This reflects the low purity of the isolated antigen-specific B cell population or a low proportion of antigen binding B cells differentiating into antibody-secreting cells.

Although immunoadsorbent techniques have been used to isolate antigen-specific B cells by many other research groups, the specificity of the isolated cells often has not been determined by confirmatory testing.^{25–28}

Rosetting techniques (Table 2)

Rosetting with antigen-coated red blood cells

The rosetting technique for isolation of antigen-specific cells was first reported by Brody in 1970.²⁹ The basic principle of this technique is to fractionate antigen-specific lymphoid cells by incubating cells with antigen-coated red blood cells to form rosettes. The rosettes can then be separated from non-rosetted cells by sedimentation on a density gradient. In the initial experiments, unprimed mouse spleen and bone marrow cells were incubated with sheep red blood cells (SRBC), and rosettes were separated using a zonal sedimentation technique. The specificity of the isolated B cells was determined by haemolytic plaque assays. Plaque assays do not permit direct assessment of purity, since only a proportion of antigen-specific cells secrete sufficient antibody to form plaques. This study demonstrated synergy between antigen binding cells and the cells that did not directly bind the antigen. This was not effective as a purification method however, because non-rosetting cells were not separated effectively from rosettes in the sedimentation method used.

In 1973, Wilson described a velocity sedimentation procedure to isolate rosette forming cells.³⁰ Sheep red blood cell immunized murine spleen cells were incubated with SRBC to form rosettes. The rosettes were purified by velocity sedimentation, and rosette enriched and rosette depleted cell populations were injected into irradiated mice. After 7 days, haemolytic plaque assays were performed using spleen cells of these mice to determine the presence of antibody-forming cells. It was estimated that enrichment of SRBC-specific B cells in the rosette-forming population was about 12-fold.

Walker *et al.* in 1977 reported a negative selection method for purifying antigen-specific cells by rosetting and removing non-antigen-specific cells from cell populations.³¹ Turkey gamma globulin (TGG) immunized mouse B cells were initially incubated with large amounts of TGG to trigger endocytosis of the surface receptor Ig of antigen-specific B cells by capping with the antigen. The non-antigen-specific B cells were removed from the antigen-specific B cell population by rosetting with red cells coated with anti mouse F(ab')₂ Ig. Following *in vitro* culture for 5–6 days, only 0.1% of the isolated B cells produced anti TGG plaques. This suggests a very low purity or a very low proportion of antigen-specific cells transforming into antibody-secreting cells in culture. However, the antigen-specific cells were enriched 64–132-fold. The potential advantage of this indirect method is the ability to purify antigen-specific cells without binding to another cell population for rosette formation.

Table 2 Rosetting techniques

Authors	Year	ASC separation technique	Antigens	Species	Cell population	Comments
Brody ²⁹	1970	Rosetting with SRBC	Not used	Mouse	Unprimed spleen marrow cells	Yield <0.1%, non-specific binding
Walker <i>et al.</i> ³¹	1977	Rosetting non-ASC (Neg. selection)	TGG	Mouse	Immunized B cells	Purity 0.1%, enrichment 64–132-fold
Kenny <i>et al.</i> ³²	1978	Rosetting with SRBC	SRBC	Mouse	Immunized spleen cells	Purity > 50%,* non-specific binding
Snow <i>et al.</i> ³³	1983	Rosetting with Ag coated HRBC	TNP	Mouse	Unprimed spleen cells	Yield 70%, purity 40%
Myers <i>et al.</i> ³⁴	1986	Rosetting with Ag coated HRBC to separate virgin memory B cells	TNP	Mouse	Primed/unprimed spleen cells	Purity virgin 71%,* memory 60%,* after culture purity 56%* and 30%*
Egeland <i>et al.</i> ³⁵	1988	Immunomagnetic rosetting	RF	Human	RA patient PBMC	Purity 92%, enrichment 10 ³ –10 ⁴ -fold
Oshiba <i>et al.</i> ¹⁵	1994	Immunomagnetic rosetting	TT	Human	Immunized PBL	Yield 2%, purity 78%, T cell binding
Irsch <i>et al.</i> ¹⁶	1995	MACS and flow cytometric analysis	PLA ₂	Human	Allergic patient PBMC	Purity up to 75%, enrichment PLA ₂ 55-fold, <i>ParoI</i> 120-fold
Leyendeckers <i>et al.</i> ³⁶	1999	MACS and flow cytometric analysis	TT	Human	Immunized B cells	Yield 98%, purity 10%, enrichment 20 000-fold

* Not determined by confirmatory testing. ASC, antigen-specific cell; HRBC, horse red blood cells; MACS, magnetic activated cell sorting; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cells; PLA₂, phospholipase A2; RF, rheumatoid factor; SRBC, sheep red blood cells; TGG, turkey gamma globulin; TNP, trinitrophenyl; TT, tetanus toxoid.

A two step density centrifugation combined with a velocity centrifugation procedure was developed by Kenny *et al.* in 1978.³² Sheep red blood cell immunized mouse spleen cells were incubated with SRBC, and rosette-forming antigen-specific cells were then separated from the non-rosetting cell population by density centrifugation on Ficoll-Hypaque medium. In the second step, the rosettes were further purified on the basis of the size of the rosettes. Between 0.8% and 1.6% of spleen cells formed rosettes with SRBC and 80–90% of these were B cells. Haemolytic plaque assays were performed at each stage in the separation procedure to determine the specificities of the isolated populations. It was found that 70–90% of the plaque-forming cells bound non-specifically to glass or cotton wool columns that were used to remove dead cells and macrophages from the cell population. However, the purity of the rosette-forming cell population from immunized mice was between 50 and 100%, and from unprimed mice was around 40%, expressed in terms of ability to form rosettes. An independent test of purity was not performed and only 0.2% of these B cells produced plaques in haemolytic plaque assays.

In 1983, Snow *et al.* used the rosetting technique to fractionate thymus independent antigen binding B cells from unprimed mouse spleen cells.³³ They isolated TNP-specific B cells by using trinitrophenylated-horse red blood cells (TNP-HRBC) to form antigen-specific rosettes. Several purification steps were performed to increase the purity of the rosette-forming cells. At the end of five short centrifugation steps, 40% of cells were rosette-forming cells. After centrifugation through a Percoll gradient the proportion of cells forming rosettes increased to as much as 70%. The isolated cells were cultured for 18 h and stained with TNP-FITC to assess antigen binding ability. About 40% of the cultured cells stained with TNP-FITC, and the cultured cells did not bind unrelated antigens. In the presence of free antigen, 95% of the cultured cells did not stain with TNP-FITC. This study was important in that it used an independent test for purity, and

also used inhibition of binding by free antigen to confirm specificity. Purity was about 40%, a major improvement on earlier studies.

In 1986, Myers *et al.* described a modified rosetting method to isolate antigen binding virgin and memory B cells.³⁴ The method described by Snow *et al.* in 1983 was modified further, using different buffering systems, changes to the centrifugation steps and use of red cells with different haptenation densities to separately isolate virgin and memory B cells. The purity was assessed in terms of ability to form rosettes. After the final purification step, the mean purity of TNP antigen binding virgin B cells from unprimed mouse spleen cells was 71%. The mean purity of TNP antigen binding memory B cells from immunized mouse spleen cells was nearly 60%. Following overnight culture of isolated B cells, re-rosetted B cell percentages decreased to 56% for virgin cells and 30% for memory cells. Myers *et al.* also demonstrated a direct correlation between the haptenation density of red cells and the purification and functional activity of B cells. It was suggested that the shorter incubation period with the antigen and lower hapten densities prevented B cell activation.

Rosetting with antigen-coated magnetic particles

More recent studies have reported the use of antigen-coated immunomagnetic beads to isolate specific B cells from human peripheral blood. In 1988, Egeland *et al.* isolated rheumatoid factor (RF) positive B cells from patients with rheumatoid arthritis and from healthy blood donors by incubating peripheral blood mononuclear cells (PBMC) with HSA and anti HSA IgG coupled supraparamagnetic particles to form RF specific B cell rosettes.³⁵ Non-rosetting cells were separated by placing the mixture in a magnetic field. Although the numbers of RF-specific B cells in peripheral blood are small, a high yield of RF positive cells was achieved with 10³–10⁴-fold enrichment. When RF positive

Table 3 Staining with fluoresceinated antigen and isolation by flow cytometric cell sorting

Authors	Year	ASC separation technique	Antigens	Species	Cell population	Comments
Julius <i>et al.</i> ⁴²	1972	Flow cytometric cell sorting	KLH HSA, HGG	Mouse	Immunized spleen cells	Purity KLH 40–52%* HSA, HGG 55–65%*
Greenstein <i>et al.</i> ⁴³	1980	Flow cytometric cell sorting	TNP	Mouse	Unprimed spleen B cells	Purity 90%, enrichment 15-fold
Hayakawa <i>et al.</i> ⁴⁴	1987	Flow cytometric cell sorting	PE	Mouse	Immunized spleen cells	PE binding up to 0.05%
Hoven <i>et al.</i> ⁴⁵	1989	Flow cytometric cell sorting	HPH, OA	Mouse	Immunized spleen cells	Purity HPH 84%, OA 42% 20–50% ASC secreted Ig in ELISPOT
Lalor <i>et al.</i> ⁴⁶	1992	Flow cytometric cell sorting IgG + IgM- NP + B cells	NP	Mouse	Immunized spleen cells	Purity 2.5%
McHeyzer-Williams <i>et al.</i> ⁴⁷	2000	Flow cytometric cell sorting	NP	Mouse	Immunized spleen cells	55% ASC secreted Ig in ELISPOT
Townsend <i>et al.</i> ⁴⁸	2001	Flow cytometric cell sorting by single epitope multiple staining	HEL	Mouse	Transgenic mouse spleen cells	Highly sensitive Yield, purity not reported

* Not determined by confirmatory testing. ASC, antigen-specific cell; HEL, hen egg white lysozyme; HGG, human gammaglobulin; HPH, helix pomatia haemocyanin; HSA, human serum albumin; KLH, keyhole limpet haemocyanin; NP, 4-hydroxy-3-nitrophenyl acetyl; OA, ovalbumin; PE, phycoerythrin; TNP, 2,4,6-trinitrophenyl.

cells were transformed by Epstein-Barr virus, more than 92% of isolated cells produced RF antibodies in ELISA plaque assay.

Oshiba *et al.* described the isolation of tetanus toxoid (TT) binding B cells from blood from immunized donors by incubating cells with TT coated immunomagnetic beads and detecting rosette formation under a microscope.¹⁵ The results indicated that 0.34% of peripheral blood mononuclear cells bound with TT antigen. This mean percentage increased to 2% following booster immunization. Pre-incubating the cells with free antigen inhibited 90% of rosette formation. About 12% of rosette forming cells were identified as T cells.

Irsch *et al.* in 1995 developed a new method to isolate low frequency antigen-specific B cells by high gradient magnetic activated cell sorting (MACS).¹⁶ Peripheral blood mononuclear cells from patients with severe allergy to bee venom or *Parietaria officinalis* were incubated either with digoxigenin conjugated phospholipase A2 or *ParoI* (phospholipase A2 [PLA₂] and *ParoI* are major allergens of bee venom and *Parietaria officinalis*, respectively). The cells were then labelled with antidigoxigenin antibodies coupled to supra-paramagnetic microbeads and phycoerythrin (PE) conjugated anti digoxigenin antibodies. Allergen-binding cells were separated in magnetic columns and analysed by multiparameter flow cytometry. According to flow cytometric analysis, the frequencies of PLA₂ and *ParoI* binding cells were 1.2% and 0.3% in the blood of allergic donors. These values increased to 66% and 39%, respectively, following MACS enrichment. The purities of the allergen-binding cell populations were reported as 75%. Unlike the data published by Oshiba *et al.* in 1994, the isolated allergen-binding cells in this experiment did not contain T cells. They consisted of B cells, plasma cells and basophilic granulocytes with bound allergen-specific IgE antibodies.

Leyendeckers *et al.* in 1999 combined two step immunomagnetic enrichment (MACS) with multiparameter flow cytometry to isolate and characterize TT specific memory B cells from blood from immunized human adult donors.³⁶ Immunomagnetically preselected CD19+ B cells were incubated with

TT conjugated microbeads to isolate TT binding B cells. These B cells were enumerated and further characterized by multiparameter flow cytometric cell sorting. On average, 10% of the enriched TT binding B cells were positive when stained with TT conjugated PE. Results for inhibitory assays were not presented, but were stated to show specificity of the TT binding B cells. An indirect assessment of yield suggested that 98% of antigen-specific cells in the sample were recovered. They obtained up to a 20 000-fold enrichment of TT specific B cells. Furthermore, Leyendeckers *et al.* also showed similar results for isolation of PLA₂ specific B cells from blood from patients with severe allergy to wasp venom.

The authors have described this combined technique as a highly efficient, very reliable and extremely specific method for isolating antigen-specific B cells. The combination of magnetic cell sorting with flow cytometric evaluation, and the use of non-fluorescent antigen to compete out the binding of fluorescent antigen, make this one of the most thorough analyses of antigen-specific B cell purification in the literature. The results, with purities at best around 10%, show how difficult it is to obtain really pure antigen-specific B cells. The flow cytometric analysis presented by Leyendeckers *et al.* suggests that fluorescence activated cell sorting (FACS) after MACS would yield a higher purity.

Immunomagnetic rosetting methods have been widely used to separate antigen-specific B cells, resulting in satisfactory yields. However, in most instances, confirmatory tests have not been performed to assess the purities of the isolated specific B cell populations.^{37–41}

Staining with fluorescent antigen and isolation by flow cytometric cell sorting (Table 3)

The cell sorting technique to isolate antigen-specific cells was first described by Julius *et al.* in 1972.⁴² Mouse spleen cells primed for keyhole limpet haemocyanin (KLH), HSA or human gammaglobulin (HGG) were labelled either by a direct or by an indirect immunofluorescence method, and were then separated using flow cytometric cell sorting to

isolate antigen binding cells. Sorted cell fractions contained 40–52% fluorescein conjugated KLH antigen binding cells. This was a 400–500-fold enrichment of antigen binding cells compared to the original cell populations. Antigen binding cell percentages for HSA and HGG antigens in the sorted populations were 55–65%. However, the enrichment factors for these two antigens were only 42–62-fold, as there were greater numbers of fluorescent cells in the starting populations. No independent test of purity, or inhibition by unlabelled antigen, was described, leaving open the possibility of undetected non-specific binding.

Greenstein *et al.* in 1980 demonstrated the use of flow sorting techniques to isolate TNP binding B cells in studies of functional B cell heterogeneity.⁴³ In this experiment, mouse spleen B cells primed *in vitro* were stained with fluoresceinated trinitrophenylated bovine serum albumin (FL-TNP-BSA) and fluorescence positive cells were sorted. Fluorescence positive cells formed about 6% of the purified B cell population. When fractionated cell populations were examined by fluorescence microscopy, 90% of the positively sorted cells were fluorescence positive whereas the negatively sorted cells were 5% fluorescence positive. Nearly 0.5% of the unstained control population was also fluorescence positive. This could be due to cell auto-fluorescence, and needs to be considered as a background fluorescence level. Performing inhibitory staining assays by preincubating B cells with TNP-BSA showed that there was nearly 9% non-specific fluorescence staining. Therefore, the purity of the FL-TNP-BSA positive B cell population could be estimated to be approximately 90%.

In 1987, Hayakawa *et al.* reported the isolation of PE binding memory B cells from immunized mouse spleen cells by cell sorting.⁴⁴ Three months after immunization, 0.02–0.05% of the spleen cells were positive for PE binding. Although a PE binding memory cell subpopulation was not detected in unprimed control spleen cells, non-specific binding cannot be ruled out. Inhibitory assays were not performed to determine the purity.

Hoven *et al.* described an immunofluorescence and cell sorting method to isolate ovalbumin (OA) and helix pomatia haemocyanin (HPH) specific splenic B cells from immunized mice.⁴⁵ The yields and purities of the isolated ovalbumin and HPH binding B cell populations were 1%, 3.5% (yield) and 42%, 84% (purity), respectively. However, only 20–50% of the isolated antigen binding B cells resulted in antibody production in ELISPOT assays. This was explained as possibly being due to the antigen binding B cell population consisting of memory B cells, lymphoblasts and plasma cells. It was noted that thorough washing of the B cell suspension prior to fluorescein labelling was necessary to prevent non-specific binding, since antibodies present in the cell suspension against the vaccine antigen bound to almost all B cells through Fc receptors and formed immune complexes with the antigen.

In 1992, Lalor *et al.* used multiparameter flow cytometric cell sorting to isolate antigen-specific memory B cells from 4-hydroxy-3-nitrophenyl acetyl (NP) immunized mouse spleen cells to functionally characterize NP specific B cell clonal involvement.⁴⁶ When IgG⁺ IgM⁻ NP binding B cells were sorted into limiting dilution cultures, only 2.5% of input cells secreted anti NP IgG antibodies.

In order to quantitatively study memory B cell development upon antigen rechallenge and maintenance of the memory B cell compartment, McHeyzer-Williams *et al.* isolated NP specific memory B cells, based on B220 and CD138 expression from immunized mouse spleen cells by flow cytometry.⁴⁷ In their experiments, only 55% of the isolated NP specific CD138⁺ B cell subpopulation secreted anti NP antibodies in ELISPOT assays. Flow cytometry assays with unlabelled antigens to determine the specificity of the isolated B cell population were not performed. However, if the results of the ELISPOT assay are considered to represent the purity of the isolated NP specific CD138⁺ B cell subpopulation, the purity of the isolated B cells appears low. The yield and enrichment factors were not reported.

A single epitope multiple staining immunofluorescence technique was developed to detect very small numbers of antigen-specific B cells by Townsend *et al.*⁴⁸ Spleen cells from mice transgenic for hen egg white lysozyme (HEL) specific immunoglobulin were stained with FITC conjugated HEL and biotin conjugated HEL, and were then analysed by flow cytometry. Double positive B cells were sorted as antigen-specific cells. This reduced the chances of detection of non-specific antigen binding leading to inclusion of false positive cells which causes high background. However, independent confirmatory tests were not performed to determine the purity of the isolated population. The sensitivity of this method was estimated as one antigen-specific B cell in 10 million splenocytes. This method could be considered as extremely sensitive and specific, and is worthy of wider application.

Despite the very high cost of the instrument, flow sorting has been a popular technique for isolating antigen-specific B cells. However, most publications have not reported independent assessment of the purities of the selected populations.^{49–51}

Conclusion

Some important publications describing isolation of antigen-specific B cells using three principal techniques have been reviewed. Each method discussed has advantages and disadvantages.

Techniques using cell capture on an antigen-coated solid matrix have isolated antigen-specific B cells with purities ranging from 0.5% to 88%. Rosetting techniques have provided purities from 0.1% to nearly 100%. The purities achieved by staining with fluorescent antigen and isolation with flow cytometric cell sorting have been 2.5% to 90%. This huge discrepancy of purities within each technique may be due to the isolation of B cell populations specific for different antigens and the use of differing methods for assessing specificity of the isolated B cells. The lowest purities have been recorded when the specificity was tested by plaque-forming assays from cultures of isolated B cells. This may not reflect the true purity of the isolated population however, since only a proportion of antigen-specific cells may differentiate into antibody-secreting cells in culture. The small proportions of antibody-secreting cells enumerated in plaque assays have been shown to increase in some experimental situations by use of combinations of cytokines and other stimulatory factors in the cell cultures.⁵²

Furthermore, different subpopulations of B cells have differing abilities to generate antibody-secreting cells. For example, the marginal zone B cells in the spleen have been reported to proliferate and differentiate into plasma cells more rapidly than circulating B cells.⁵³ The ability of marginal zone B cells to generate antibody-secreting cells in response to T-independent polyclonal stimuli also is greater than that of follicular B cells.^{53,54} Therefore, when comparing and analysing methods for isolation of antigen-specific B cells in terms of yield and purity, it is important to consider the cell populations and the type of antigen used in the isolation.

This review focuses on isolation methods based on cell surface Ig expression. Plasma cells have been purified by methods based on capturing secreted antibody at the cell surface.^{55,56}

Purity and enrichment are the two key factors that determine the efficiency of any isolation technique. The other important features of a good purification method are the maintenance of cell viability and function, reproducibility and general applicability.

The degree of purity required will depend on the intended use of the cells. Some of the methods reviewed were designed primarily to remove cells reacting with a particular antigen, and were successful for that purpose. Other studies required enriched antigen-specific populations for functional studies. However, recovery of pure antigen-specific cells has proved to be difficult. When the properties of antigen-specific cells are the subject of the study, it is necessary to determine the purity of the isolated population. It is therefore essential to test for purity by a process independent of the purification method. Inhibition of binding of the labelled antigen by unlabelled antigen is a useful control in fluorescence-based assays. Specific functional assays, such as plaque assays, can ensure specificity, but may underestimate purity, since some antigen binding cells may not secrete antibody in the plaque assay.

The main limitation in isolating antigen-specific B cells in the techniques reviewed above has been the non-specific binding of cells, which in turn decreases the purity of the isolated population. There is no single method, which provides a high yield of antigen-specific B cells with very high purity.

This review of the literature shows that some studies have not performed specificity controls or functional assays. Where it is possible to assess the purity of the final population, it is often quite low. The method that, in our analysis of the literature, shows the most promising isolation is purification by magnetic beads followed by flow cytometric analysis.^{16,36} Examination of the analytical flow cytometric patterns suggests that, if magnetic separation was followed by flow sorting a high purity could be achieved. Use of flow cytometry in isolation also provides an important tool to study and analyse the properties and characteristics of the isolated cells.

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