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Review article

SELECTION OF HYBRIDOMAS AND HYBRIDOMA VARIANTS USING THE FLUORESCENCE ACTIVATED CELL SORTER [1]

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INTRODUCTION

Monoclonal antibody reagents of extreme specificity, available in potentially unlimited quantities, have caused a revolution in immunology, cell biology, molecular genetics, medicine, and essentially all levels of biomedical science since the initial report of Köhler and Milstein (1975). Hybridoma production is now routine in many laboratories, and promises to increase in coming years as the spectrum of uses for monoclonal antibodies spreads. Concomitant with this revolution in the application of monoclonal antibodies is the requirement for rapid development of hybridoma selection technology. It is often the case that hundreds of primary fusion cultures, derived from many hybridizations, require rapid screening, cloning, and characterization before the desired reagent is recovered. Thus, large scale generation of hybridomas requires a great deal of forethought, labor, and capital. This is especially true in searches for antibodies reactive with poorly represented antigens, such as those associated with specific tumor types, polymorphic determinants, or antigens that are weak immunogens. General fusion techniques and hybridoma production are the subject of a number of recent reviews and will not be considered further (Goding, 1980; Oi and Herzenberg, 1980).

In this review, we address a specific aspect of hybridoma production aimed at substantially speeding the process of isolating stable hybridomas from early fusion cultures, or of selecting hybridoma variants with desired structural and functional properties from already existing hybridoma clones.

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This growing methodology is based on the ability of the fluorescence activated cell sorter (FACS) (Bonner et al., 1972; Herzenberg and Herzenberg, 1978) to analyze for and then to clone directly desired hybridoma cells. Analytic powers of the FACS (Becton-Dickinson, Mt. View, CA) and other commercial and home built flow cytofluorimetric sorters are well established in many areas of immunobiology, genetics, cell biology, and cytology (Loken and Herzenberg, 1975; Herzenberg et al., 1976; Fulwyler, 1980; Miller et al., 1981). Used as cell sorters, their highly refined ability to viably separate cells for a variety of functional and structural characterizations has been an important tool provided by these instruments. However, the power of cell sorters to enhance and refine an investigator's control in the production of hybridomas remains largely untapped.

The work of Parks et al. (1979) using antigen coated fluorescent microspheres to label desired antigen binding hybridomas, combined with direct FACS cloning of these cells, merged 2 important facets of hybridoma production-selection of the rare immunoglobulin producing hybridomas from a large fusion population early after hybridization and reliable cloning of the selected cell. Workers in Klaus Rajewsky's laboratory in Cologne have used FACS enrichment to isolate variant clones of myelomas (of unknown antigen binding specificity) expressing new 'switched' immunoglobulin heavy chain isotypes (classes) while retaining the parental idiotypic (variable region) determinants (Liesegang et al., 1978; Radbruch et al., 1980). We have recently used improved FACS analytic procedures and direct FACS cloning, to construct a 'family' of antigen binding heavy chain switch variants (Dangl et al., 1982). Each of the linearly derived clones produces immunoglobulin molecules with identical antigen binding sites and identical light chains in conjunction with IgG1, IgG2b, IgG2a, and IgE heavy chain isotypes. Using a different parent hybridoma, we have also isolated a 2-member switch variant family, as has Rajewsky's group (Neuberger and Rajewsky, 1981). Our antigen binding families have proven useful in studies of 2 constant region associated properties of the immunoglobulin molecule, complement fixation and segmental flexibility. Another interesting use of FACS selection of rare cells relevant to this review comes in the selection of clones exhibiting structural mutation in their H-2K^k molecules (Holtkamp et al., 1981). These experiments used 2 cross blocking monoclonal antibodies and FACS enrichment and cloning to isolate structural variants defined by the loss of one of the determinants recognized by the selecting reagents.

The majority of this review, then, will be devoted to detailed analysis of the 3 types of FACS selection of hybridomas and hybridoma variants described above. Mention will be made, however, of other instances of FACS selection of rare cells, notably of antigen binding B cells and T cell hybrids, in terms of their effect on the evolution of these methods. Due to the relative infancy of this facet of FACS technology and the paucity of relevant literature, we detail here methods designed to increase the reader's general

awareness of FACS selection of hybridomas, as well as pointing out the current limitations of the selection systems. It is our hope that isolations of the type described here will simplify and speed the 2 often limiting steps in specific hybridoma production: selection of a rare antibody producing cell and its subsequent efficient cloning, and selection of hybridomas expressing a desired isotype as well as antigen specificity.

EARLY SELECTIONS OF RARE CELLS BY FACS

Use of directly fluoresceinated soluble antigen to label antigen binding B cells, and FACS sorting for the enrichment of these cells was demonstrated in 1972 (Julius et al., 1972). These investigators sorted spleen cells from mice primed with keyhole limpet hemocyanin (KLH) stained with directly fluoresceinated KLH. After either 1 or 2 enrichment sorts, the percentage of cells binding the fluorescent antigen had increased from approximately 0.1% to 40%, an enrichment of 400-fold. When these viably sorted antigen binding cells were transferred to non-lethally irradiated hosts, they remained able to elicit a secondary anti-KLH response when presented with T cell help. These experiments established that antigen binding B cells are the precursors of antibody secreting plasma cells. Recently, Greenstein and co-workers (1980) employed fluorescein conjugates of the hapten trinitrophenol (TNP) coupled to various carriers as a staining reagent to sort B cells specific for TNP. Though antigen binding B cells were recovered in both sets of experiments, the large degree of non-specific staining seen with fluorescein-labeled soluble antigen hampered these data.

Another group has devised an interesting strategy relying on FACS selection to isolate T cell hybrids. Taniguchi and Miller (1978) stained and sorted primary fusion cultures for the presence of surface markers found only on the T cell partner of the hybridization in order to enrich for desired T cell hybrids. However, non-specific staining as low as 0.5%, combined with low hybridization frequencies (approximately 10^{-6}), made their selections painstaking. In a subsequent report (Arnold et al., 1979), the same group followed a similar enrichment sort, for surface markers unique to the T cell, with a period of culture growth and subsequent selection of tetraploid hybrids on the basis of staining with the dye Hoechst 33258. This reagent labels cells according to their DNA content (Arndt-Jovin and Jovin, 1977). Hence, hybrids were expected to exhibit nearly tetraploid amounts of DNA, and were easily sorted. Even after this double sorting procedure, however, only 15—25% of clones finally obtained were the desired hybrids. This result is most likely explained by non-specific sorting of hybrids between 2 lymphoma cells in the first round of selection, followed by specific sorting on the basis of increased DNA content. Nonetheless, this method is especially useful when no means of chemical selection (e.g., HAT medium) is available, since it allows for the potential use of parents which are not metabolically selectable in hybridizations.

ANTIGEN COATED FLUORESCENT MICROSPHERES AND HYBRIDOMA SELECTION

For selection of antigen binding hybridomas by FACS, investigators from this laboratory (Parks et al., 1979) have used fluorescent microspheres coupled to myeloma protein antigens to select hybrids producing monoclonal antibodies to mouse allotypes. The use of microspheres labeled with a variety of ligands is not new to immunology (Molday et al., 1975), and is the subject of a recent review (Rembaum and Dreyer, 1980). Briefly, microspheres of 0.8 μm diameter were covalently coupled to the myeloma protein by a carbodiimide reaction (Hoare and Koshland, 1967). These microspheres emit fluorescence at 485 nm and are excited at 465 nm, permitting easy analysis with normal fluorescein detecting FACS systems. Important details of the staining reaction may be found in Parks et al., 1979. Using antigen coupled microspheres, a mean staining ratio of 100 : 1 for specific : non-specific binding of beads to hybridomas was observed. When sorting premixed samples of hybridomas with these reagents, an enrichment factor of 250 was readily obtained. The most important reasons for this specific enrichment are as follows. First, the high ratio of specific to non-specific staining allows for easy discrimination between positive and negative populations, and, therefore, higher yields of the desired cell type. Related to this is the finding that the average labeling of hybridomas with fluorescein conjugated soluble myeloma antigen was only 7% as bright as labeling with 1 microsphere. As well, an average hybridoma was found to specifically bind a mean of 21 microspheres per cell, an increase of nearly 300-fold in staining intensity over soluble antigen. Other aspects of these reagents worthy of note are that microspheres are not internalized by hybridomas, since pronase digestion removes 98% of specifically bound microspheres; and that binding of microspheres is stable for many hours at room temperature or 37°C. One possible drawback of the antigen coated microsphere method is that of inactivating the antigenic determinant in the coupling reaction. Optimal conditions of direct or indirect coupling need to be determined in many cases.

The above discussions outline 2 crucial points in the rapid isolation of desired hybridomas by FACS. First, when possible, enrichment for the desired hybridoma (either antigen binding or presence of a unique surface antigen) should be coupled to FACS selection or cloning. The enrichment, however, must be done with reagents which stain the desired cell with a high signal to noise ratio, i.e., in a very specific manner. And second, even when relatively specific enrichment procedures are carried out, overgrowth by undesired cells, sorted non-specifically, will lower the frequency of desired clones significantly. This problem, as well as the laborious, low efficiency tasks of limiting dilution or soft agar cloning have been largely eliminated by the use of direct FACS cloning. Since the FACS analyzes samples on a cell by cell basis, single desired viable cells can be detected and deflected into individual microculture wells.

Such a modification to the FACS has been described (Stovel and Sweet, 1979) and commercial versions are available from Becton-Dickinson (Sunnyvale, CA) and Coulter Electronics (Hialeah, FL). This modification was initially used in the experiments of Parks et al. (1979) to directly clone viable hybridomas from a primary fusion. One can combine single cell FACS cloning with selection for hybridomas bound to antigen coated fluorescent microspheres, as described above. As well, cells carrying any one of a number of desired surface antigens can be cloned at the time of selection, as will be discussed. The power of this modification lies in the merger of immediate cloning with selection for the desired marker labeled with one or more fluorescent tags. Hundreds of single cell clones can be initiated in a matter of minutes, with or without FACS selection for other desired properties of the cell in question.

Often cloning of viable hybrids from preliminary fusions without specific enrichment for other characteristics is very useful in increasing the yield of stable antibody producing hybridomas. Dead cells and debris exhibit both lower forward angle light scattering properties and/or greater red autofluorescence than live cells. These properties can often be used to select gates in the cloning of viable hybrids present in fusion culture containing large numbers of dead and dying cells (Fig. 2 of Parks et al., 1979). When light scatter and red autofluorescence alone are not sufficient to resolve live and dead populations, viability dyes such as propidium iodide (Krishan, 1975; Yeh, 1981) can be used to increase the red fluorescence of dead cells significantly, allowing for their easy removal during FACS analysis. In our laboratory, propidium iodide is now routinely used to eliminate dead cells from FACS analysis. In this manner, dead and dying cultures can be rescued by directly cloning the few live cells present. Cultures with a few as 1 live cell in 3200 have been saved in this manner. This type of cloning procedure can also be used to save infected cultures. Cloning efficiency with the FACS varies, but is typically between 40—80% for a healthy culture.

ISOLATION OF RARE HYBRIDOMA SWITCH VARIANTS BY FACS

FACS can be a powerful tool in the analysis and cloning of rare hybridomas. This is vividly illustrated in the recent isolations of variant hybridomas exhibiting new immunoglobulin heavy chain isotypes or idiotypes on their cell surface, as mentioned in the Introduction. Heavy chain class switching represents a normal and important process in development of B cell clones, and is the phenotypic reflection of DNA rearrangements which can increase the isotypic spectrum of an antibody response (for a detailed recent discussion of these events, see Müller, 1981). We have generated 'families' of such heavy chain switch variants, each expressing the same heavy chain variable region gene and light chain in conjunction with a different one of the 8 possible murine isotypes. It is hoped that these variant 'families' will lead to a more refined understanding of the molecular genetic

events involved in the class switch, as well as the functional properties rendered unto the immunoglobulin molecule by each isotype in combination with an identical variable region. It was first shown that myeloma (Liesegang et al., 1978; Radbruch et al., 1980) and later hybridoma (Neuberger and Rajewsky, 1981; Dangl et al., 1982) heavy chain switch variants could be isolated with a FACS. The rarity of these events, on the order of 10^{-5}—10^{-7}, mandated technical revisions of the FACS hybridoma selection procedure. We outline these techniques and results in detail below with the hope that this methodology will become a generally useful one, aimed at further tailoring the antibody products of hybridoma technology.

In the initial variant selections of Liesegang et al. (1978), myeloma MPC-11, which carries surface immunoglobulin of class IgG2b, was reacted with a 'cocktail' of heterologous guinea pig antisera to both IgG1 and IgG2a, followed by a fluoresceinated second step reagent. From initial cultures of several million, the brightest 0.5—2.0% of cells were sorted and maintained in culture for 4 weeks before the subsequent round of FACS enrichment. The assumption, of course, is that any rare cells expressing a switched isotype will be contained in this brightly staining fraction. After 3 rounds of enrichment sorting, positively staining cells were present in high enough frequencies (60%) for efficient isolation by limiting dilution cloning. Clones were isolated which expressed the switched isotype, while retaining the idiotypic determinants present on immunoglobulin produced by the parental cell. In these selections, only an IgG2a heavy chain variant and a non-producing variant were isolated. It is worth noting that selection of non-producing myeloma variants is a useful way of generating new fusion partners. Negative selection from a commonly used myeloma, P3-X63-Ag8 (Köhler and Milstein, 1975), using 3 rounds of FACS selections has been described (Kearney et al., 1979). Of 23 clones isolated, 15 were non-producers as defined by their lack of cytoplasmic immunoglobulin, which retained the ability to generate viable hybrids.

Radbruch et al. (1980) enlarged upon these original selections to create a 'family' of heavy chain switch variants derived from the IgG1 producing myeloma X63 (see above). Again, they used a 'cocktail' of heterologous antisera to select variants by indirect immunofluorescence and FACS enrichment. From the IgG1 myeloma, only IgG2b variants were selected, although the reagent contained activity to 5 murine isotypes. This is not surprising in light of evidence at the DNA level that switching occurs in a 5′—3′ manner (with respect to the transcribed strand) and that the IgG2b heavy chain gene is the next gene 3′ to the IgG1 gene (Shimizu et al., 1981). Selections from this IgG2b variant clone, however, gave 3 new classes of variants: (1) those expressing the IgG2a isotype, (2) those expressing both IgG2b and IgG2a, and (3) clones showing reversion to IgG1 expression. The latter 2 phenomena are not explained by simple deletion models for heavy chain class switching (Honjo and Kataoka, 1978), but can be used as examples of possible sister chromatid exchange mediated switching, as proposed by Obata et al.

(1981). However, the aneuploidy of these myelomas (approximately 70 chromosomes) clouds interpretation of DNA rearrangements seen.

The above studies required between 3 and 7 rounds of enrichment sorting, and months of culture growth before variant frequencies were high enough to permit feasible cloning by limiting dilution. As well, the myeloma variants, though idiotypically identical, bind an unknown antigen. Thus, there is the possibility that a different variable region is expressed within the 'family'. We therefore wished to enlarge upon this work by isolating a 'family' of antigen binding hybridoma variants. To do this, we have combined various modifications of the enrichment sorting protocols, including direct FACS cloning, aimed at greatly speeding the generation of useful hybridoma variants.

Ideally, one would like to select and directly clone hybridomas expressing the desired isotype without prior enrichment sorting. We, however, have found that efficient FACS selection at frequencies below 10^{-5} is at the border of sensitivity (since deflections occur approximately once every 2 min, thus jeopardizing the sterility of the procedure) and have concentrated instead on improvements in analytic technique designed to minimize the number of enrichment sorts. In the isolation of rare hybridomas, then, use of very specific staining reagents and efficient analytic procedures is imperative, in order to choose the most discrete sorting parameters. We discuss below 3 such additions to the general selection protocol in the context of our isolation of a 'family' of antigen binding heavy chain switch variants (Dangl et al., 1982).

The most important of these analytic modifications are aimed at reducing the number of non-specifically sorted cells. Dead and dying cells tend to stain non-specifically with immunofluorescent reagents, and it is crucial to exclude such artifacts from potential sorting windows. In our protocol, the viability dye propidium iodide (PI) is used in the last wash at 0.5 μg/ml (1 : 1, v/v) to label dead cells (Krishan, 1975; Yeh, 1981). In this manner, dead cells which may exhibit false positive green fluorescence are easily identified by their red fluorescence, and removed from further FACS analysis. Fig. 1 illustrates this effect. Each dot of the FACS dot plot represents measurements on 1 cell. In the first panel, the boxed area represents cells selected as PI negative (live) by their lack of red fluorescence and proper forward angle light scatter. Without this added window, gating on scatter alone in the same sample gives the green fluorescence distribution seen in the second panel. Note the large number of false positives which are removed after combination gating on scatter and PI fluorescence, and subsequent analysis of fluorescein fluorescence, as illustrated in the boxed area of the final panel.

Fluorescein and PI are excited by an argon-ion laser, 400 mW at 488 nm wavelength. A Corning 3-69 longpass filter and a 580 nm longpass reflector (Zeiss, part 46 63 05) are used to block scattered laser light and divide red and green fluorescence essentially as described for dual detection of rho-

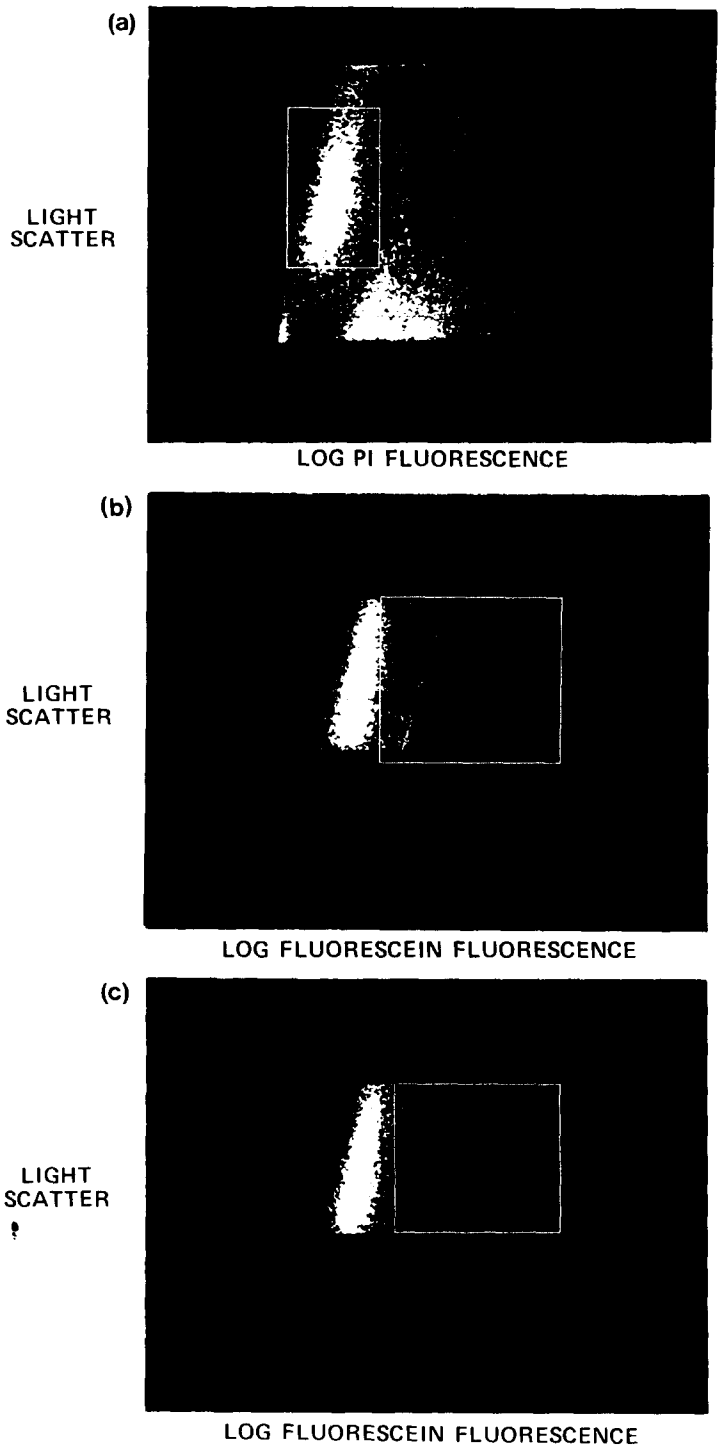

Fig. 1. Logarithmic FACS dot plot illustrating the use of propidium iodide labeling of dead cells in the isolation of rare hybridoma switch variants. See text for details.

damine and fluorescein (Loken et al., 1977). Fluorescein emits primarily below 580 nm and PI mostly above.

The second addition to our protocol, also designed at reducing non-specific staining, is the use of directly conjugated fluorescent reagents, both heterologous and monoclonal, for FACS selections. Non-specific staining as low as 0.1% is enough to skew fluorescence distributions and undermine the choice of sorting parameters for rare cell selections. One may have to sacrifice the higher staining intensity of indirect immunofluorescence for the ability to choose discrete sorting parameters. This argues for the use of highly absorbed, affinity purified heterologous antibodies, or monoclonal reagents. As well, tolerance induction at the time of immunization for isotype specific antibodies (Henney and Ishisaka, 1966) may be worthwhile, and was used effectively by Liesegang et al. (1978).

Monoclonal antibodies have the obvious drawback that they see at most 2 identical determinants on each target immunoglobulin molecule. Two approaches have been used to overcome this drawback. First, we have used a mixture of 2 non-crossblocking monoclonal antibodies in our selection of an IgG2a producing variant, and have found the staining intensity of this mix to be within 2-fold of that of an affinity purified conventional antibody with the same isotypic specificity. This mixture proved to be adequate in selection of artificial mixtures of hybridomas at ratios of 1 : 2500. Other selections with monoclonal 'cocktails' as selecting reagents, however, have not been successful. One possible reason for this is the comparative lack of immunoglobulin on the surface of the cell being selected. Three approaches have been employed to overcome this lack. First, the use of new dyes which have higher quantum yields of fluorescence emission, such as phycoerythrin (Oi et al., 1982b) coupled to the selecting antibody can effectively label cells expressing low quantities of the desired surface antigen. Second, workers in our laboratory have conjugated monoclonal antibodies to fluorescent microspheres, which, as discussed above, greatly increases their staining brightness. Finally, Holtkamp et al. (1981) used the FACS to enrich for a brightly staining clone before beginning variant selections with directly fluoresceinated monoclonal antibodies to be described later. As well, workers in our laboratory have successfully isolated stable clones exhibiting nearly 10-fold increases in staining after repeated FACS selection (R. Hardy, personal communication).

The final analytic improvement used to speed the isolation of rare variants is logarithmic collection and display of FACS fluorescence profiles. The somewhat normalized distributions generated in this manner have proven useful in identifying subpopulations of T cells with quantitatively differing amounts of T cell surface antigens (Ledbetter et al., 1980), and in monitoring ontogeny of both T and B cell subsets (Haaijman et al., 1980; Hardy et al., 1982; Haaijman et al., 1982). Fig. 2 illustrates the use of logarithmic amplification in selection of one of our variants, clone 27-35, which expresses IgG2b. The left hand panel shows analysis of a twice enriched

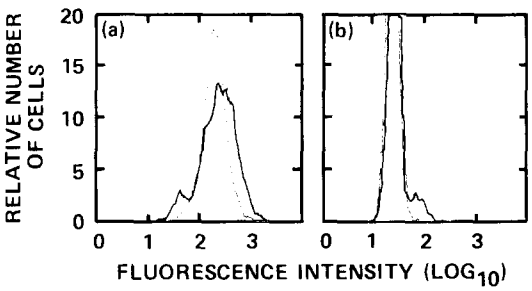

Fig. 2. FACS histograms of enrichment sorting for isotype switch variant 27-35 (IgG2b) from 27-44 (IgG1). a: Staining of 4.4 (light line) and a population twice enriched for IgG2 (dark line) with an anti-IgG1 reagent. Note the negative subpopulation in the twice enriched sample. b: Staining for expression of the switched isotype (in this case with a reagent for either subclass of mouse IgG2). Note a positively staining subpopulation corresponding to that in a. The parent clone, 4.4, stained with the same anti-IgG2 reagent is negative (light line).

population stained for IgG1 (heavy line) versus staining of the IgG1 parent with the same reagent (light line). The right hand panel gives reciprocal staining results for IgG2. Note that the IgG2b positive subpopulation is easily distinguishable from the parental type cells in either case, even though the mean brightness difference between positive and negative is only 2—4-fold.

Both of our variant 'families' are schematically diagrammed in Table 1. Variants expressing IgG2b, IgG2a, and IgE isotypes have been isolated from the 27-4.4 (IgG1) parent. As well, an IgG1 producing revertant (27-4F5) was isolated from the IgG2b clone, and a very interesting IgG2a variant (27-1B10) was derived from this revertant. 27-1B10 is a 'short chain' variant; its heavy chain is approximately 10,000 daltons lighter than a normal IgG2a. This appears to be due to a deletion of the CH1 domain of the IgG2a heavy

TABLE 1

Derivation of hapten binding switch variant families.

27-4.4 Family:				
Clones:	27-4.4 →	27-35 →	27-13 →	27-74
Isotype:	IgG1	IgG2b	IgG2a	IgE
	↙			
Clones:	27-4F5 →	27-1B10 →	27-7B7	
Isotype:	IgG1	IgG2a	IgE	
44-10.5 Family:				
Clones:	44-10.5 →	44-32		
Isotype:	IgG1	IgG2b		

chain gene (C. Hsu et al., unpublished results). Notably, an IgE variant (27-7B7) derived from 27-1B10 produces normal sized IgE heavy chains. In the second 'family', only an IgG2b variant has been isolated from a second IgG1 parent.

Details of each isolation may be found elsewhere (Dangl et al., 1982). Importantly, these selections required only 3—5 weeks to isolate stable clones expressing the preselected switched isotype. A typical selection required the bulk sorting of the brightest 0.1—1.0% of cells from initial populations of 3×10^6—2×10^7 cells. In order to diminish the chance of loss of variants due to overgrowth by non-specifically sorted parental cells, we grew the once enriched culture for as short a time as possible. Usually, 5—9 days of culture gave 2—5×10^6 cells (doubling time of the hybridoma parents used was approximately 22 h). At this point, cells were analyzed and sorted again, with essentially the same sorting thresholds. Most importantly, cells were directly cloned at the time of this second 1000-fold enrichment. They were also bulk sorted for further analysis and cloning as needed. Thus, a total enrichment of between 10^4 and 10^6 was achieved in 2 rounds of selection, and clones carrying the desired trait were already established, well before a positive subpopulation is easily discernible in FACS analysis. Clones are subsequently tested for antigen binding capabilities and isotype in solid-phase radioimmunoassay.

Immunoglobulin molecules produced by members of the variant families diagrammed in Table 1 have been particularly useful in studies of isotype associated functions, since each apparently employs an identical heavy chain variable region and the same light chain. Thus, each binds the hapten dansyl (5-[dimethylamino]naphthol-1-sulfonyl) in precisely the same binding site as determined by fluorescence spectroscopy of the bound dansyl (Oi et al., 1982a). Two other characteristics of these molecules have been investigated (Oi et al., 1982a). The parent IgG1 molecules and the variant IgE molecules all fix complement weakly, the IgG2b molecules fix complement extremely well, while both of the IgG2a molecules exhibit nearly identical complement fixing ability. The latter result is especially interesting in that the 27-1B10 short chain molecule appears to lack all of CH1 and possibly the hinge region as well. The other functional feature we have investigated is segmental flexibility of the immunoglobulin molecule about its hinge. The technique used assays the decay of fluorescence polarization (anisotropy) of the bound dansyl hapten in the nanosecond time range, and can be comparatively correlated to motion of the fab′ arms about the hinge of the molecule (Reidler et al., 1982). The IgG1 parent 27-4.4 is essentially rigid, as are the IgE variants. The IgG2b variant is nearly as flexible as free fab′, and the IgG2a molecules are both intermediate. The IgG1 parent 44-10.5 is slightly flexible, though much less flexible than the IgG2b variant derived from it. Only a 'family' of immunoglobulin molecules derived in this manner is amenable to unambiguous interpretation of these types of structural and functional studies.

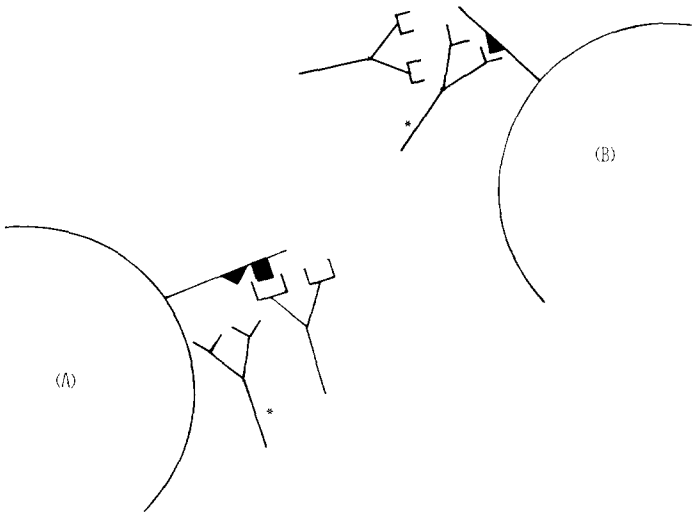

Fig. 3. Selection of mutants expressing loss of an antigenic determinant. In A, 2 cross-blocking monoclonal antibodies, one fluorochrome labeled (*), are used as selecting reagents. Note that in such a selection scheme, the higher affinity reagent is unlabeled. If the determinant defined by the unlabeled reagent is lost, the labeled reagent is bound, and the desired variant sorted. This scheme summarizes the selection of H-2K^k variants by Holtkamp et al. (1981) as described in the text.

The last example of selection of variants with FACS enrichment and cloning is the isolation of T lymphoma clones exhibiting structurally altered H-2K^k molecules (Holtkamp et al., 1981). The variants have lost the determinant defined by a given monoclonal antibody. Their novel selection scheme employs 2 cross-blocking monoclonal antibodies, one unlabeled which completely blocks the binding of the second, fluorescein tagged, reagent (Fig. 3). This mixture, with a 1000-fold excess of the unlabeled antibody, was used to stain the HK13 T lymphoma. Thus, only variants which no longer express the determinant defined by the unlabeled antibody are expected to be stained. After 3 enrichment sorts, 78% of cells were stained. Thirteen of 14 clones tested also stained with the fluoresceinated reagent, even in the presence of 1000-fold excess of unlabeled antibody. The benefits of rapid generation of variants with determinant deletions comes in the definition of determinants necessary for a particular cellular function. Citing unpublished reports of J. Neuerburg and H.-W. Vohr, Holtkamp et al. (1981) state that cells exhibiting the altered H2-K^k molecule function efficiently as targets for alloreactive T cells. These same cells, however, are deficient as restricting elements for hapten specific, H-2 restricted cytotoxicity.

CONCLUSION AND PERSPECTIVES

Through the above examples, it is evident that FACS enrichment and cloning can be a generally useful tool in the rapid isolation of rare primary hybridomas, hybridoma variants, and other rare cells. Combining direct FACS cloning with selection for another desired property (e.g., antigen binding and/or desired surface antigens) succeeds in merging 2 often limiting steps in hybridoma production-rapid generation of hundreds of clones in conjunction with preliminary screening via FACS selection for a desired trait.

We feel that this methodology can be of practical use in many areas of research. Mention was made of the power inherent in a 'family' of immunoglobulin molecules, each bearing the same variable region combined with different constant regions. These molecules have allowed us to discretely analyze functional properties of native immunoglobulins in terms of their constant regions, independent of possible differences due to variable region effects on the function in question. Further dissection of the relationship of immunoglobulin fine structure and function can now be gleaned from new families of variants, lacking either whole domains, as in the case of the IgG2a variant mentioned above, or determinants, as in the structural variants of Holtkamp et al. (1981).

As well, the switching of a hybridoma producing immunoglobulin of one isotype with reactivity against a tumor antigen to immunoglobulin of a functionally more effective isotype, with retention of specificity, would engineer a very powerful therapeutic reagent. Moreover, an entire family of immunoglobulins with anti-tumor activity would be an even more appropriate therapeutic weapon. These molecules may be able to recruit natural elements of the immune response better than monoclonal antibody of a single isotype. Furthermore, isotype families of antibodies coupled to toxins, drugs, or radioisotopes could be employed therapeutically. FACS selection with monoclonal antibodies can speed the isolation of hybridomas with missing, recombinant, or inter-species heavy chain domains, adding another dimension to the engineering of a more perfect antibody molecule. In this regard, it is worth noting that switch variant selection seems to be a general phenomenon. Other workers in our laboratory have succeeded in 'switching' yet a third IgG1 parent to IgG2b (R. Hardy, personal communication). The procedures we have developed were aimed at making the isolation of variants, or any rare cell to which a specific antibody exists, both rapid and efficient.

The emergence of FACS selection and cloning of hybridomas of any sort expands the possibilities available to the creative researcher in custom designing both cellular and molecular reagents for a myriad of uses. This kind of genetic engineering bypasses many laborious aspects of recombinant DNA technology, and is easily made routine. We hope that the applications of hybridoma selection by FACS, as presented here, hastens progress in any field requiring easy availability of such reagents.

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