Chapter 5

Switching the Isotype of Monoclonal Antibodies

THOMAS J. KIPPS

1. Introduction

Isotype switch variant selection is an important adjunct to monoclonal antibody technology. Through the production of hybridomas, we can now harness the specificity of the humoral immune response to generate virtually unlimited amounts of monoclonal antibody of desired specificity. Unfortunately, a selected hybridoma may fail to secrete specific immunoglobulin with an isotype of desired functional activity. This problem may be alleviated through the selection of isotype switch variants that secrete antibody of a different immunoglobulin isotype but with preserved antigen-binding specificity. In addition, by selecting hybridomas that produce monoclonals differing from one another in isotype only, comparative studies can be performed on the functional activities of the different murine immunoglobulin isotypes. Such studies may increase our understanding of potential uses of monoclonal antibodies in cell biology and medicine.

This chapter will review current concepts of immunoglobulin isotype switching, discussing several advances that facilitate the selection of switch variant hybridomas.

2. Mouse Immunoglobulin Isotypes

The capacity to change the isotype of an existing murine monoclonal antibody may greatly enhance its utility. It has long been recognized that major differences exist in the biological activities of the different mouse immunoglobulin isotypes.[1] Pentameric IgM, for example, is particularly efficient in hemagglutination and in directing complement-mediated cytolysis. IgE, on the other hand, is unable to fix complement, but is extremely cytophilic for mast cells and basophils, providing for the specific triggering of anaphylaxis. Even among the subclasses of mouse IgG, namely IgG1, IgG2a, IgG2b, and IgG3, major

THOMAS J. KIPPS • Department of Genetics, Stanford University School of Medicine, Stanford, California 94305.

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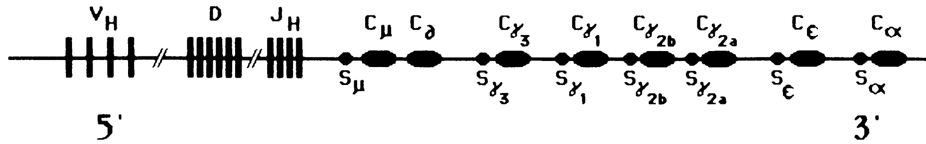

Figure 1. The murine heavy chain immunoglobulin gene complex. The distances between the constant region loci (C) are not drawn to scale, which in kilobases (kb) are: 5'-J_H-(6.5 kb)-$C\mu$-(4.5 kb)-C_δ-(55 kb)-$C\gamma_3$-(34 kb)-$C\gamma_1$-(21 kb)-$C\gamma_{2b}$-(15 kb)-$C\gamma_{2a}$-(14 kb)-C_ϵ-(12kb)-C_α.[27] (S, Switch region; V_H, variable region gene; D, diversity segment; J_H, joining segment.

differences exist. IgG2a and IgG2b fix complement through the conventional pathway, whereas most IgG1 and IgG3 do not.[1–3] Although IgG2a and IgG2b, but not IgG1, are capable of directing passive cutaneous anaphylactic (PCA) reactions in the guinea pig, only IgG1 is able to direct PCA in the mouse.[1–4] More recently, we have detected differences between these three classes of murine antibody in their capacities for directing antibody-dependent cell-mediated cytotoxicity (ADCC) by human K cells.[5] IgG2a functions best in human ADCC. IgG2b directs only intermediate levels of activity, but functions significantly better than IgG1. Furthermore, mouse immunoglobulin isotypes of antitumor antibodies apparently differ in their ability to influence tumor growth in experimental animals.[6–9] Thus, by defining the methodology for isotype switch variant selection, a hybridoma producing a monoclonal antibody of desired specificity may be modified to secrete antigen-specific immunoglobulin with an isotype of desired biological activity.

3. The Genetics of Immunoglobulin Isotype Expression

The immune system has evolved complex genetic machinery for immunoglobulin isotype expression, allowing it to link a usefully permuted variable region gene to any one of the different heavy chain constant region genes through the process of isotype switching. The immunoglobulin heavy chain isotypes are coded for by individual gene loci arranged in tandem along a chromosome region that is 200 kilobases long and 3′ of the variable region gene complex of mouse chromosome 12 (Fig. 1).[10–12] Prior to immunoglobulin gene expression, at least two DNA rearrangements are required to form a functional VDJ gene, composed of the variable (V_H), diversity (D), and joining (J_H) gene segments that together code for the variable region of the antibody heavy chain.[13,14] The VDJ gene is subsequently transcribed with one of the several heavy chain isotype genes to produce a single mRNA transcript that is translated to form the antibody heavy chain. Subsequent rearrangements apparently occur to produce an isotype "switch," allowing for the expression of the same VDJ gene with a different heavy chain isotype gene.

Several models have been proposed to describe the molecular events involved in the isotype switch. One theorizes that selective processing of a large RNA transcript may allow for translation of the variable region with different

constant region isotypes.[15,16] This model may account for the coordinate expression of IgM and IgD.[17–19] A second model proposes that the genes on a single DNA strand internally recombine, deleting the gene coding for the formerly expressed isotype and bringing into juxtaposition the VDJ gene with the newly expressed isotype gene.[20,21] Another model theorizes that this type of DNA rearrangement results instead from unequal recombination between newly formed sister chromatids.[22–25]

Theories postulating that isotype switching results from rearrangement of the heavy chain constant region genes have received considerable support from the analysis of isotype genes in plasmacytomas.[10,13,18,20,24,26] These studies have demonstrated that the expressed heavy chain isotype gene is repositioned immediately downstream, or 3′, of the VDJ gene through DNA rearrangement(s) that delete intervening constant region exons. Rearrangement of the DNA is noted to occur at so-called "switch regions" (S), located 5′ of each heavy chain locus except delta (Fig. 1). These regions share short repetitive sequences [i.e., TGAGC and TGGG(G)],[27] which possibly allow for pairing of different switch region loci for S–S recombination. These regions also may act as binding sites for postulated switch recombinases that catalyze the DNA rearrangements necessary for isotype switching.

These features of the immunoglobulin gene complex have important implications for the design of an isotype switch variant selection. Analyses of the DNA from several hybridomas have demonstrated rearrangements of isotype genes similar to that noted in myelomas, with deletion of the genes coding for isotypes located 5′ of the expressed heavy chain isotype gene.[28,29] Such deletions make switching to these isotypes impossible, unless switch recombination could occur with the upstream isotype genes positioned on the nonexpressed homologous chromosome. Indeed, we have observed interchromosomal recombination between isotype genes in cultured hybridomas.[30] Variants have also been selected that express an upstream isotype gene[31,32]; however, this has been observed only when cloned switch variant cell populations were selected for "back-switch" variants producing antibody of the original parental isotype. As such, these examples of "back-switch" variants may not be representative of hybridomas obtained through a hybrid fusion. It should be noted that rearrangements and deletions of constant region genes frequently occur in the nonexpressed heavy chain gene complex.[24,28,33,34] Thus, before attempting to select variants expressing an isotype gene located 5′ of the originally expressed heavy chain gene, it is best to determine whether the gene coding for the desired isotype is still present in the hybridoma's genome.

4. Spontaneous Isotype Switching in Myelomas and Hybridomas

In vitro isotype switching was first observed in the IgG2b-secreting MPC 11 myeloma.[35] After mutagenesis with melphalan or ICR 191, approximately 0.5% of the cells cloned in soft agar expressed myeloma protein with altered

heavy chains, a few of which reacted with antibodies specific for mouse IgG2a. Subsequent characterization of these variants confirmed that some of these clones had indeed undergone an isotype switch from IgG2b to IgG2a, while retaining expression of the same light chain genes and heavy chain VDJ rearrangement.[36–38] Although spontaneously occurring switch variants of MPC 11 were also noted, these were relatively rare and difficult to isolate by soft agar cloning techniques. Staining the myeloma cells with fluorescein-conjugated antibody specific for mouse IgG2a, however, Rajewsky and his associates demonstrated that these relatively rare spontaneous switch variant cells could be isolated from MPC 11 using the fluorescence activated cell sorter (FACS).[39] By selecting cells based on variant surface immunoglobulin expression, these investigators increased the efficiency of isolating variant myeloma cells two- to threefold over the previously employed technique of antibody–agar overlay.

Spontaneous isotype switching subsequently was observed in monoclonal antibody-producing hybridomas.[3,5,32,40–43] Switch variant hybridomas are detected at frequencies similar to those found in myeloma cell populations (see Section 6). Importantly, such hybridoma variants produce antibodies with idiotypic specificities, antigen-binding activities, and antibody light chains that are identical to that of the antibodies produced by the parent hybridoma.

In vitro isotype switching apparently results from DNA rearrangement(s) similar in many ways to those found in plasmacytomas.[29] The newly expressed heavy chain gene is found generally 3′ of the expressed VDJ gene, this resulting from excision of DNA coding for the formerly expressed isotype. Although the sites of recombination responsible for such rearrangement(s) may be outside the physiological switch regions (S), switch variants generally produce antibodies that are analogous to physiologically occurring antibodies of the same isotype in their molecular weight, glycosylation, and primary protein sequence.

5. Techniques of Switch Variant Selection

5.1. Cloning in Soft Agar

The first method used for selecting variant hybridomas involved cloning the cells in soft agar.[44] After discrete colonies are detected, heterologous isotype-specific antibody is added to the agar plates. Either the presence of a surrounding precipitin ring (positive selection) or lack thereof (negative selection) signals the presence of a variant clone. Since this method screens for alteration(s) in the secreted antibody, it can potentially be used to select variants from myelomas having little or no surface immunoglobulin. Although this technique has been used successfully to isolate a number of interesting variants of MPC 11,[35,36] it may be limited to selecting variants that arise at relatively high frequencies. This is because one can easily screen only a limited number of cells with this method, usually $(1-5) \times 10^4$.

A permutation on the soft agar cloning technique that may be useful in limited settings is that of clonal abortion. Köhler and Shulman used this method for

selecting variants from a hybridoma-secreting IgM specific for the hapten trinitrophenol.[45] Hybridoma cells were conjugated with the hapten so as to make their secreted antibody self-reactive. The cells were then plated in soft agar to which guinea pig complement was added. Only variants producing antibody that could not fix complement or bind antigen would survive under such selective pressure. Although this group did not isolate isotype switch variants, they succeeded in selecting a variety of variant hybridomas secreting variant IgM molecules. It remains to be seen whether this method may be successful in selecting noncomplement-fixing isotype variants when the probability of isotype switching is higher, i.e., from IgG2a to IgE (see Section 6).

5.2. Sequential Sublining

A more recent method of switch variant selection is through sequential sublining[42] or sib selection (see Spira *et al.*, this volume, Chapter 4). Hybridomas are plated into microtiter plates at approximately 10^4 cells per well. After 3–4 days of culture, the supernates are harvested and assayed for the presence of variant isotype using a sensitive solid phase radioimmunoassay. Wells identified as possibly containing switch variant cells are grown up in culture and the cycle is repeated. Through several rounds of such sequential sublining, variant cells may become sufficiently enriched to allow cell cloning.

5.3. Fluorescence Activated Cell Sorting

A powerful way to isolate switch variant hybridomas is to use the fluorescence activated cell sorter (FACS). As mentioned previously, this method selects cells based on their expression of surface immunoglobulin, which for hybridomas is of the same isotype as the secreted antibody. Cells are stained with fluorochrome-labeled antibodies specific for the variant isotype and the most brightly fluorescent cells are selected. In less than 1 hr of sorting, one can easily screen 10^7 hybridomas, enriching them for switch variants more than a thousandfold. Of course, operational knowledge of the FACS is required. A detailed description of this is outside the scope of this chapter and the reader is referred to a recent review.[46]

Although selection of the first switch variants required many weeks with successive rounds of enrichment sorting,[39] several improvements have been made that significantly lessen the time and effort required for variant isolation. Currently, it is possible to detect, and in many cases clone, switch variant cells in only a single sort.[5,47] The following sections will describe several important measures either necessary or helpful for rapid detection of isotype switch variant hybridomas using the FACS.

5.3.1. Staining Reagents

Efficient variant selection is predicated on the quality of the fluorescent reagent(s) used to label variant cells. Close attention must be given to both the

specificity and staining intensity of a given antibody preparation. The fluorescence conferred upon the variant cell by the staining reagent should be bright enough to allow for easy discrimination from cells having bright autofluorescence. Since the autofluorescence of individual cells within a given hybridoma population may vary over nearly an order of magnitude in brightness, staining reagents ideally should increase the mean fluorescence of cell populations bearing desired isotype(s) tenfold.

5.3.1a. Heterologous Antibody. Fluorescein-conjugated heterologous anti-isotype antibodies can serve as excellent selective reagents. These are generally prepared in the goat, since immunoglobulins of this animal tend not to be cytophilic for murine cells. Antibodies purified from the immune sera must be extensively absorbed with other mouse antibody isotypes to render them uniquely specific for the heavy chain isotype of interest prior to fluorescein conjugation.

Heterologous immunoglobulin preparations are composed of heterogeneous antibodies with many different binding affinities for mouse immunoglobulin. As such, the staining intensity may be quite sensitive to changes in antibody concentration.[3] Titrations with control hybridomas expressing the desired isotype should be performed at several different concentrations of antibody to determine not only the amount of antibody required for saturation staining of a given number of cells, but also the optimal antibody concentration for effective staining (usually 25–50 μg/ml). For selecting hybridoma variants present at 10^{-4}–10^{-7} within a hybridoma population, the same antibody concentration is used; however, the cell concentration is increased fivefold, so that only one-fifth the total amount of antibody required for saturation staining of positive control hybridomas is used.

Heterologous anti-isotype antibodies are commercially available. Many reputedly specific reagents unfortunately do not have the specificity required for rare cell sorting. Exceptions are the FITC-conjugated goat anti-isotype antibodies recently made available from Southern Biotechnology Associates (Birmingham, Alabama). We have tested several and found them well suited for selecting isotype switch variants.

5.3.1b. Monoclonal Antibody. Monoclonal antiallotypic antibodies may have several advantages over heterologous anti-isotype antibodies for selecting switch variants. Many are isotype-specific, thus obviating the need for absorption.[48,49] Most bind with high affinity, making staining intensity less sensitive to changes in antibody concentration. Also, by mixing two fluorescein-conjugated antiallotype antibodies that noncompetitively bind to different sites on immunoglobulins of a selected isotype, the staining intensity achieved with monoclonal antibodies may be comparable to that of heterologous antibody preparations.[50,51]

5.3.1c. Fluorochromes. Fluorescein isothiocyanate (FITC) is the most widely used fluorescent molecule for labeling specific antibody, partly because

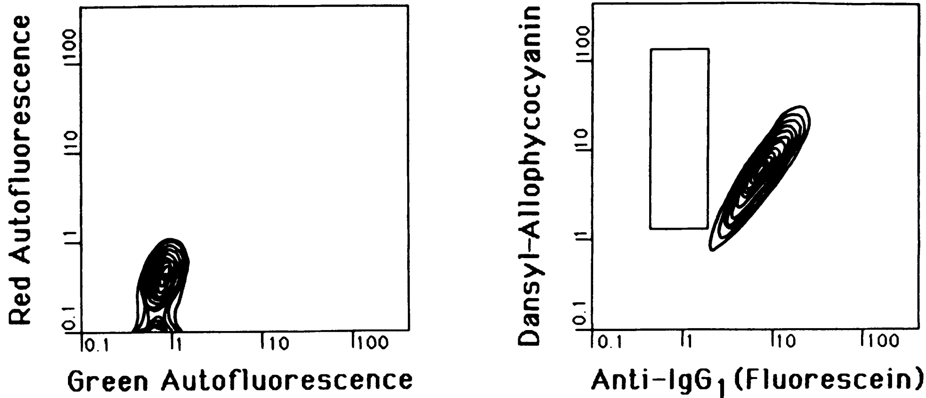

Figure 2. Fluorescence staining of IgG1 antidansyl-antibody-producing hybridomas. Hybridomas were stained with FITC–goat anti-mouse IgG1 and dansyl-allophycocyanin. Histograms depict fluorescence intensity of (right) stained and (left) unstained hybridomas when excited at 488 and 600 nm with a dual-laser FACS-II equipped with logarithmic amplifiers. The numbers on the ordinate and abscissa refer to the relative fluorescence intensity. Boxed area in the histogram on the right circumscribes the sorting gate used for switch variant selection.

it is so readily conjugated to proteins via the isothiocyanate moiety.[52] For variant sorting, however, it is important to monitor the average number of fluorescein molecules covalently attached to the isotype specific antibody. This fluorescein to protein ratio (F/P) can be calculated from the measured light absorption of the conjugated protein at 280 and 495 nm.[52] Antibodies with F/P ratios less than two may not stain cells with sufficient intensity for variant sorting. On the other hand, overconjugation may make the antibodies nonspecifically "sticky." Therefore, for variant sorting, antibodies with F/P ratios of three to four generally work best.

The advent of phycobiliproteins may allow for new dimensions in hybridoma variant selection.[53,54] These brightly fluorescent proteins may be coupled with haptens, allowing for specific staining of hybridomas producing antihapten antibodies. One can stain with a combination of one fluorochrome linked to antigen and a second linked to anti-isotype antibody, and carry out multiparameter analysis and sorting using a dual-laser FACS. Seen in Fig. 2, for example, are histograms of stained and unstained hybridomas producing IgG1 monoclonal antibody specific for the hapten dansyl (5-dimethylaminonaphthalene-1-sulfonyl). Staining for dansyl is achieved with the hapten conjugated to allophycocyanin, a phycobiliprotein with bright fluorescence at wavelengths between 640 and 680 nm when excited at 600 nm. By concommitantly staining with FITC–anti-IgG1, one can select for variants that have lost surface IgG1 expression but retain antigen-binding activity (boxed area of Fig. 2). Variants so selected are primarily isotype switch variants producing IgG2b antidansyl antibody.

5.3.2. Exclusion of Dead Cells

For efficient rare cell sorting, it is necessary to exclude dead and dying cells. Such cells increase the background fluorescence and decrease the staining reagent's effective concentration by nonspecifically absorbing the fluorescein-labeled antibody. Thus, cells should be greater than 90% viable prior to sorting. Even so, the proportion of dead cells usually will exceed that of switch variants. For this reason, we include propidium iodide at 1μg/ml in the antibody staining mixture. This dye labels dead and dying cells, making them brightly red fluorescent when excited by the argon dye laser at 488 nm. As such these cells can be electronically excluded from analysis and sorting.[46,51]

5.3.3. Paucipopulation Sorting

Enhanced sensitivity for detecting switch variant hybridomas can be achieved through paucipopulation sorting.[47] Even with the best of staining reagents and sorter conditions, the maximum enrichment that can be achieved with the FACS is on the order of 10^3–10^4. Thus, when sorting for cells present at frequencies of 10^{-6}–10^{-7}, the sorted sample will consist primarily of parent-type hybridomas. By sorting 5, 25, or 125 cells into individual wells of a 96-well microtiter plate, however, a limiting dilution of switch variant cells is achieved. After 1 week of culture, the supernates from the separate wells can be assayed for the variant isotype using a solid phase radioimmune assay (RIA). Using the Poisson distribution, the number of switch variant cells actually sorted can be calculated from the proportion of wells found positive for the desired isotype.

This method has several advantages. First, it permits direct determination of the proportion of variants found within a given population without the need for collating data from successive rounds of sorting. Second, it increases the speed at which variants can be isolated. For, after the first sort, paucipopulations of cells that are greatly enriched for variants can be identified. Thus, approximately 2 weeks after the initial sort, the switch variants in these populations can be cloned.

6. Kinetics of Isotype Switching

The analysis of the kinetics with which spontaneously arising switch variants emerge within a culture of hybridomas is basically a problem in population genetics. The proportion of variants within a given hybridoma population will depend on the intrinsic rate(s) of spontaneous isotype switching per cell division, the growth rate(s) of the hybridomas and their newly generated switch variant progeny, and the per generation rate(s) of subsequent switching in individual switch variant cells.

Luria and Delbruck formulated an analysis of the rate at which mutant-type cells emerge within a microbial population[55] that may apply to switch

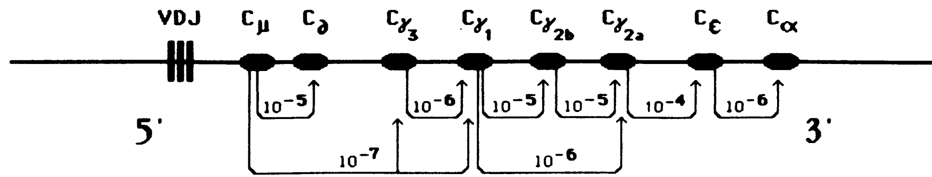

Figure 3. Approximate spontaneous isotype switch frequencies per cell division in cultured murine hybridomas. VDJ, Rearranged variable region gene complex; C, constant region loci.

variant hybridomas. For logarithmically growing cultures, they concluded that the proportion of mutants will increase linearly with time, provided that the mutation rate is low, backmutation is neglible, the growth rates of mutant and wild-type cells are equal, and the proportion of mutants to wild-type cells remains relatively small. Although exceptions are observed, these same conditions are met by many hybridoma switch variant families. Thus, for most hybridomas, the relative proportion of switch variants should increase with the number of generations spent in culture. This has been verified experimentally.[47]

Catcheside[56] derived a formula useful for calculating the actual mutation rate of cells within a logarithmically growing culture prior to achieving mutational equilibrium:

$$m = 2(\ln 2)\,\frac{M_2/N_2 - M_1/N_1}{G}$$

where m is the mutation rate per cell per generation, 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.

By determining the proportion of variants within a given hybridoma population at two separate times, one can use the above formula to obtain approximations of the switch variant frequency per cell per generation. Figure 3 depicts the generalized spontaneous isotype switch frequencies in cultured hybridomas (T. J. Kipps, unpublished observations). These frequencies may not apply to all hybridomas and are meant to serve only as approximations. Several points, however, are worth emphasizing. The rates of spontaneous isotype switching are comparable to conventional spontaneous mutation frequencies, these rates ranging from 10^{-4} to 10^{-7}. The probabilities of isotype switching vary, depending on the isotypes of both parent and switch variant hybridomas. Switch events occurring with the highest probability exchange the expression of one isotype gene to that of its nearest 3′ neighbor. Switching to isotypes located farther downstream apparently can occur directly, however. The probability of such spontaneous events is much greater than the product of probabilities for successive switching through an isotype variant interme-

diate. Spontaneous switching to isotype genes located 5′ of the heavy chain gene expressed by the hybridoma after fusion has not been detected.

7. Effects of Mutagens on the Rate of Isotype Switching

Several mutagens have been found to increase the number of antibody heavy chain variants. Cells generally are cultured for one cell cycle with a mutagen at concentrations that reduce the viability of the treated cells by 40%. At this dose, melphalan or ICR 191 significantly augments the number of antibody heavy chain variants of the MPC 11 myeloma.[35] Most of these, however, are not switch variants and secrete altered heavy chains of the parent isotype.[57] We have observed four- to eightfold increases in the number of isotype switch variants found in newly subcloned hybridomas so treated with hydroxyurea.[47] The relative increase in the proportion of variants found in drug-treated hybridoma populations that previously had been cultured for several weeks, however, is not as apparent, due to the accumulation of spontaneous variants within the culture, increasing the switch variant "background" of matched untreated controls. Recently, it has been reported that UV induces high rates of spontaneous isotype switching in a human lymphoblastoid cell line.[58] Whether this is also true for murine hybridomas remains to be tested.

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