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Rapid Isolation of Cloned Isotype Switch Variants Using Fluorescence Activated Cell Sorting[1]

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We have used highly specific, directly fluorescein-conjugated heterologous (conventional) and monoclonal antibodies directed against mouse immunoglobulin isotypes in conjunction with the fluorescence activated cell sorter (FACS) to enrich and clone hybridoma cells producing new immunoglobulin heavy chain constant regions. Each variant retains the parental heavy chain variable region and the parental immunoglobulin light chain; thereby each variant binds the same dansyl (DNS) hapten. These isotype switch variants occur at frequencies of approximately 10^{-5} to 10^{-6}. We were able to isolate the variants by first sorting for an approximate 1000-fold enrichment of the desired immunoglobulin-producing cells, growing these cells for five to nine days, followed by a second 1000-fold enrichment and direct cell cloning into 96 well culture trays. Clones were screened only 3-5 weeks after the original selection for secretion of dansyl-binding immunoglobulin of the selected isotype. Judicious combination of existing methods permits improved analytical techniques using the cell sorter. These include: first, "red" fluorescence staining of dead cells with ethidium bromide or propidium iodide and using the red fluorescence measurement to exclude dead cells from the green fluorescence selection; and second, the use logarithmic amplification of fluorescence signals, allowing for more succinct selection of fluorescence parameters for sorting.

Key terms: Fluorescence activated cell sorting, immunoglobulin heavy chain switching, single cell cloning, monoclonal antibodies

The ability to use the fluorescence activated cell sorter (FACS) to identify and sort viable functional immunocytes is well documented (4, 13). Recent advances in FACS instrumentation have made it possible to select antigen-binding hybridoma cells and to clone them directly into 96 well culture trays (20). The use of logarithmic amplification of fluorescence signals greatly increases the FACS capability to discriminate between cells with different amounts of cell surface markers (12). It has remained difficult, however, to analyze and sort small subpopulations because of nonspecific dead cell fluorescence within the fluorescence intensity range expected from specifically stained cells.

Since dead cells often stain nonspecifically with fluorescent reagents, it is important to exclude them from sorting windows to allow for correct interpretation of staining distributions and optimal selection of the fluorescence range for sorting rare events in a population of cells. We have used the DNA intercalating dyes, propidium iodide (PI) and/or ethidium bromide (EB), to stain dead cells (11). Live cells are identified by the lack of red fluorescence from PI or EB and by appropriate forward light scatter signals. These parameters allow only live cells to be considered in the gated green fluorescence measurements and in sorting for rare cells.

Combining these methods, we have rapidly isolated and cloned rare variants of hybridomas which have switched production of antibody molecules from one immunoglobulin class to another, while retaining the original antigen-binding specificity of the immunoglobulin produced by the parental hybridoma cell line.

Immunoglobulin molecules are composed of heavy (H) and light (L) polypeptide chains held together by disulfide bonds in an H2L2 tetramer. The eight classes (isotypes) of murine immunoglobulin, IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE,

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and IgA are defined by their H chain constant region which is coded for by the H chain gene complex. The tandem arrangement of these genes is illustrated in Figure 1 (25). The phenotypic expression of a H chain results from two distinct DNA rearrangements (16). The first rearrangements bring together a variable region (V) gene and two shorter segments, the D, and J segments (for "diversity" and "joining" respectively) to complete the V gene which codes for the H chain half of the antigen combining site of the molecule (2, 3, 24). These segments are joined probably by excision and deletion of intervening DNA sequences (5). This rearranged gene lies 5′ to the C gene cluster, with respect to the direction of transcription. V, D, J, and C are all located on chromosome 12 in the mouse. Immunoglobulin L chains are formed by similar DNA rearrangements involving a V, J, and either the kappa (κ) or lambda (λ) constant region genes (1). The L chain genes are not linked to the H chain gene complex. During normal B cell development, a given cell first will produce IgM antibody molecules. As the immune response matures antibody production switches to other H chain isotypes, *i.e.*, production of IgG antibody molecules. A second DNA rearrangement is associated with this H chain class switch (5, 17). The composite VDJ gene originally located 5′ to the mu (μ) constant region gene is translocated to a position 5′ to another constant region gene which is then phenotypically expressed (Fig. 1). Highly homologous switch region (S) sequences located in the 5′ flanking region of each constant region gene are thought to mediate a looping out, excision and deletion of intervening constant region gene sequences (8, 9).

The H chain class switch has been studied using the FACS (12, 23) by isolating switch variants from myeloma and hybridoma cell lines. Sorting for these variants required up to seven rounds of selection and months of culture. By combining methodologies designed to refine FACS analysis, we have rapidly isolated these rare variant cells. We have generated an entire "family" of anti-dansyl antibody molecules. The same H chain V region and the same L chain are expressed in stable cell lines producing IgG1, IgG2b, IgG2a, and IgE anti-dansyl antibodies. We present here the application of these methods in the isolation of rare (10^{-5} to 10^{-6}) H chain switch variants in 3 to 5 weeks using both monoclonal and conventional antibodies as selecting reagents.

These antigen-binding switch variant cell lines provide a new vehicle for studies of the molecular events involved in H chain switching. Furthermore, immunoglobulin produced by these variant cell lines will be useful in structural and functional studies of immunoglobulin molecules. They represent a family of immunoglobulin isotypes sharing identical antigen-combining sites.

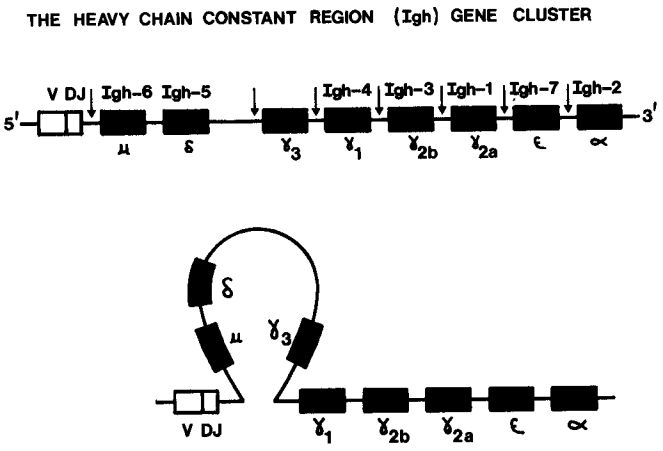

FIG. 1. Illustration of the Ig heavy (H) chain locus and mechanism of the H chain class switch. Names of Igh loci are listed *above*, with heavy chain Ig class listed *below*. The direction of transcription is referred to by 5′; arrows 5′ to each locus refer to switch regions described in the text. *Bottom line* shows the proposed mechanism of the H chain class switch. Looping out of intervening DNA mediates juxtaposition of the composite V gene with a new constant region gene. See text for further explanation (after refs. 6, 8, 25).

Materials and Methods

Cell lines and culture conditions: The parent mouse hybridoma used, 27-4.4, was generated by classical somatic cell hybridization techniques (10, 19). It produces antibody of the IgG1 subclass which reacts with the hapten dansyl (5-[dimethylamino]naphthalene-1-sulfonyl). 27-4.4 and the variants described were maintained in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, and 10% horse serum at 37°C in a 7% CO2-in-air atmosphere.

Fluoresceinated antibody reagents: Goat antibody to MOPC 21 Fc (mouse IgG1) was affinity purified from a MOPC 21-Sepharose 4B column. The final fluoresceinated reagent had a fluorescein-to-protein (mole-to-mole) ratio (F/P) of 4.35. Goat antibody to MPC 11 Fc (mouse IgG2a) was also affinity purified from a MPC 11-Sepharose 4B column and had an F/P of 3.2. This reagent retained reactivity with both IgG2a and IgG2b. Rabbit anti-mouse IgE was affinity purified and had an F/P of 3.7. Mouse monoclonal anti-Ig(4a) 18.1 (anti-IgG1) antibodies and antibodies anti-Ig(1a) 8.3, 14.4, and 15.3 (anti-IgG2a) were purified from sera of hybridoma tumor-bearing mice (18). These antibodies had F/P ratios of 4.0, 4.9, 6.5, and 1.3, respectively.

Fluorescence staining: Cells for analysis or selection were spun out of culture medium and washed once with RPMI-1640 supplemented with 0.1% sodium azide and 3% horse serum (staining medium). Cells were resuspended at 10^6 cells/50 μl. Fluoresceinated antibody was added and allowed to react for 30 min at 4°C. At that point, a 1:1 (vol:vol) mixture of staining medium plus 0.5 μg/ml EB or PI was added to stain dead cells. This mixture stood for 1 min, then the suspension was washed with five volumes of medium to remove free EB or PI and excess antibody. Cells were resuspended at 3×10^6 cells/ml for analysis and sorting.

To examine cells using fluorescence microscopy, cells were spun onto a microscope slide with a cytocentrifuge. Pelleted cells were immediately fixed with ethanol and stained essentially as described above. The amount of fluoresceinated antibody required to stain a cell population completely was determined by FACS analysis of hybridoma cells known to exhibit the appropriate immunoglobulin on their surface. This amount of staining reagent should not exhibit any nonspecific staining of hybridoma cells expressing the inappropriate immunoglobulin class. Reagents that nonspecifically stained inappropriate cell types within the fluorescence intensity range considered to be positive staining were discarded. The amount of reagent used for selection of rare variants was 1/4 to 1/2 that needed for maximal staining of a 100% positive cell population.

Fluorescence sorting: Cells sorted for bulk enrichment were collected directly into a single well of a 24-well culture tray (Costar, Cambridge, MA) containing 1 ml culture medium, or a 3-ml glass conical tube containing 1 ml horse serum. In the latter case, cells were centrifuged and resuspended in 1 ml culture medium and then grown in a single well of a 24-well tray.

"Sorter cloning" of single cells was done directly into 96-well culture trays (Costar) containing 0.1 ml culture medium (20). Only the central 60 wells of the tray were used. When necessary, BALB/c thymocytes at 5×10^5 cells/well were used as a feeder layer.

FACS Parameters for analysis and sorting: Fluorescein, PI and EB dyes were excited by an Argon-ion laser providing 400 mW at 488 nm wavelength. Scattered laser light was blocked by a Corning 3-69 longpass filter (Corning Glass, Corning, NY), and a Zeiss 580-nm longpass reflector (part 46 63 05) (Carl Zeiss Inc., New York, NY) was used to divide the fluorescence signals between two phototubes (EMI 9524A for below 580 nm and EMI 9798B for above 580 nm). Fluorescein fluorescence occurs primarily below 580 nm while PI or EB fluorescence is mostly above 580 nm. The two fluorescence outputs were then linearly combined to yield the independent fluorescein and viability dye (PI or EB) signals. The rationale and electronics for the linear combinations are essentially the same as those previously reported for fluorescein-rhodamine dual immunofluorescence detection (15).

Radioimmunoassay (RIA): Solid-phase RIA was used to screen clones for the production of antibody in culture supernates as described previously (19).

Results

Viability staining and removal of dead cells from FACS analysis: To optimize variant selection efficiency it was necessary to exclude debris, clumped cells and dead cells from live cell fluorescence signals. With lymphocyte populations, dead cells give smaller forward light scatter signals than live cells, allowing good discrimination on this basis alone (14). With tissue culture cells such as hybridomas, however, there is considerable overlap of the forward light scatter signals from dead and live cell populations.

To overcome this difficulty, we have used nucleic acid intercalating dyes to selectively stain dead cells (10). EB and PI dyes are excited by the same 488 nm argon-ion laser line used to excite fluorescein, but their fluorescence emission is easily distinguished from fluorescein fluorescence. Therefore, both low angle light scatter and fluorescence above 580 nm (red fluorescence) are used to distinguish live from dead cells. Although the earlier selections reported here were carried out with EB, we have found the use of PI preferable. EB labels live cells about 1% as brightly as dead cells while live cells labeled with PI are indistinguishable from unstained cells.

Red fluorescence of PI-labeled hybridoma cells shows distinct populations of unstained live and brightly stained dead cells with a few signals falling in between. Most of such "in between" cells were found to be dead when sorted and examined microscopically with acridine orange-EB (20).

An example of the use of this protocol is shown in Figure 2. In panel *a* the single live cells are boxed by windows on scatter and PI fluorescence. Analysing only PI negative signals eliminates most of the false positive signals from the fluorescein staining of dead cells. Panels *b* and *c* illustrate this effect. Hybridoma cells labeled with these viability dyes show no detectable decrease in cloning efficiency.

Rapid isolation of immunoglobulin switch variants: Combining the technical advances described above, we have selected and cloned rare variants which have switched expression of their immunoglobulin constant region while retaining the same antigen-binding specificity. Figure 3 shows the lineage of the family of anti-DNS antibody-producing variants

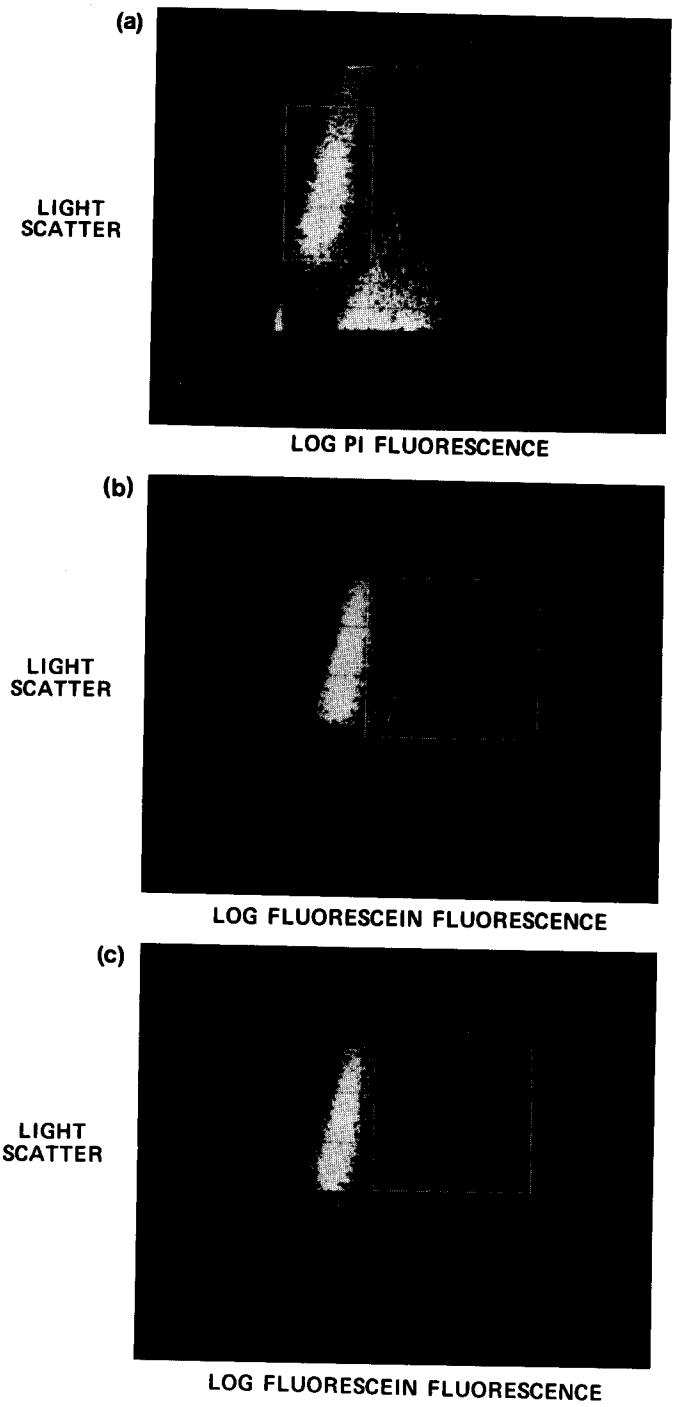

FIG. 2. FACS dot plot distributions illustrating the use of propidium iodide (PI) for dead cell rejection in the analysis of clone 27-42 (IgG1) stained with fluoresceinated antibody to IgG2. Each dot displayed represents the measurements on one cell. *a*, Light scatter *versus* log PI fluorescence. The box encloses the area selected as live cells. *b*, Light scatter *versus* log fluorescein fluorescence with light scatter windowing but no PI windowing. Note the significant number of cells in the box from which we would hope to sort IgG2 producing variants. *c*, Light scatter *versus* fluorescein fluorescence with both light scatter and PI windowing. Note the reduction in false positives cells in the box.

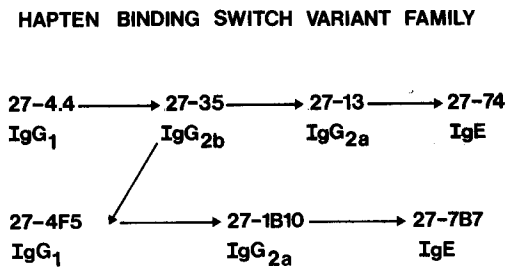

FIG. 3. Lineage of antigen binding hybridoma variants. Nomenclature is based on the 27- of the parent 27-4.4; the number after the dash refers to the Igh locus number of the H chain produced (see Fig. 1), followed by a clone designation.

derived from hybridoma 27-4.4. The parental IgG1 anti-DNS producing cell population first was stained with an antibody recognizing both subclasses of IgG2. After two rounds of enrichment, clones secreting IgG2b were identified. This phenotype switch represents the expression of a new constant region gene located 3′ to the parental H chain gene (Fig. 1). It is noteworthy that although the selecting reagent reacts with both IgG2a and IgG2b immunoglobulin subclasses, all clones identified in this selection expressed the IgG2b H chain gene.

One of these clones, 27-35, then was stained with anti-IgG1 antibodies and after two rounds of enrichment, clones were identified which had reverted to IgG1 expression. The protein products of the 27-4.4 parent and this revertant are identical as determined by two-dimensional gel electrophoresis. These two clones do, however, contain slightly different rearrangements at the DNA level (Oi, *et al.*, in preparation), showing that the revertant, 27-4F5, is a unique variant cell line. The revertant phenotype cannot be explained at the genotype level by current molecular models of H chain class switching (Fig. 1 and references. 6, 17).

From a population of 27-4F5 cells, a variant expressing IgG2a was isolated with the same anti-IgG2 antisera used to select for the 27-35 variant cell line. This variant, 27-1B10, is unusual in that the apparent molecular weight (MW) of its H chain is 10,000 daltons smaller than the normal IgG2a H chain. An IgE producing variant, 27-7B7, isolated from a population of 27-1B10 cells produces normal sized IgE heavy chains.

Selection from 27-35 cells with monoclonal antibodies directed against allotypic determinants on the IgG2a heavy chain gave clones producing normal size IgG2a molecules. From one such clone, 27-13, additional IgE producing variants (27-74) were isolated after staining with a rabbit anti-mouse IgE and FACS selection.

Each variant was selected after only two rounds of FACS enrichment and subsequent growth which took approximately 4 weeks. The biochemical and biophysical analyses of this antibody family will be presented elsewhere (Oi, *et al.*, in preparation). Briefly, characterization of these antibodies shows that each variant produces antibody that binds dansyl in the same binding site as determined by fluorescence spectroscopy of the bound DNS. Each variant, except 27-1B10, produces H chains of the appropriate apparent MW and each has identical L chains. Each variant cell line contains nearly the same number of chromosomes. Examination of 25 metaphase spreads from each stable cell line gave a range of 69–71.

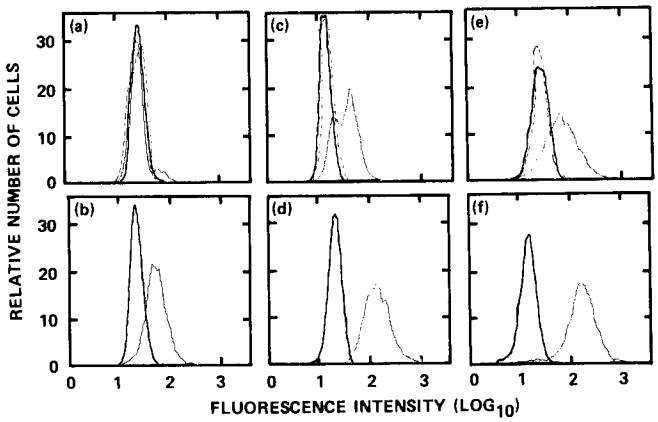

FIG. 4. Logarithmic FACS histograms analyzing variant selections. *Thick solid line* indicates original population; *dashed line*, once enriched population; *thin solid line*, final analysis of either a bulk sorted population (panels *a,c,e*) or a clone derived from the second enrichment and subsequently analyzed (panels *b,d,f*). Panels: *a* and *b*, selection of IgG2b variant (clone 27-35) from IgG1 (27-4.4) with goat anti-mouse IgG2; *c* and *d*, selection of IgG1 revertant (clone 27-4F5) from IgG2b (27-35) with goat antimouse IgG1; *e* and *f*, selection of IgE variant (clone 27-74) from IgG2a (27-13) with rabbit anti-mouse IgE.

Figure 4 shows fluorescence histograms of cell populations stained with appropriate antibodies for three different variant selections. Panels *a*, *c*, and *e* each illustrate the outcome of two enrichment selections. (Note that fluorescence intensity is shown on a logarithmic scale.) The dark line in panel *a* is the first analysis 27-4.4, an IgG1-bearing cell line stained with a directly fluoresceinated antibody reactive with both subclasses of IgG2. From this population, the brightest 0.3% of cells was sorted from 2.5×10^6 cells and grown for 10 days to 1.6×10^6 cells. The dotted histogram in panel *a* is the analysis of these cells stained with the selecting reagent. The brightest 0.3% of this cell population was both bulk sorted and cloned into 96-well tissue culture trays. The bulk sorted cells were cultured until enough cells were available for reanalysis, which showed that 7.7% of this twice-enriched population now stained specifically for IgG2 (*light line*, panel *a*). Culture supernates from clones selected in the second enrichment were tested to determine anti-DNS antibody isotype. Ten of 62 clones were found to produce IgG2b anti-DNS antibody.

FACS histograms of IgG1 anti-DNS selection from the 27.35 cell line also are shown in Figure 4, panels *c* and *d*. 3.2×10^6 Cells were stained to select for reversion to IgG1 expression. The brightest 1.2% of cells were sorted in the first enrichment and the brightest 0.23% of cells in the second; 61% of the final culture stained specifically for IgG1 and did not stain with anti-IgG2. Fourteen of 24 clones selected in the second enrichment sort produced IgG1 anti-DNS antibody.

The final set of histograms in Figure 4 (panels *e*, *f*) shows the selection of an IgE anti-DNS antibody-producing variant. This variant, 27-74, was derived from an IgG2a variant, 27-13 (Fig. 3). From an initial population of 9×10^6 cells stained with fluoresceinated anti-IgE antibody, the brightest 0.4% of cells were selected, grown up and sorted again for IgE expression. The brightest 0.38% of cells were selected. Subsequent analysis (Fig. 4, panel *e*) is hard to interpret, but 35 of 36 clones from the second enrichment produced IgE anti-DNS

antibody. Therefore, we interpret the FACS histogram in panel *f* to show nearly 100% positive staining for IgE and attribute the overlap with the IgG2a parent to the dullness of the rabbit anti-IgE antibody used for the analyses. Clone 27-74, however, stains well for IgE.

Frequencies of H chain switch variants: We have estimated the frequencies of each isotype switch variant in long term cultures in the 27-4.4 family by two methods. Table 1 shows each variant, its parent, and its frequency. Frequency estimations of this type are useful in determining selection procedures, and are not meant to state anything about the rate of mutation associated with the H chain class switch.

The simplest method to determine frequency is to count variants by cytoplasmic staining using the same H chain specific reagents used for FACS selections. Only cells exhibiting clear staining and correct morphology were taken as positive. Examination of slides was done blindly and multiple slides were prepared from each cell line.

The other method for frequency determination relies on accurate counting of the number of cells sorted and the number of live cells passed during a FACS separation. The number of cells sorted is divided by the number of live cells passed to give a fraction representing the reciprocal of an enrichment factor (1/E). For each enrichment sort or cloning, a 1/E term was generated.

The frequency of positive variants in the multiply enriched population was determined by either FACS analysis of a bulk culture or the frequency of positive clones. The 1/E values for first and second bulk selections and the final fraction positive are multiplied together to give the frequency of the variant in the original population.

The two frequency estimates agreed well, not varying more than seven fold. The frequency of the revertant, 27-4F5, is approximately 10 times higher than any other variant by both methods. It should be noted that both the parent clone, 27-4.4 had been in culture for approximately 2 months, while the revertant, 27-4F5, had been cloned 1 month before these frequency determinations. This frequency difference between "forward" and "revertant" variants also was found in variants of myeloma MOPC-21 (23).

Use of monoclonal antibodies to select rare variants: Monoclonal antibodies reactive with defined determinants have proven to be powerful analytical probes of molecular structure (22). We compared the utility of monoclonal antibodies with affinity-purified conventional heterologous antibodies as directly fluoresceinated reagents in the isolation of rare variants.

Figure 5 shows control staining of an IgG2a cell line with a single monoclonal antibody, a mixture of two monoclonal antibodies reactive with distinct antigenic determinants, and affinity-purified goat antibody reaction with both subclasses of mouse IgG2. The mean fluorescence values of these histograms do not differ more than two-fold. The mixture of monoclonal antibodies exhibits additive staining of 16-fold over background compared to 10- and 7.6-fold above background for each monoclonal antibody alone (data not shown).

We used the antibodies with which we generated the histograms in Figure 5 to select for variants from artificial mixtures of cells (Table 2). Each antibody was directly fluoresceinated and used to select variant cell 27-13 (IgG2a) from a population of 27–42 (IgG1) in mixtures of 1:500 or 1:2500. These mixtures were chosen because we believe they represent typical high and low expected frequencies of isotype variants in populations that have undergone one enrichment selection of approximately 10^3-fold; *i.e.*, if the frequency of a variant were initially 10^{-6}, the expected frequency after a 10^3 enrichment would be near 10^{-3}. A sorting threshhold for each of the staining reagents was set to directly clone the brightest 0.1% cells. With this selection parameter, each clone in the 1:500 mixtures and 40% of clones in the 1:2500 mixture would be expected to be 27-13.

As shown in Table 2, in the 1:500 mixtures 76% of clones selected with either a single monoclonal antibody or a mixture

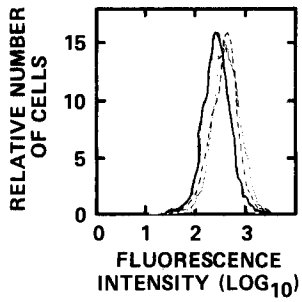

FIG. 5. Logarithmic FACS histogram of comparative staining with directly fluoresceinated McAbs or goat anti-mouse IgG2. IgG2a cell line 29/B1 stained with Ig1a8.3 (*thick solid line*), Ig1a8.3 and Ig1a14.4 (*thin solid line*) and goat anti-IgG2 (*dashed lines*).

Table 1
Frequency of Heavy Chain Switch Variants[a]

Parent		Variant		Frequency ($\times 10^{-6}$) by Analysis of:		
Isotype	Name	Isotype	Name	Population	Clones	Cyto. stain
IgG1	27-4.4	IgG2b	27-35	0.74	3.0	4, 5, 7
IgG1	27-4F5[b]	IgG2a	27-1B10[c]		16.3	
IgG2b	27-35	IgG1	27-4F5[b]	170	160	78, 93
IgG2b	27-35	IgG2a	27-13		1.7	7
IgG2a	27-13	IgE	27-74	13.0	11.0	
IgG2a	27-1B10[c]	IgE	27-7B7		0.9	

[a] See text for explanation of derivation of frequencies and symbols cited above.
[b] Denotes IgG1 revertant.
[c] Denotes IgG2a short chain Ig producer.

Table 2
Monoclonal Antibodies as Selecting Reagents in the Isolation of Rare Variants Isolation of IgG2a Cells from an IgG1 Population

Selecting Reagent	+/Total Clones from 80 Wells at Dilutions of:	
	1:500	1:2500
Monoclonal antibody Ig1a8.3	16/20	2/6
	18/25	5/10
Monoclonal antibodies Ig1a8.3 + Ig1a14.4	29/40	12/20
	34/42	17/29
Goat anti-mouse IgG2	38/38	4/9
	37/41	4/11

of monoclonal antibodies were positive for IgG2a production. Ninety-five per cent of those selected with the heterologous antibody were positive. This difference is probably not statistically significant. With 1:2500 mixtures, each of the three selecting reagents yielded IgG2a-positive clones in nearly the expected 40% frequency. In the derivation of the variant family seen in Figure 3, 27-13(IgG2a) was selected from 27-35(IgG2b) by staining with monoclonal antibodies Ig(1a)8.3 and 74.4.

These data show that the fluorescence distribution of negative cells overlaps the positive distribution, since in a 1:500 mixture selected at thresholds defining 1:1000 there are still some cells of the negative type cloned nonspecifically. Thus, it argues strongly for use of highly purified, brightly staining antibodies to minimize nonspecific binding and to clearly resolve negative and positive cell distributions. Logarithmic amplification of fluorescence signals is useful in this regard, since it exaggerates small differences.

We also have directly selected IgE anti-DNS variants from variant 27-13 without prior enrichment sorting. From previous selections of an IgE variant (Table 1), we estimated the frequency of IgE producing variants in the 27-13 population at approximately 10^{-5}. Of 24 clones selected with an affinity purified rabbit anti-IgE, 3 produced IgE. In this experiment, selection thresholds were set to clone one cell per 10^5 live cells analyzed.

Discussion

We have shown that rapid and efficient isolation of rare (frequencies of 10^{-5} to 10^{-6}) H chain switch variants by FACS selection is feasible. Refined analytical capabilities combining existing methods such as viability staining and gating of dead cells from live cell analysis, and the use of monoclonal antibodies as selecting reagents, have greatly accelerated the process of enrichment and cloning of rare variant cells.

Ideally, one should be able to select reagents and sorting parameters that allow for direct isolation of rare cells without prior enrichment. Such was the case for one of our variant selections ("Results") where an IgE producing hybridoma was directly cloned at a frequency of approximately 10^{-5}. This level of sorting accuracy requires the precise analytic manipulations available through the use of directly fluoresceinated monoclonal antibodies and the ability to choose distinct sorting parameters. In our enrichment procedures, sorting gates of approximately 0.1% were chosen as the lowest acceptable with our reagents. At this point, each cell is deflected in a reasonable period of time. In the case described for the one step isolation of an IgE variant, each deflection required approximately 2 min, making the time required prohibitive. In this regard, staining with viability dyes to remove dead cells that may have retained live cell forward light scattering properties was a crucial addition to our protocol. Logarithmic amplification of the fluorescence signals also increases sorting efficiency through normalized distributions which assist in the interpretation of immunofluorescence distributions, thus allowing for clear resolution of small positive populations of cells carrying low amounts of surface antigen (Fig. 4, panel *a*).

Use of directly fluoresceinated monoclonal antibodies as selecting reagents adds another dimension to the isolation of rare biological events (5). Although we were successful in isolation of the 27-13(IgG2a) variant with monoclonal antibodies, back selections using monoclonal antibodies to isolate IgG1 variants from an IgG2a cell line (27-1B10) or variants expressing IgG2a from an IgE cell line (27-74) were unsuccessful. Revertant variants, when they have been isolated, have occurred in frequencies easily detectable with monoclonal antibodies in our system as determined by both the reconstruction experiment described above (Fig. 5) and the isolation of 27-13 with monoclonal antibodies at an approximate frequency of 3×10^6 (Table 1). Our failure to isolate revertants in these instances probably reflects an extremely rare or absent biological event.

It is important to note that each isotype switch variant described here was isolated in a matter of 3 to 5 weeks, and required only one round of bulk enrichment before direct cloning at the time of a second enrichment. In our system, continual enrichment sorting to a point where variants comprised a population large enough for efficient cloning by limiting dilution was unnecessary. Direct FACS cloning was performed before a distinct variant population could be observed on FACS histograms. Through the ability to clone efficiently in conjunction with an enrichment of at least 10^3, we have greatly decreased the time required to isolate rare variants. Radbruch, *et al.* (23), using linear fluorescence distribution and limiting dilution cloning required between 3 and 9 rounds of selection with 50-fold maximum enrichment, and months of culture maintainence to isolate myeloma switch variants occuring at frequencies similar to those reported here.

We feel that these technical improvements make selection of any rare cell very feasible. Others in our laboratory have succeeded in isolating cells transformed with genes coding for H-2 and HLA antigens using FACS selection at frequencies of approximately 10^{-3} (P. Kavathas, personal communication). Two new families of variants are being developed, each deriving from an IgG parent, giving rise, thus far, to IgG2b producing clones (Dangl, *et al.* unpublished; R. Hardy, personal communication). Previous isolation of rare fetal lymphocytes from maternal peripheral blood (7, 21) also made use of the refined analyses detailed here. Other applications of these techniques will come in any system requiring rapid, precise isolation of rare events.

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