Biotechnology in Diagnostics,
H. Koprowski, S. Ferrone and A. Albertini editors



# HYBRIDOMA VARIANTS AFFECTING ISOTYPE, ANTIGEN BINDING AND IDIOTYPE

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## Introduction

Since 1976 there has been an explosion of numbers of hybridomas generated by the technique of Kohler and Milstein (1) or modifications of this technique (2,3). Often, however, the monoclonal antibodies produced by these hybridomas have had the desired specificity that belonged to isotypes which did not permit them to be used for the physiological or in vitro function desired by investigators. Therefore, the finding by Radbruch, Rajewsky and colleagues that rare variants of hybridomas which had switched isotype could be selected using the FACS (4,5) opened up the possibility of employing hybridoma switch variant selection as a useful tool in "biotechnology". We have employed and made more routine switch variant selection to such diverse problems as: 1) segmental flexibility and complement fixation as a function of isotype (6), and 2) the role of isotype in therapy with monoclonal antibodies (7,8). Some of the improvements in selection methods have been reported previously (9-11). In this paper, we describe the importance of isotype in antibody dependent cellular cytotoxicity (ADCC) using families of switch variants to HLA antigens.

The second part of this paper is a "progress report" on obtaining variants affecting antibody affinity, specificity, and/or idiotypy, using the FACS. In obtaining such presumed variable region mutants, we have also found a new variety of constant region variants. These are producing heavy chains which appear to have lost approximately two domains and no longer permit secretion of immunoglobulin molecules. Instead, these shortened heavy chains may have kept the membrane binding domains giving four chain molecules in the membrane which bind the antigen but have no detectable isotype specific epitopes (determinants).

Isotype switch variants

Immunoglobulin isotype switch variant cells can be isolated readily using the fluorescence activated cell sorter. These switch variants, present at frequencies of 1 in $10^5$ TO $10^6$ in newly subcloned populations of antibody producing hybridomas, are cells that have spontaneously changed in their expression of heavy chain isotype while retaining expression of the same light chain and variable region as expressed by the parent hybridoma cell. As such they produce immunoglobulin that retains the same antigen binding specificity as the original monoclonal antibody. Using highly specific heterolgous antibody for the variant isotype or combinations of isotype specific monoclonal antibodies, these cells can be stained for variant immunoglobulin present on their cell surface. Using the technique of pauci-population sorting, these cells can be isolated and identified after one round of sorting. Thus whole families of immunoglobulin isotype switch variants can be fashioned, allowing for comparison of the biochemical, physical and physiological properties of the murine immunoglobulin isotypes, independent of other antibody variables.

We used the technique of pauci-population sorting to select families of hybridomas producing monoclonal antibodies specific for polymorphic determinants of class I HLA molecules that are of different immunoglobulin isotypes. Starting with an $IgG_1$ producing hybridoma, one, five, twenty-five or fifty cells staining with anti-$IgG_2$ antibody were sorted into individual wells of a microtiter plates. After nine days culture, wells were assayed for the presence of variant $IgG_2$ isotype using a sensitive solid phase radioimmune binding assay. The $IgG_{2b}$ variant of ME1 was present at high frequency, allowing us to obtain $IgG_{2b}$ producing clones after the first round of sorting. (Table 1) Other variant isotypes, namely the $IgG_{2a}$ of ME1 and the $IgG_{2b}$ and $IgG_{2a}$ isotypes of MA2.1 were sorted from the pauci-populations producing variant immunoglobulin as detected in the radioimmune binding assay.

The $IgG_{2a}$ and $IgG_{2b}$ variants of ME1 and MA2.1 retain the same binding activity for HLA as the $IgG_1$ antibodies produced by the respective parent hybridomas. Each member of the ME1 variant family secretes antibody with high affinity to HLA-B7, Bw22, Bw42, and B27, and with low affinity for HLA-B14 (12). Each member of the MA2.1

family secretes immunoglobulin with high affinity for HLA-A2 and B17. When compared with each other in either radio-immune or fluorescence-immune cell binding assays, all antibodies of a given family retain the same binding activity for cells bearing the appropriate HLA specificity (Figure 1). In contrast to the $IgG_1$ antibodies produced by the respective hybridomas, however, the $IgG_{2b}$ and $IgG_{2a}$ antibodies of each of these families can be used in standard complement dependent cytotoxic tissue typing assays.

NUMBER OF ISOTYPE SWITCH VARIANTS

DETECTED IN HYBRIDOMA POPULATIONS ($\times 10^{-6}$)

| HYBRIDOMA CELL LINE | $IgG_{2b}$ | $IgG_{2a}$ |
|---|---|---|
| ME1 | 1600 | 64 |
| MA2.1 | 32 | 8 |

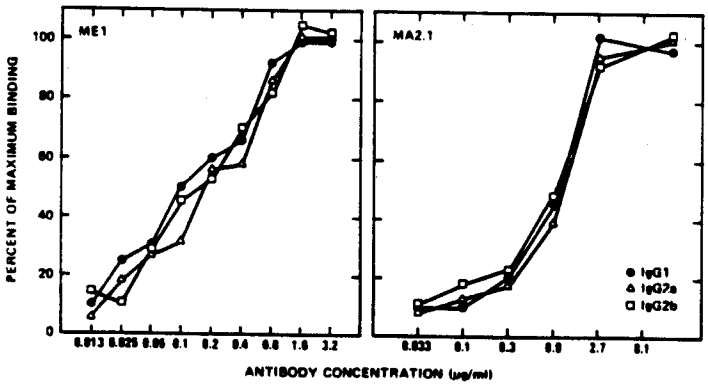


Fig. 1. Titration of antibodies from switch variant families with JY: Antibodies of the MA2.1 switch variant family (right) or the ME1 family (left) were titered with a fixed number of JY. Specifically bound antibody was detected with FITC-labeled goat anti-mouse Ig (right) or with $^{125}I$-labeled goat anti-mouse Ig (left) for the fluorescence-immune or radioimmune cell-binding assays, respectively.

Having families of immunoglobulin with shared binding activities but with different immunoglobulin isotypes allowed us to compare the capacity for each antibody isotype to direct antibody-dependent cellular cytotoxicity (ADCC) by effector cells of the human immune system. We noted that the $IgG_{2a}$ ME1 can direct significant ADCC of a human B cell line, JY, by freshly isolated human PBL. (Figure 2) This lymphoblastoid cell line expresses the HLA-B7 determinant recognized by the ME1 antibody (13). The $IgG_{2a}$ ME1 antibody, however did not direct lysis of Daudi cells lacking the HLA-B7 in the presence of human PBL, indicating that specific binding of antibody is required for cytolysis. In addition, the $IgG_{2a}$ antibody did not direct lysis of JY in the absence of PBL, confirming that ADCC was being oberved. Because the $IgG_{2a}$ ME1 antibody demonstrated 30% specific lysis of JY after 270 minutes incubation with the PBL (Figure 2), we choose a four hour incubation time for subsequent ADCC experiments comparing the activities $IgG_1$, $IgG_{2b}$ and $IgG_{2a}$ ME1.

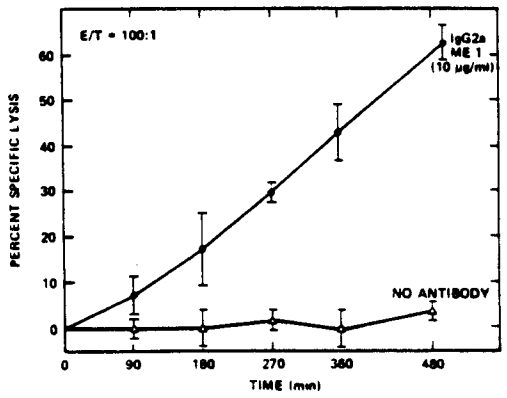


Fig. 2. ADCC directed by $IgG_{2a}$ ME1 antibody: $Cr^{51}$-labeled JY was incubated with or without $IgG_{2a}$ ME1 at 10 micrograms/ml. PBL were added to an E/T ratio of 10:1. Bars indicate standard deviation (+/-SD) of percent specific lysis for triplicate samples.

Although both classes of the $IgG_2$ molecules are active in ADCC, $IgG_{2a}$ ME1 antibody provides a higher maximal (25% ±2%) ADCC activity than $IgG_{2b}$ (10% +2%) (Figure 3). The concentration at which the $IgG_{2b}$ ME1 achieves maximal ADCC, however, is identical to that noted for $IgG_{2a}$ ME1, consistent with these antibodies having identical binding affinities for the JY target cell. In contrast, no specific

lysis of JY sensitized with $IgG_1$ ME1 is detected, even at high antibody concentrations. A similar heirarchy of capacities for directing ADCC was found using the MA2.1 switch variant family of antibodies which recognizes the HLA-A2 determinant expressed by JY. (Figure 4).

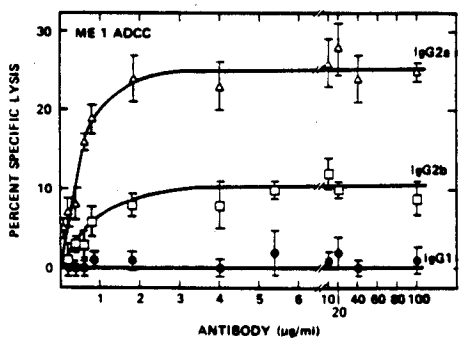


Fig. 3. Comparison of ADCC activities of different antibody isotypes of ME1. Antibody at various concentrations was added to $^{51}$-labeled JY. Percent specific lysis (+/- SD) is of JY with PBL after 4 hours incubation with an E/T ratio of 50:1.

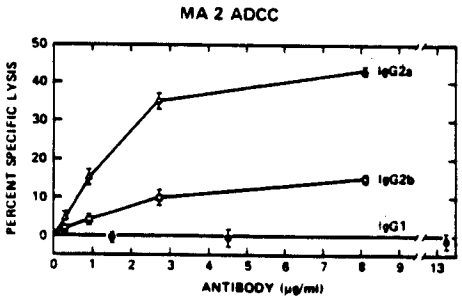


Fig. 4. Comparison of ADCC activities of different antibody isotypes of MA2.1. Conditions are as described in figure 3.

Because longer incubation with PBL increased the specific lysis of $IgG_{2a}$-coated JY, we compared ADCC activities at incubation times greatsr than four hours, seeking to enhance detection of target cytolysis directed by either $IgG_{2b}$ or $IgG_1$ ME1 antibodies. The percent level of cell killing directed by the $IgG_{2b}$ ME1 remained

significantly less than that of the $IgG_{2a}$-coated JY at all time points tested (FIGURE 5) and $IgG_1$ ME1 directed no ADCC up to at least 19 hours.

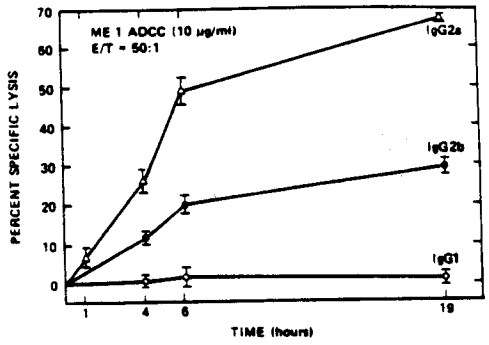


Fig. 5. Kinetics of ADCC directed by the different isotypes of ME1. $^{51}Cr$-labeled JY was incubated with antibody of each isotype at 10 micrograms/ml. ADCC (+/- SD) of PBL at an E/T ratio of 50:1 was assayed at times indicated.

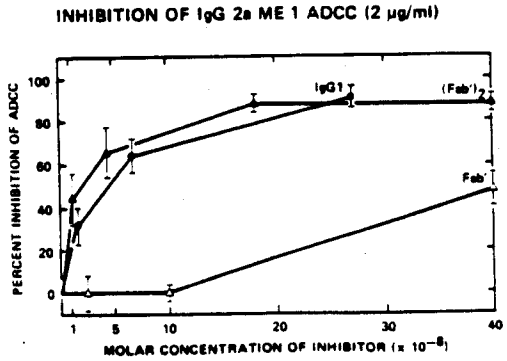


Fig. 6. Inhibition of $IgG_{2a}$ ME1 ADCC: The $IgG_1$, $F(ab')_2$, or Fab' of ME1 was added at various concentrations to separate wells containing $^{51}Cr$-labeled JY. $IgG_{2a}$ ME1 was subsequently added to 2.5 micrograms/ml. After a 20 minute incubation at room temperature, PBL were added to an E/T ratio of 50:1. ADCC wa measured after 4 hr. incubation at 37 degrees C. Samples without inhibitor had 30% (+/-1%) specific lysis of JY with the $IgG_{2a}$ ME1. Percent inhibition of ADCC was determined by comparing the percent specific lysis of each sample with this value.

The $IgG_1$ ME1 antibody, however, could competively inhibit the capacity of the $IgG_{2a}$ antibody to direct ADCC (Figure 6). Such inhibition is comparable to the $F(ab')_2$ fragment of ME1, demonstrating that the Fc of the antibody molecule is critical for directing effective ADCC. Futhermore, $IgG_{2a}$ ME1 directed ADCC of JY is not affected by staturating concentrations of MA2.1 $IgG_1$, although the later antibody could effectively inhibit MA2.1 $IgG_{2a}$ directed ADCC. These results demonstrate that the inhibition of ADCC by the $IgG_1$ antibody of a given switch variant family is due to competition with the effective $IgG_{2a}$ molecule for binding the HLA target.

Frequency of Isotype Switching:

Using the technique of pauci-population sorting, we assessed the frequency of isotype switching in generating several different switch variant families and noted the frequencies of isotype variants found within hybridoma populations over time. Figure 7 displays the generalized spontaneous isotype switch frequencies in cultured hybridomas. Although these frequencies may not apply to all hybridomas, they may serve as useful approximations for those attempting to isolate isotype switch variants. In this light, several points are worth emphasizing. The rates of spontaneous isotype switching range from 10-4 to 10-7, comperable to conventional spontaneous mutation frequencies. The probabilities of isotype switching vary, depending on the isotypes of both parent and switch variant hybridomas. The highest probability events exchange the expression of one isotype gene to that of its nearest 3' neighbor. This has been confirmed using two-color fluorescence cell sorting to select variant cells that have lost expression of the parent surface immunoglobulin but retain the capacity to bind antigen. In these cases, $IgG_{2b}$ switch variants most frequently occur from $IgG_1$ producing hybridomas and IgE switch variants most frequently occur from $IgG_{2a}$ producing hybridomas. Thus, without directly selecting for a particular isotype, the switch variant isotype from a particular hybridoma population is that of the nearest downstream isotype gene. Switching to isotypes located farther downstream apparently can occur directly, however, because the probability of spontaneous switching two, and even three, genes downstream is much greater than the product of probabilities for successive switching through isotype

variant intermediates. This is even clearer if one multiplies the probability of successive switching by the number of intermediate switched cells, which is always small. Furthermore, we generally find that the frequencies of successive spontaneous isotype switching from one given isotype to another is the same regardless of whether the hybridomas constitute a switch variant population or an original cell line. This is in contrast, however, to a few exceptional variant families that manifest successively higher switch frequencies with each successive switch (Rajewsky, personal communication). Spontaneous switching to isotype genes located 5' of the heavy chain gene expressed by the hybridoma after fusion has not been detected. Finally, in accord with the principles of population genetics elucidated by Luria and Delbruck for assessing the rates of spontaneous mutations within microbial cultures (14), the number of switch variants within a hybridoma culture generally increases with the number of generations spent in culture without cloning. This last principle has great practical significance. Indeed, long term cultures of hybridomas should not be subcloned prior to selecting switch variants as the frequency of accumulating variants within such cultures may be two to three orders of magnitude higher than the frequency of variants found within a newly cloned hybridoma population.

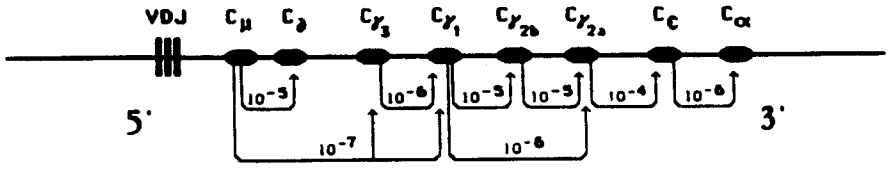


Fig. 7. Generalized spontaneous isotype switch variant frequencies.

Variant selected for altered antigen binding properties

As part of a project to investigate the relationship between antigen binding properties of hybridoma antibodies and combining site sequences and structures we are selecting spontaneous hybridoma variants for altered binding affinity or fine

specificity. This is done by labeling the cells with two colors of fluorescent reagents in a way that is dependent on the binding properties and then selecting on the fluorescence-activated cell sorter (FACS) for cells that fall outside the main parental population. Often several cycles of sorting and growth are required to obtain a sufficiently enriched population of variants to allow them to be cloned.

We are using two antigen systems: dansyl (DNS) and digoxin. In the dansyl system, cells of a hybridoma secreting antibody that binds dansyl are surface stained with multivalent dansyl on protein (e.g. DNS20-BSA) with a fluorescent label. This staining can be inhibited by free hapten (DNS-lysine or Di-dansyl cadaverine). The extent of inhibition is a function of the affinity of the antibody and of the concentration of the inhibitor. In particular, high affinity antibodies are more easily inhibited by free hapten than low affinity antibodies. The cells are also stained with an antibody to their surface immunoglobulin which serves to normalize the antigen binding since the amount of surface antibody is quite variable among the cells.

Thus, as shown in Figure 8, panels (a) and (b), DNS-BSA binding to the high affinity 27-4.4 cells can be easily inhibited by Di-dansyl cadaverine. The mean DNS-BSA fluorescence without inhibitor was 11.1 (based on the logarithmic data as displayed) while with inhibitor it went to 1.0 (over unstained cell fluorescence of 1.0). On the other hand, exposure of the low affinity BV107 cells to the same concentration of Di-dansyl cadaverine results in little inhibition. As illustrated in panel (c) BV107 cells with no inhibitor had mean staining 100 while with inhibitor (panel (d)) they had mean staining 88.

To select for lower affinity variants of 27-4.4 we sort rare cells from the region marked by hatch lines in Figure 8, panel (b). To select for higher affinity variants of BV107 (or variants with low enough affinity to bind dansyl poorly) we sort from the hatched region of panel (d). So far the only unambiguous affinity change variants have been lower affinity variants of BV107.

The digoxin system works similarly except that the inhibitor is digitoxin, a molecule which lacks one hydroxyl group found in digoxin. Some monoclonal antibodies to digoxin bind digitoxin more-or-less equally while others bind digitoxin quite poorly (19). We therefore use the ability or inability of digitoxin to inhibit digoxin binding to cells as a fine specificity marker for sorting. Again we use antibody to the cell surface immunoglobulin to normalize the antigen labeling.

As shown in Figure 9 the 40-160 cells bind digoxin, but the binding is almost totally inhibited by digitoxin. Sorting cells from the hatched region of panel (b) we obtained variant 40-160A1.5 whose digoxin binding is quite resistant to inhibition by digitoxin [panels (c) and (d)].

We have obtained variants from two anti-digoxin cell lines. One variant which secretes antibody that binds digoxin poorly was used in a second cycle of selection to yield a second order variant with good digoxin binding. The detailed binding properties of the variants are being investigated to select clones for variable region sequencing, etc.

Surface Antibody Positive, Non-secreting Variants

A fascinating by-product of the selections for antigen binding variants has been the appearance of variants that have surface antibody but do not secrete. From three of the four cell lines we have used we have obtained nonsecreting variants whose cell surface immunoglobulin binds antigen and stains for kappa chains but is negative for ALL heavy chain isotypes. The parent cell lines are all $IgG_1$s. Some of these variants have distinctly altered antigen binding properties (40-160A1.5 illustrated above is one of these) while others seem to bind about like the parent implying that they were selected on the basis of altered staining with the anti-Ig reagent rather than for altered antigen binding. The genetic or regulatory event leading to this type of variant must be fairly generally available to hybridoma cells and not too rare since we find it from several parent lines. Perhaps there has been a deletion of the whole region of the heavy chain bearing the isotypic determinants with loss of the ability to produce secreted form Ig but maintenance of a membrane attaching form. We are investigating the origin of this type of variant.

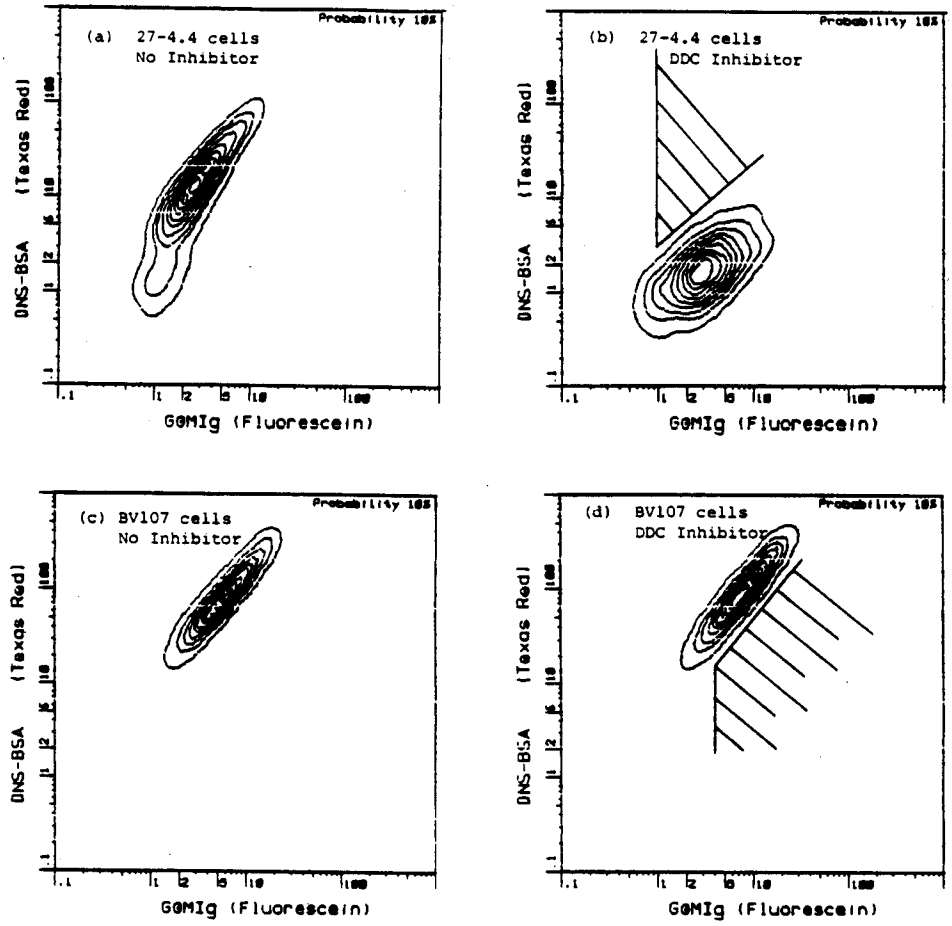


Fig. 8. Two color FACS measurements on two anti-dansyl producing cell lines (27-4.4 and BV107). The cells were stained with biotin conjugated DNS-BSA revealed with Texas Red conjugated avidin and with fluorescein conjugated goat anti-mouse Ig. The cells were preincubated with control medium or medium with di-dansyl cadaverine (DDC) inhibitor for 10 minutes before the DNS-BSA was added. Data for the contour plots was pre-gated on forward light scatter and large angle light scatter to include only single live cells. The hatched regions mark the areas in which cells producing variant antibody with altered antigen affinity should be found.

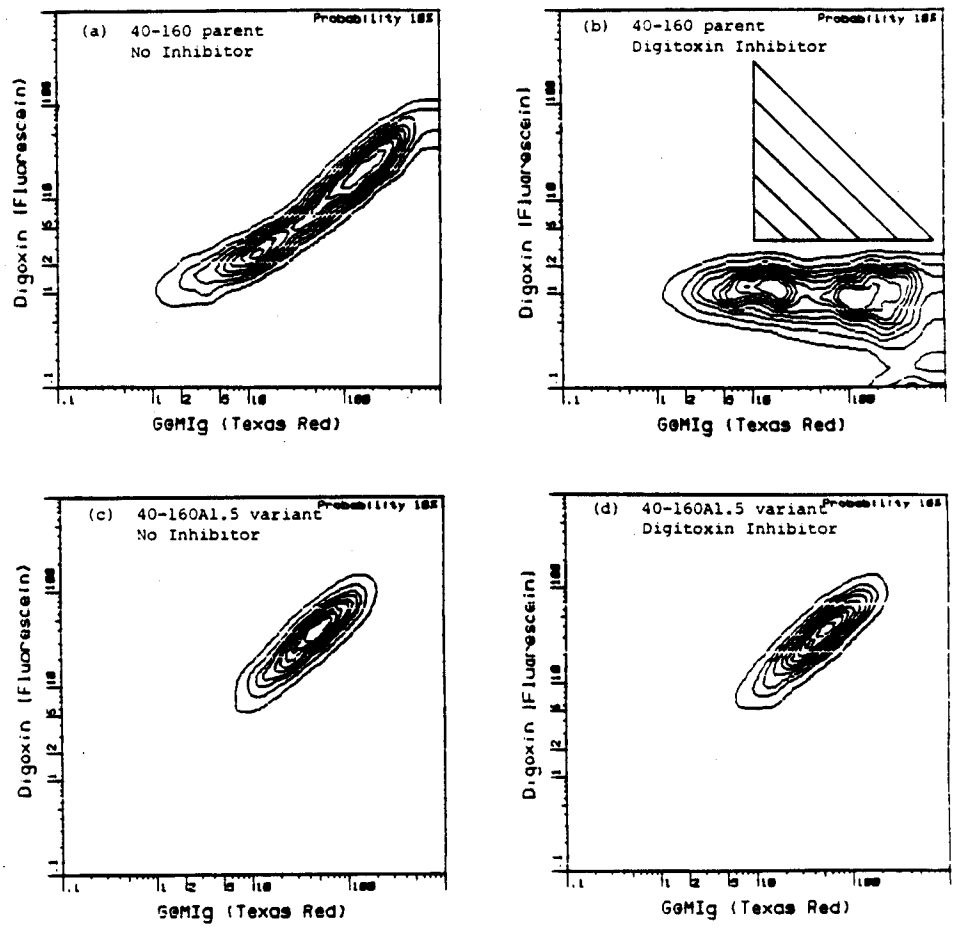


Fig. 9. Two color FACS measurements on an anti-digoxin cell line (40-160) and a fine specificity variant (40-160A1.5). The cells were stained with digoxin-fluorescein and with goat anti-mouse Ig with the biotin/Texas Red avidin technique. The cells were incubated with control medium or digitoxin medium for 10 minutes before starting the fluorescent staining procedure. Data for the contour plots was pre-gated on forward light scatter and large angle light scatter to include only single live cells. The hatched region marks the area from which cells were sorted to obtain the variant.

Idiotype Variants:

We have also recently applied two-color immunofluorescence cell sorting to select for other variable region variants of hybridomas, namely those expressing new or altered idiotypic determinants. In collaboration with Drs. Katheryn Meek and J. Donald Capra of the University of Texas Health Science Center, we selected idiotype variants from the 93G7 hybridoma. This cell line produces anti-p-azophenylarsonate (anti-Ars) antibodies bearing the A/J Ars-A cross reacting idiotype (CRI) (15). Several monoclonal anti-idiotypic antibodies to the family of CRI expressed on A/J antibodies raised to azophenylarsonate have been characterized. Comparison of the primary amino acid sequences of several different monoclonal anti-Ars along with their differential reactivities with a panel of these monoclonal anti-idiotypic antibodies has allowed for the tentative designation of $V_H$ region sites that are major contributors to a particular idiotope (16,17). Using fluorochrome conjugated anti-isotype antibodies and biotinylated anti-idiotypic monoclonal antibodies developed by the subsequent staining with Texas-Red-Avidin, we selected for variants of 93G7 that had lost idiotypic determinants tentatively mapped to the D segment of the 93G7 $V_H$ but retained surface immunoglobulin expression. These variants were subsequently found to secrete monoclonal antibody capable of binding p-azophenylarsonate but lacking the idiotypes selected against. Preliminary sequence data suggests, surprisingly, that such variants may arise from isolated point mutations outside of the D segment within the $V_H$ region, indicating the difficulty in defining serological epitopes solely through comparisons of the primary sequences of related proteins. The frequencies at which such variants occur is similar to that noted for point mutations, namely one in $10^5$ to $10^6$. This frequency is in contrast to the reportedly high rates of somatic mutation within the $V_H$ region during early B cell ontogeny (18), suggesting a different mechanism of mutation during B cell development.

Acknowledgements

The authors would like to acknowledge Drs. E. Haber and M. Hunter for providing the anti-digoxin hybridomas used in these studies (19). This work was supported in part by NIH grants GM-17367, AI-19512, and CA-04681. T.J.K. is a Special Fellow of the Leukemia Society of America.

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