

# **Biotechnology in Diagnostics**

H. Koprowski, S. Ferrone  
and A. Albertini  
Editors



# BIOTECHNOLOGY IN DIAGNOSTICS

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Proceedings of the International Symposium on The Impact of Biotechnology on  
Diagnostics held in Rome, Italy, April 16-18, 1985

*Editors*

H. Koprowski

S. Ferrone

*and*

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## PREFACE

In recent years much progress has been made in the area of biotechnology. The cellular and molecular cloning methodology to develop monoclonal antibodies and DNA probes have been extensively utilized in basic and clinical research. These investigations have provided the necessary information to apply these reagents to diagnostic problems.

The RIA 85 meeting has focused on the application of monoclonal antibodies and DNA probes in laboratory medicine. The papers presented at this meeting clearly indicate that biotechnology has already had a significant impact on clinical medicine. We hope that this book will give the reader a picture of the status of the art in this field and will stimulate additional investigations in this area of research.

We thank the contributors for having managed to find time in their already busy schedules to write their chapters and for having helped us to meet the deadline.

The editors

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# CONTENTS

Preface	v
NEW APPROACHES IN HYBRIDOMA TECHNOLOGY	
Hybridoma variants affecting isotype, antigen binding and idiotype L.A. Herzenberg, T.J. Kipps, L. Peterson and D.R. Parks	3
Isolation of class and subclass switch variants by sib-selection and ELISA A. Bargellesi, G. Spira, E. Fishberg, E. Cosulich and M.D. Scharff	17
MONOCLONAL ANTIBODIES FOR TUMOR DIAGNOSTICS	
Monoclonal antibodies in diagnostic tumor pathology D.J. Ruiter, G.N.P. Van Muyen and S.O. Warnaar	31
Monoclonal antibodies for tumor diagnosis: application <i>in vitro</i> M.K. Schwartz	45
Non-DR molecules present on monocytes participate in the development of autologous mixed lymphocyte reaction in humans G. Damiani, E. Zocchi, M. Fabbi, O. Sacco, F. Patrone, G. Rossi and A. Bargellesi	57
Monoclonal antibody to 5-bromodeoxyuridine: a sensitive and rapid method for estimating the amount of S-phase cells S. Sacchi, A. Donelli, P. Cocconcelli, G. Emilia, A. Messerotti, L. Piccinini, L. Selleri, G. Torelli, G. Rinaldi and U. Torelli	65
Production of monoclonal antibodies specific for differentiation antigens on B cells and large granular lymphocytes F. Malavasi, A. Funaro, G. Bellone, Y. Bushkin, C. Tetta, E. Berti, F. Caligaris-Cappio, A.P.M. Cappa and A. Bonati	71
Monoclonal antibodies in diagnosis and monitoring of myeloid leukemic cells G. Rovera and B. Lange	77
Acute leukemias: B or T lineage commitment detected by HD 37 (pan B) and HD 49 (T reactive) monoclonal antibodies B. Dörken, A. Pezzutto, E. Thiel, G. Moldenhauer, R. Schwartz and W. Hunstein	89
HD 39: different cytoplasmic and surface expression of a B-specific antigen useful in diagnosis and therapy monitoring of hairy cell leukemia A. Pezzutto, B. Dörken, G. Moldenhauer, R. Schwartz and W. Hunstein	95
Development and application of monoclonal antibodies for the detection of TSH in thyroid carcinomas S. Ghielmi, S. Archetti, P. Panina, F. De Simone, E. Brocchi and A. Albertini	101
Circulating adenocarcinoma-associated antigen detected by a monoclonal antibody A. Yachi, K. Imai, T. Endo, H. Hoshi and Y. Hinoda	109

Glycolipid and glycoprotein markers of gastrointestinal cancers Z. Steplewski and H. Koprowski	117
Application of monoclonal antibodies in the recognition of tumor-associated antigens in human breast M. Nuti, M. Castagna and F. Squartini	123
Radioimaging of prostatic carcinoma with prostatic and phosphatase – specific antibodies P. Vihko, J. Heikkilä, M. Kontturi, O. Lukkarinen and R. Vihko	131
Monoclonal antibodies to human melanoma associated antigens with special emphasis on their clinical relevance S. Ferrone, G. Buraggi, M. Temponi, C.V. Hamby, M. Matsui and P.G. Natali	135
<b>MONOCLONAL ANTIBODIES AND DNA PROBES IN DIAGNOSIS OF INFECTIOUS DISEASES</b>	
Infectious disease probes P.J. Olsiewski and D.L. Engelhardt	149
Improved detection by monoclonal antibodies of circulating viral antigens despite immune complexes formation: the hepatitis B experience J.P. Heyraud, C. Pichoud, L. Vitvitski, M. Gassin, P. Chevallier, P. Debeau, L. Cova, O. Hantz and C. Treppe	155
Detection of hepatitis B virus (HBV) DNA by molecular hybridization, clinical significance F. Bonino, E. Chiaberge and F. Negro	163
<b>MOLECULAR MECHANISM OF DISEASE</b>	
DNA probes as diagnostic reagents G. Milanesi	175
Molecular genetics of B-cell neoplasia C.M. Croce	183
The human major histocompatibility complex: genes and proteins J.L. Strominger	189
Detection of cytolytic complexes assembled by complement and by killer lymphocytes E.R. Podack	201
Liver specific expression of cloned human genes R. Cortese, G. Ciliberto, V. Colantuoni, G. Raugi, L. Dente, C. D'Onofrio, G. Bensi, M. Colombo, F. Palla and G. Paonessa	213
The technology of carrier erythrocytes: a versatile tool for diagnosis and therapy A. De Flora	223

**NEW APPROACHES IN HYBRIDOMA  
TECHNOLOGY**





## 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 idiotype, 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 heterologous 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  $\text{IgG}_1$  producing hybridoma, one, five, twenty-five or fifty cells staining with anti- $\text{IgG}_2$  antibody were sorted into individual wells of a microtiter plates. After nine days culture, wells were assayed for the presence of variant  $\text{IgG}_2$  isotype using a sensitive solid phase radioimmune binding assay. The  $\text{IgG}_{2b}$  variant of ME1 was present at high frequency, allowing us to obtain  $\text{IgG}_{2b}$  producing clones after the first round of sorting. (Table 1) Other variant isotypes, namely the  $\text{IgG}_{2a}$  of ME1 and the  $\text{IgG}_{2b}$  and  $\text{IgG}_{2a}$  isotypes of MA2.1 were sorted from the pauci-populations producing variant immunoglobulin as detected in the radioimmune binding assay.

The  $\text{IgG}_{2a}$  and  $\text{IgG}_{2b}$  variants of ME1 and MA2.1 retain the same binding activity for HLA as the  $\text{IgG}_1$  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<sub>1</sub> antibodies produced by the respective hybridomas, however, the IgG<sub>2b</sub> and IgG<sub>2a</sub> 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 <sub>2b</sub>	IgG <sub>2a</sub>
ME1	1600	64
MA2.1	32	8

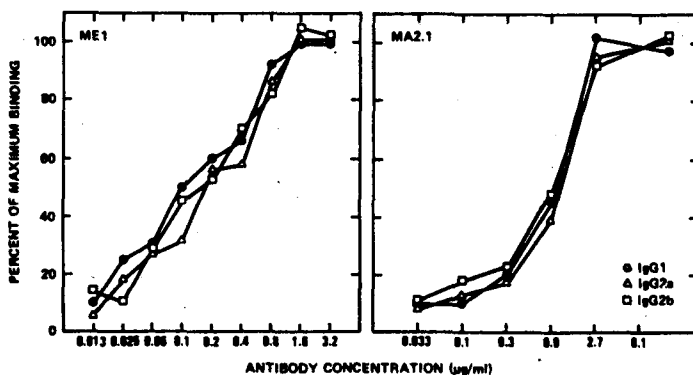


Fig. 1. Titration of antibodies from switch variant families with JY: Antibodies of the MA2.1 switch variant family (right) or the ME1 family (left) were titrated with a fixed number of JY. Specifically bound antibody was detected with FITC-labeled goat anti-mouse Ig (right) or with <sup>125</sup>I-labeled 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<sub>2a</sub> 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<sub>2a</sub> 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<sub>2a</sub> antibody did not direct lysis of JY in the absence of PBL, confirming that ADCC was being observed. Because the IgG<sub>2a</sub> 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<sub>1</sub>, IgG<sub>2b</sub> and IgG<sub>2a</sub> ME1.

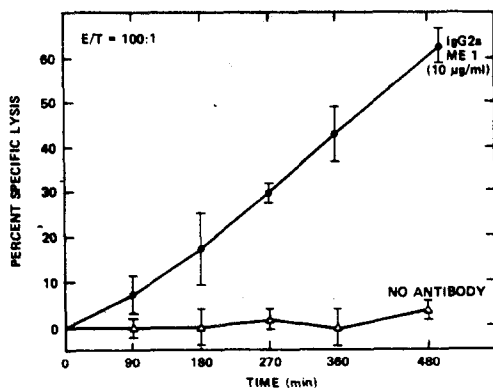


Fig. 2. ADCC directed by IgG<sub>2a</sub> ME1 antibody: Cr<sup>51</sup>-labeled JY was incubated with or without IgG<sub>2a</sub> ME1 at 10 micrograms/ml. PBL were added to an E/T ratio of 10:1. Bars indicate standard deviation (+/-SD) of percent specific lysis for triplicate samples.

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

lysis of JY sensitized with IgG, ME1 is detected, even at high antibody concentrations. A similar hierarchy 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).

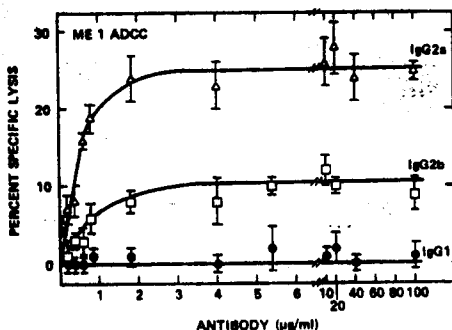


Fig. 3. Comparison of ADCC activities of different antibody isotypes of ME1. Antibody at various concentrations was added to  $^{51}$ -labeled JY. Percent specific lysis ( $\pm$  SD) of JY with PBL after 4 hours incubation with an E/T ratio of 50:1.

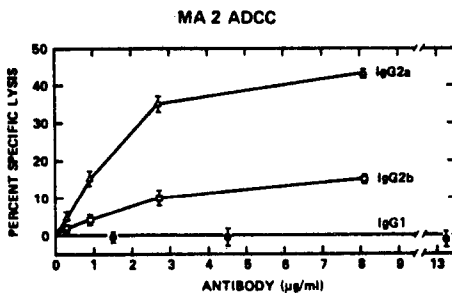


Fig. 4. Comparison of ADCC activities of different antibody isotypes of MA2.1. Conditions are as described in figure 3.

Because longer incubation with PBL increased the specific lysis of IgG<sub>2a</sub>-coated JY, we compared ADCC activities at incubation times greater than four hours, seeking to enhance detection of target cytolysis directed by either IgG<sub>2b</sub> or IgG<sub>1</sub>. ME1 antibodies. The percent level of cell killing directed by the IgG<sub>2b</sub> ME1 remained

significantly less than that of the IgG<sub>2a</sub>-coated JY at all time points tested (FIGURE 5) and IgG<sub>1</sub> ME1 directed no ADCC up to at least 19 hours.

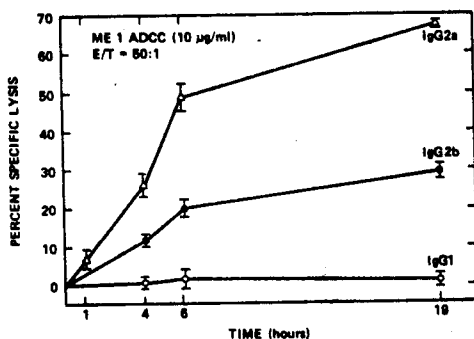


Fig. 5. Kinetics of ADCC directed by the different isotypes of ME1. <sup>51</sup>Cr-labeled JY was incubated with antibody of each isotype at 10 micrograms/ml. ADCC (+/- SD) of PBL at an E/T ratio of 50:1 was assayed at times indicated.

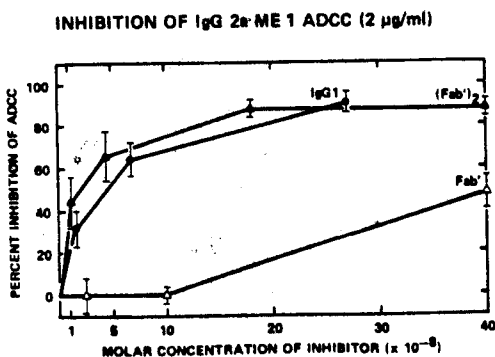


Fig. 6. Inhibition of IgG<sub>2a</sub> ME1 ADCC: The IgG<sub>1</sub>, F(ab')<sub>2</sub>, or Fab' of ME1 was added at various concentrations to separate wells containing <sup>51</sup>Cr-labeled JY. IgG<sub>2a</sub> ME1 was subsequently added to 2.5 micrograms/ml. After a 20 minute incubation at room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

The IgG<sub>1</sub> ME1 antibody, however, could competitively inhibit the capacity of the IgG<sub>2a</sub> antibody to direct ADCC (Figure 6). Such inhibition is comparable to the F(ab')<sub>2</sub> fragment of ME1, demonstrating that the Fc of the antibody molecule is critical for directing effective ADCC. Furthermore, IgG<sub>2a</sub> ME1 directed ADCC of JY is not affected by saturating concentrations of MA2.1 IgG<sub>1</sub>, although the later antibody could effectively inhibit MA2.1 IgG<sub>2a</sub> directed ADCC. These results demonstrate that the inhibition of ADCC by the IgG<sub>1</sub> antibody of a given switch variant family is due to competition with the effective IgG<sub>2a</sub> 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<sup>-4</sup> to 10<sup>-7</sup>, comparable 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<sub>2b</sub> switch variants most frequently occur from IgG<sub>1</sub> producing hybridomas and IgE switch variants most frequently occur from IgG<sub>2a</sub> 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.

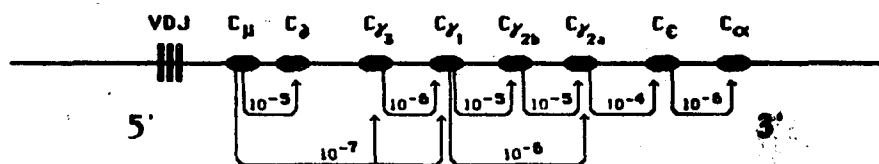


Fig. 7. Generalized spontaneous isotype switch variant frequencies.

#### 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