

# **Fluorescence-Activated Cell Sorter and Monoclonal Antibodies: Complementary Tools in Immunodiagnosis and Immunotherapy**

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## **Introduction**

### **The Fluorescence-Activated Cell Sorter and Monoclonal Antibodies as Complementary Tools**

The fluorescence-activated cell sorter (FACS) and monoclonal antibodies are complementary tools in cell biology. Monoclonal antibody technology has made generally available many well-characterized, fluorochrome-conjugated antibodies to cell surface antigens that are required for discriminating FACS analysis and sorting. The FACS, on the other hand, has greatly aided the production, characterization, and utilization of monoclonal-producing hybridomas. A recent extension of this has been the use of the FACS to select from existing hybridomas ones producing monoclonal proteins of different antibody isotype. This paper will focus on this most recent application, discussing hybridoma isotype switch variant selection using the FACS.

### **Monoclonal Antibody Isotype and the Need for Change**

The ability to change the isotype of monoclonal antibodies may greatly enhance their application to cell biology and medicine. Most screening techniques for newly developed hybridomas focus on selecting those producing monoclonal antibodies of desired antigen-binding specificity. It is the immunoglobulin isotype, however, that largely directs the functional activity of the selected monoclonal antibody [1]. Thus, while a monoclonal antibody may have the desired binding specificity, it may still fail to satisfy the requirements for a given biologic assay or application. Many monoclonal antibodies to cell surface antigens, for example, are of the IgG<sub>1</sub> isotype, a class of antibody generally unable to fix complement through the conventional pathway. Because of this, many of these antibodies are not suited for complement-mediated cell depletion experiments or standard tissue-typing assays. The ability to change the isotype of these monoclonal antibodies to either IgG<sub>2a</sub> or IgG<sub>2b</sub>, antibody isotypes that are very efficient in fixing complement, may enhance their application to these fields [2].

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### **Isotype Switch Variants**

As first demonstrated by Rajewsky and his associates, the FACS can isolate rare isotype switch variant cells from large cultures of hybridomas [3-6]. The cell surface immunoglobulin of hybridomas is of identical isotype and antigen-binding specificity as the secreted monoclonal antibody. Using fluorescence-conjugated antibody specific for an immunoglobulin isotype not expressed by the parent hybridoma cell line, one can stain and subsequently sort rare switch variant cells that produce antibodies of desired isotypes, while retaining variable region idiotype(s), binding specificity, and light chain identical to antibodies produced by the parent hybridoma line [5-8]. Switch variants thus apparently switch from the expression of the gene encoding the constant part of one heavy chain to the expression of another gene, while using the same heavy-chain variable region gene and L-chain gene which together encode the antibody's antigen-combining site.

### **Techniques for Rare Cell Sorting**

#### **Improvements in Switch Variant Selection**

Since the first variants were isolated with the FACS, we have made several improvements in the sorting of rare cells which greatly facilitate switch variant selection. The first switch variants were isolated only after repeated rounds of FACS sorting, involving months of tissue culture and many hours of FACS time. Currently, it is possible to detect and, in many cases, directly clone switch variant cells with only one round of FACS selection.

#### **Staining Reagents**

The specificity and brightness of the staining reagent are critical for variant isotype sorting. Conventional polyclonal anti-isotype antibodies must be absorbed extensively. After affinity purification and subsequent conjugation to fluorescein, such preparations may still nonspecifically stain hybridomas, making them unacceptable for switch variant selection. Many recently developed monoclonal anti-mouse allotype antibodies, however, react with only one isotype [9, 10], making them useful reagents for variant sorting [11, 12]. Staining with two such monoclonal antibodies labeled with fluorescein that bind to different sites (epitopes) on the variant isotype specifically labels variant cells more brightly than many fluorochrome-conjugated polyclonal antibody preparations.

#### **Exclusion of Dead Cells**

When selecting for rare cells (i.e., at frequencies of  $1-10/10^6$  cells), it is important to exclude dead and dying cells. Such cells nonspecifically absorb the fluorescein-

labeled antibody, decreasing the reagent's effective staining concentration and increasing background fluorescence. For these reasons, cell populations should have a greater than 90% viability. Even with optimal viability, however, a given population will have many more dead cells than true variants. Therefore, we use a dye, propidium iodide (PI), in our cell-staining experiments. This dye intercalates with RNA and DNA but is excluded from living cells. Because the membranes of dead and dying cells are freely permeable to PI, this dye specifically labels these cells brightly red fluorescent (above 580 nm) when excited by the argon dye laser at 488 nm. We also monitor forward-angle light scatter measured from 3°–12° on the FACS, since the scattered light of most dead cells is less than that of living cells. Thus, by electronically excluding (gating out) cells with bright red fluorescence and low forward-angle light scatter, dead or dying cells can be largely eliminated.

### Cell Flow Rates

To select rare cells such as isotype variants, we use flow rates of 2,500–3,500 cells per second. To minimize cell loss due to "coincidence" gating, in which the drop containing the desired cell is not deflected because of the possible inclusion of unwanted cells, we deflect an average of 1.5 drops per sorted cell. Using an 80- $\mu$  nozzle driven at 28–32 kHz, excellent recovery of sorted cells can be obtained even at these high flow rates. Setting the number of deflected drops to average 1.5, however, provides only a  $\pm 0.25$  drop cycle latitude for error in the drop deflection. Therefore, it is particularly important to determine carefully the optimal intrinsic sort delay setting for each particular nozzle and FACS apparatus. A recent review on fluorescence-activated cell sorting discusses the problem of optimization [13].

### Limiting Dilution Analysis of Sorted Populations

In order to detect directly rare switch variant cells, we devised "pauci-population sorting." With this technique, we sort 5, 25, 50, or 100 selected cells into each well of 96-well microtiter plate. After 1–2 weeks' culture, we test the supernates for antibodies of a desired isotype, using a sensitive, solid-phase radioimmunoassay. The cells from positively identified wells are then expanded in culture and subjected to another round of FACS sorting for individual cell cloning.

This technique has several advantages over sorting into a single large well. First it allows us to assess directly the number of variants present in a given hybridoma population. After establishing the number of wells positive for the variant isotype on the microtiter plate, we use limiting dilution analysis based on the Poisson distribution to calculate the number of true switch variants that were actually sorted onto the plate. Dividing this by the total number of cells sorted onto the plate gives the fraction of cells sorted that were truly switch variants. Multiplying this fraction by the actual fraction of cells sorted with the FACS yields a good approximation of the proportion of variants present in the original population.

A second advantage is the speed at which switch variants can be isolated. One to two weeks after the first round of FACS selection, positive wells will have switch variant cells at frequencies equal to or greater than the reciprocal of the number of cells sorted per well. As the FACS can accurately sort individual cells that have been enriched to fractions greater than  $10^{-3}$ – $10^{-4}$ , switch variants can be readily cloned from positive wells during a second round of FACS sorting.

## Switch Variant Frequency Analysis

### Mutagenesis Studies

Using pauci-population sorting to quantitate the proportion of switch variants within a given hybridoma population, we tested several mutagens for their abilities to increase the rate of isotype switching. Exponentially growing hybridoma cells were maintained in culture in the presence of a mutagen for one cell cycle period of 18 h. A concentration of the drug which reduced the viability of the treated population by 30%–40% was used. Treatment with higher drug levels resulted in populations of cells unacceptable for variant sorting because of excessive debris and poor cell cloning efficiencies. After washing cells free of the mutagen, the cells were cultured for 72–96 h to allow for cell recovery and cell surface expression of variant immunoglobulin.

Table 1 lists a few early results from this investigation. Since we expect variants to accumulate with the length of time elapsed since cloning, we tabulate the approximate time in weeks elapsed since the subcloning of the IgG<sub>1</sub>-producing hybridoma cell lines. The accumulation of variants that occurs as time passes limits the sensitivity of mutagen testing for switch variants that arise acutely. Mitomycin C, a potent inducer of sister chromatid exchange [14], appears not to in-

**Table 1.** Accumulation of switch variants over time after subcloning of IgG<sub>1</sub>-producing hybridoma cell lines

Treatment	Frequency of IgG <sub>2</sub> Variants	
	Experiment 1 3–4 weeks after cloning	Experiment 2 4–8 weeks after cloning
Mitomycin C (50 ng/ml)	$1.2 \times 10^{-5}$	$7.0 \times 10^{-5}$
Untreated	$8.4 \times 10^{-6}$	$5.0 \times 10^{-5}$
	Experiment 3 2–3 weeks after cloning	Experiment 4 8–10 weeks after cloning
	Hydroxyurea (1 µg/ml)	$8.3 \times 10^{-6}$
5-Azacytidine (25 ng/ml)	nt	$1.2 \times 10^{-4}$
Untreated	$1.3 \times 10^{-6}$	$8.3 \times 10^{-5}$

nt, not tested

crease the proportion of switch variants with the limitations stated. With hydroxyurea, however, the frequency of variants increased more than sixfold (Table 1, Experiment 3). However, on the same cell line that had been maintained in culture for several weeks, we did not find a similar proportionate increase in variants compared to the untreated population (Table 1, Experiment 4). Similarly, 5-azacytidine appears to cause only a modest increase in the generation of variants. Note that the frequency of variants found in the untreated population greatly increased after a few weeks in tissue culture.

### The Kinetics of Spontaneous Isotype Switching

The increases in the proportion of variants in control populations stimulated investigation into the rate at which isotype switch variants emerge in a logarithmically growing culture. A newly subcloned population of an IgG<sub>1</sub>-producing hybridoma was maintained in continuous culture, and at various times, aliquots were assayed for IgG<sub>2</sub> variants by pauci-population sorting. The fraction of variants increased with time in culture (Table 2). Although increases in the proportions of both IgG<sub>2b</sub> and IgG<sub>2a</sub> occurred, IgG<sub>2a</sub> variants were more frequent.

Switch variants should accumulate with time, since they arise by spontaneous events (DNA or RNA rearrangement) similar to spontaneous mutations. As initially described in microbial cells by Luria and Delbruck [15], the proportion of mutant-type cells increases linearly with the number of generations through which the culture is maintained, provided the growth rates of normal and mutant cells are equal, back-mutation is negligible, and the proportion of mutant-type cells remains small. These same conditions apply to many of the switch variant families that we have analyzed.

Catcheside derived a formula to calculate the actual mutation rate from the increase in the proportion of mutants noted with time in a logarithmically growing bulk culture assayed prior to achieving mutational equilibrium [16].

$$M = 2(\ln 2) \left[ \frac{\left[ \frac{M_2}{N_2} \right] - \left[ \frac{M_1}{N_1} \right]}{G} \right]$$

Where  $m$  is the mutation rate,  $M_1$  and  $M_2$  are the numbers of mutants at times 1 and 2,  $N_1$  and  $N_2$  are the numbers of cells at these two time points, and  $G$  is the number of generations elapsed between times 1 and 2.

**Table 2.** Increase in variants compared to length of time in culture

Cell generations since cloning	Variants 10 <sup>6</sup> IgG <sub>1</sub> Cells ( $n$ )		
	IgG <sub>2b</sub>	IgG <sub>2a</sub>	Total
25	0.8	0.2	1
36	13	3	16
50	30	90	120
125	50	350	400

The natural logarithm of 2 appears in the formula because the mutation rate is per cell per generation rather than the rate per cell division. Since we are dealing with unsynchronized, exponentially growing populations, an event observed to occur within one generation to produce  $n$  number of cells will actually be the result of  $n/(2\ln 2)$  divisions. For a mutation rate of  $m$ , the proportion of mutant cells within a culture will increase by a fixed increment,  $m/(2\ln 2)$  per generation.

Using the formula described above and the data presented in Table 2, we calculated the switch frequency of IgG<sub>1</sub> to IgG<sub>2</sub> to be approximately  $6 \times 10^{-6}$  per cell per generation.

These results have important implications for switch variant isolation. Obviously, the selective pressure applied by the FACS can not induce isotype switching but only isolate variants after they have occurred spontaneously. Thus, prior to attempting switch variant selection on a particular cell line, the line should be maintained in bulk culture for several weeks to increase the proportion of variants. If the frequency of variants within the culture drifts up to  $10^{-4}$ , then these cells may be cloned directly during the first round of FACS sorting.

## Biologic Implications of Switch Variants for Human Immunotherapy

### Switch Variant Families

Using the techniques outlined above, we have generated switch variant families from many different hybridomas producing monoclonal antibodies to polymorphic human leukocyte antigen (HLA) determinants, tumor-specific antigens, human T cell differentiation antigens, or idiotypic determinants of surface immunoglobulin of human B cell lymphomas. Because of the increase in the proportion of switch variants after propagation in culture, cells were grown for at least 3–4 weeks prior to variant selection. The proportions of variants then found in several hybridoma populations were high enough to permit direct cloning of switch variants during the first round of sorting. Many hybridoma cell populations, however, still required pauci-population sorting enrichment before cloning, perhaps reflecting lower intrinsic isotype switch rates for these cell lines.

### Comparative Studies Using Switch Variants

Because the immunoglobulins produced by individual members of a switch variant family share identical light chains and heavy-chain variable regions, valid comparative studies can be performed on the biologic activities of the various murine immunoglobulin isotypes. For example, we recently compared the IgG<sub>2a</sub>, IgG<sub>2b</sub>, and IgG<sub>1</sub> antibodies of a switch variant family derived from the IgG<sub>1</sub> producing hybridoma, ME-1, in directing antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by human K cells [17]. Although each antibody has identical binding specificity for the HLA B7 surface antigen expressed by the B lymphoblastoid target cell, the IgG<sub>2a</sub> antibody is most efficient at directing

ADCC by human effector cells. The IgG<sub>2b</sub> directs intermediate levels of target cell cytolysis, and the IgG<sub>1</sub> antibody is ineffective.

Switch variant immunoglobulins may resolve the question of whether antibody isotype influences the effectiveness of murine monoclonal antibodies in passive immunotherapy. Mouse immunoglobulin isotypes of antitumor antibodies apparently differ in their ability to influence tumor growth in experimental animal model systems [18-20]. Moreover, it is hypothesized that the isotype of a murine monoclonal antibody may largely determine how effective a reagent it is in the treatment of human malignancy [21]. In collaboration with Drs. Paul Abrams, Kenneth Foon, and Robert Oldham of the Biological Response Modifiers Program, Fredricksburg, VA; with Dr. Benedict Cosimi of the Massachusetts General Hospital, Boston, MA; and Dr. Noel L. Warner of the Becton Dickinson Monoclonal Antibody Center, Mtn. View, CA, clinical trials are in progress with several newly generated variant families, testing monoclonal antibodies of several different isotypes that have identical binding specificities for cell surface differentiation antigens, idiotypic determinants on human B cell lymphomas, or tumor-specific antigens. Determination of any different biologic activities of the murine immunoglobulin isotypes through these studies may allow for a more effective application of murine monoclonal antibodies in the immunotherapy of human disease.

## Summary

Using improved methods for rare cell detection with the fluorescence activated cell sorter (FACS), we have been able to detect and in several cases directly clone rare switch variant cells after only one round of selection. These variant cells have switched from expressing one heavy-chain constant region gene to another, while maintaining expression of the same light-chain and heavy-chain variable region gene which encodes the antibody-combining site. We have determined that various hybridomas spontaneously switch isotypes at rates ranging from  $10^{-5}$  to  $10^{-7}$  per cell per generation. We have also assessed the ability of mutagens to increase this rate.

We have developed several families of switch variant hybridomas producing monoclonal antibodies to polymorphic HLA determinants, tumor-specific antigens, human T cell differentiation antigens, or idiotypic determinants of surface immunoglobulin of human B cell lymphomas. These families have allowed us to compare directly the biologic effector functions of different immunoglobulin isotypes, including their ability to activate complement, to mediate antibody-dependent cellular cytotoxicity, and to influence tumor growth in experimental animals and humans. Such studies underscore the value of carrying out in vitro isotype variant selection in such a way that a hybridoma clone originally selected for the binding specificity of its secreted monoclonal antibody may be modified to produce specific immunoglobulins with isotypes of desired biologic activity.

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