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Chapter 109
Hybridoma immunoglobulin isotype switch variant selection with the fluorescence activated cell sorter

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In 1978, Rajewsky *et al.* utilized the powerful resolving capacity of the fluorescence activated cell sorter (FACS) to isolate isotype 'switch variant' myeloma cells from a large culture of myeloma cells [1]. Using fluorescence-conjugated antibody specific for an immunoglobulin isotype not expressed by the parent myeloma cell line, they were successful in staining and subsequently sorting rare switch variant cells. These variant cells secreted myeloma protein of a different immunoglobulin isotype but identical variable (V) region idiotype(s) as the antibody produced by the parent myeloma line [1,2]. As such, these cells apparently switched from the expression of the gene encoding the constant part of one heavy chain to that of another, while expressing the same heavy chain variable region gene which encodes the antibody's antigen combining site. Although other investigators had observed similar isotype switch variants after mutagenesis of the MPC 11 myeloma cell line using a soft agar cloning technique [3,4], these investigators demonstrated that rare spontaneous switch variant cells could be selectively stained for the variant immunoglobulin expressed on their surface membrane and subsequently sorted using the FACS [1].

More recently, the authors and others have used the FACS to isolate isotype switch variant hybridomas producing monoclonal antibodies with selected specificities and desired functional activities [5–7]. Experiments have shown that spontaneous immunoglobulin gene switching occurs in hybridomas with frequencies similar to those noted with cultured myeloma cells. Furthermore, the binding specificity of the switched monoclonal antibody is identical to that of the original parent immunoglobulin molecule. Thus, by defining the methodology required for switch variant selection, a hybridoma clone producing a monoclonal antibody with a desired specificity may be modified to secrete antigen-specific immunoglobulin with an isotype of desired biological activity.

The ability to select switch variant hybridomas producing monoclonal antibody with a particular immunoglobulin isotype may greatly enhance the application of monoclonal antibody technology. Even among the subclasses of mouse IgG—namely IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3—major differences exist. Comparison of the segmental flexibility using nanosecond fluorescence spectroscopy has demonstrated IgG_{2b} to be most flexible, IgG_{2a} slightly less flexible and IgG_1 relatively rigid [8,9]. Furthermore, each immunoglobulin subclass possesses discrete biological activity. IgG_{2a} and IgG_{2b} fix guinea-pig and rabbit complement through the conventional pathway, whereas IgG_1 and IgG_3 do not [10–12]. IgG_1 molecules, but not IgG_{2a}, IgG_{2b}, or IgG_3, are capable of mediating passive cutaneous anaphylactic (PCA) reactions in the mouse [10,13–15]. IgG_{2a} and IgG_{2b} subclasses, but not IgG_1, mediate the same reaction in the guinea-pig [11,16–18]. More recently, the authors have detected differences between these three classes of murine immunoglobulin in their ability to participate in human antibody-dependent cellular cytotoxicity (ADCC) [19]. IgG_{2a} functions better than IgG_{2b} in human ADCC, whereas IgG_1 is inactive. Furthermore, mouse immunoglobulin isotypes of anti-tumour antibodies apparently differ in their ability to influence tumour growth in experimental animal model systems [20–27]. Thus the isotype of a given monoclonal antibody may play a critical role in its biological effector function. The ability to select switch variant cells from a hybridoma originally selected for the binding specificity of its secreted monoclonal antibody may allow for the production of specific monoclonals with isotypes of desired biological activity.

Since 1978, the authors have simplified and im-

proved the process of switch variant selection using the FACS. The first switch variants were isolated only after repeated rounds of FACS variant selection, involving months of tissue culture. Currently, it is possible to detect and, in most cases, directly clone switch variant cells with only one round of FACS selection. Here the authors describe these advances which greatly facilitate the process of switch variant selection.

Directionality of *in vitro* isotype switching

In planning a switch variant selection, it is important to note some basic facts of the murine heavy chain immunoglobulin gene complex and the heavy chain class switch. The genes coding for the heavy chain constant region isotypes are arranged in tandem, 3′ of the variable region gene complex of mouse chromosome 12 [28–30]. The order in which the heavy chain immunoglobulin genes are arranged is depicted in Fig. 109.1. Prior to immunoglobulin gene expression, the variable region genes of the heavy chain gene complex must undergo rearrangement to form a functional VDJ gene composed of the variable (V), diversity (D) and joining (J) gene segments that together code for the variable region of the antibody heavy chain [31,32]. The VDJ gene is subsequently expressed with one of the several heavy chain isotypes on the same RNA transcript that is translated to form the antibody heavy chain.

Models proposed for immunoglobulin class switching must take into account the conserved expression of the same VDJ gene after the switch to another isotype. One model suggests that selective splicing of a large RNA transcript may account for the switch in the expression of the heavy chain constant region [33–35]. Another model suggests that genes on a single DNA strand can recombine internally to join the gene coding for switched heavy chain isotype with the expressed VDJ gene [36,37]. A third model proposes that unequal recombination between newly formed sister chromatids may be responsible for this genetic rearrangement [38–40].

Analyses of the DNA from several myeloma cell lines and hybridomas have demonstrated deletions of the genes coding for the isotypes located upstream, or 5′, of the expressed heavy chain isotype gene [28,41]. Such deletions may make switching to isotypes coded for by genes located 5′ of the expressed isotype gene impossible, unless there is interchromosomal switch recombination to the genes expressed on the homologous chromosome. Although interchromosomal recombination during isotype switching has been documented [42], such recombination is of much lower frequency than switching among genes located on the same chromosome. Generally, switching to genes located 5′ of the C gene expressed in the original hybridoma is rare or non-existent. Although variant cells have been selected that express isotypes located upstream of the gene expressed by the sorted hybridoma population, this has been noted only with switch variants selected for re-expression of their original parent isotype [43]. As such, these cases are not representative of hybridoma cells cloned from a hybrid fusion. Thus, in planning a variant selection, one should consider selecting for isotypes coded for by genes located 3′ of the gene coding for the expressed heavy chain isotype, unless one can analyse the DNA from the cell line of interest to confirm that the heavy chain genes 5′ of the expressed gene have not been deleted.

The technique of switch variant selection

Staining reagents

The success of switch variant selection using the fluorescence activated cell sorter depends upon the specificitity of the staining reagent for the variant isotype. In order to detect as few as one cell in several million, the fluorescence of the specifically stained variant cell should be bright enough to allow it to be discriminated from the most brightly autofluorescent cells in the population. A standard methodology employs preparations of highly absorbed, isotype-specific heterologous antibodies that have been fluorescein conjugated for variant selection. More recently, the authors have used monoclonal antibodies directed against allotypic determinants on mouse immunoglobulin for variant selection. These reagents are particularly effective when two or more types of monoclonal antibodies that have different binding specifities for a variant immunoglobulin isotype are used for surface staining, as described below.

Heterologous antibody

There are several advantages and disadvantages to using heterologous anti-isotypic antibody prep-

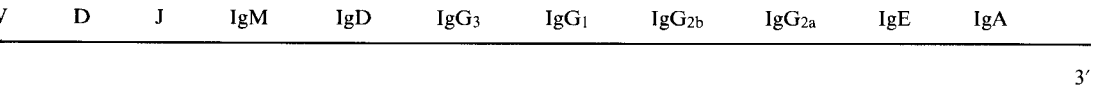

Fig. 109.1. Order of constant region genes in the murine heavy chain immunoglobulin gene complex.

arations rather than isotype-specific monoclonal antibodies. Such preparations represent heterogeneous collections of antibody molecules with multiple binding specificities for the variant immunoglobulin isotype. More than one fluorescein-conjugated antibody can bind to a single surface immunoglobulin molecule, increasing the fluorescence signal conferred upon the switch variant cell. Many preparations of heterologous anti-isotype antibodies are available commercially as fluorescein conjugates or free antibody. A disadvantage of heterologous antibody is that such preparations must be extensively absorbed with other mouse immunoglobulin isotypes on solid-phase immunoabsorbants to render them specific for the heavy chain isotype of interest. One must carefully check commercially available isotype specific heterologous antibody preparations. The authors have observed several such preparations to slightly increase the mean fluorescence of cells bearing irrelevant mouse immunoglobulin isotypes. Such non-specific staining will increase the number of false-positive variant cells sorted in a given experiment, making variant selection much less efficient.

Another problem encountered with heterologous antibody preparations is the fine dependence of optimal staining on antibody concentration. That is demonstrated in Table 109.1. This table lists the mean fluorescence values of two cell lines stained with a fluorescein-conjugated goat anti-mouse gamma 2 reagent at varying amounts and concentrations. Higher concentrations of antibody yield higher levels of specific staining at a given absolute amount of antibody used per number of cells stained. This may be due to populations of antibodies that are heterogeneous with respect to binding affinity. Thus low affinity antibodies that may specifically bind the variant surface immunoglobulin isotype and increase the fluorescence signal conferred upon variant cells may not interact efficiently with their substrate when present at low concentrations.

Table 109.1. Titration of FITC goat anti-mouse IgG_2 on hybridoma cell lines

Antibody concentration (μg/ml)	Micrograms antibody per 10^6 cells	Mean fluorescence of hybridoma cell lines 190-59.2 (IgG_{2a})	197-24.63 (IgG_1)
80	10	88	12
	3	78	11
	1	65	10
	0.3	41	10
40	10	81	12
	3	76	12
	1	63	10
	0.3	36	10
8	10	44	10
	3	42	10
	1	29	10
	0.3	19	10
	0.1	19	10
No stain	—	10	10

Staining protocols should be devised to control the concentration effect that may be observed with a heterogeneous population of heterologous antibody. The cells to be sorted are harvested and washed with staining medium consisting of phenol red-deficient RPMI-1640 medium with 4% (v/v) newborn calf serum. Sample volumes containing the desired numbers of cells are routinely aliquoted into separate tubes and centrifuged for 5 min at 200 ***g***. The supernatant is decanted and the staining reagent, diluted to the desired concentration in staining medium, is added to the cell pellet. After a 20 min incubation at 4 °C, the cells are washed twice with large volumes of staining medium prior to FACS analysis and cell sorting.

Monoclonal antibody to mouse immunoglobulin isotype

Murine monoclonal anti-allotypic antibodies have several advantages over heterologous antibody for switch variant selection. Many are isotype specific, eliminating the need for absorption with other immunoglobulin isotypes. Most bind with high affinity, minimizing the dramatic changes in staining intensity noted with slight variations in reagent concentration. Many have been well characterized with respect to the site at which they bind the immunoglobulin molecule [44–47]. Thus two fluorescein-conjugated monoclonal antibodies, with affinity for the same immunoglobulin isotype but with binding specificity to different sites along the molecule, can stain surface immunoglobulin-bearing cells with an intensity equal to or brighter than most heterologous antibody preparations.

A disadvantage of using fluorochrome-conjugated monoclonal antibody is that not all reagents stain with sufficient intensity to discriminate the majority of specifically stained cells from the most brightly autofluorescent cells of a given hybridoma population. Monoclonal antibodies to several mouse immunoglobulin isotypes may not be generally available. Finally, because most of these reagents are allotype specific, consideration must be given to the point that the hybridomas produce immunoglobulin of the appropriate allotype (see Chapter 94). A few of the monoclonal reagents that the authors found useful for variant selection are listed in Table 109.2.

Table 109.2. Monoclonal reagents useful for surface murine immunoglobulin staining

Name	Isotype/allotype	Specificity
5.7	IgG_3	CH_3 of Igh-1b
2.9	Igh-1a	CH_2 of Igh-1b
8.3	Igh-1b	CH_3 of Igh-1a
9.8	Igh-1b	CH_2 of Igh-1a
10.9	Igh-1b	CH_3 of Igh-4a
10-4.2	Igh-1b	Igh-5a
3.33	Igh-1a	Igh-5b
78	Igh-4a	Igh-6b
Bet-2 (331-12)	Rat IgG	Igh-6

Fluorescein conjugation ratios

For variant sorting, it is important to consider the average number of fluorescein molecules covalently conjugated to the isotype specific antibody. This fluorescein to protein ratio (or F:P ratio) can be easily calculated from the measured light absorption of the conjugated sample at 495 nm and 280 nm [48]. Conjugation ratios less than 2 may not stain specific cells sufficiently to allow for efficient variant sorting. Ratios greater than 4 may non-specifically bind to cells, increasing the background autofluorescence of the cell population from which variants are to be sorted. Increasing the number of fluorescein molecules bound to an antibody molecule above three does not proportionately increase the fluorescence of the antibody because of the internal fluorescence quenching of the closely coupled fluorochromes. Generally, antibody preparations for variant sorting have F:P ratios of 2–4.

Fluorescein conjugation reactions should be closely monitored (see Chapter 28 for fluorochrome conjugation techniques). If the conjugated preparation is found to have a higher F:P ratio and to have lost fine binding specificity, then isolation of antibody molecules with lower F:P ratios on columns of DEAE-Sepharose should be tried. For this the antibody preparation is dialysed against 0.01 M-phosphate buffer at pH 7.5. At this ionic strength the antibody will bind to the DEAE-Sepharose. Fractions are eluted using 0.01 M-phosphate buffers of increasing salt concentration, starting with 0.1 M-NaCl and advancing to 0.5 M-NaCl at 0.1 M increments of NaCl. The protein molecules with the lower F:P ratios will elute at the lower salt concentrations. Fractions of antibody with F:P ratios of 2–3 are pooled and subsequently retested for binding specificity and intensity of staining.

Antibody titration and variant staining concentration

It is important to titre the fluorescein-labelled reagent on cells bearing the variant isotype and compare this with the staining of the parent population. When staining for a variant present at 1 in 10^5–10^7 cells, it is not necessary to use the full saturating amount of antibody needed to stain a population of positive cells. Rather, during the first round of variant selection, the authors routinely use one-fifth this amount.

Exclusion of dead cells from variant cell sorting

Since dead cells often non-specifically bind fluorescent reagents, populations of cells with less than 90% viability are unsuitable for variant sorting. In order to exclude the small percentage of dead cells in a given population from FACS analysis and sorting, the authors include the DNA-intercalating dye propidium iodide (PI) in the staining mixture at 0.3 μg/ml [49]. Dead cells absorb PI, making them brightly fluorescent above 580 nm (red fluorescence) when excited at 488 nm. By electronically gating out cells with a bright red fluorescence, dead cells can be excluded from analysis and sorting.

Limiting dilution analysis of sorted populations

Even with the best of staining reagents and sorter conditions, the false-positive cells far outnumber the true-positive variant cells. The authors devised a cloning technique that sorts a fixed multiple number of cells into each separate well of a microtitre plate. Individual cell sorting of the brightest 0.1% of the stained hybridoma population into separate wells usually requires the assay of a multitude of individual culture supernatants for the variant isotype to identify a switch variant clone, especially for variants present at frequencies of one in several million. On the other hand, sorting all cells into a single well may result in a population of hybridomas with variant cells present at too low a percentage to generate a culture supernatant in which the variant isotype can be detected, even with the most sensitive of radioimmunoassays. This problem is alleviated by sorting 25, 50 or even 100 cells into each well of a microtitre plate. This limits the number of wells that need to be assayed. It also ensures that the frequency of variants in any given population will be greater than or equal to the reciprocal of the number of cells per well sorted. The culture supernatants of individual wells are tested after 1 week's culture to identify those containing variant cells, by a sensitive solid-phase radioimmunoassay. Then the authors expand the hybridomas from these wells and subsequently restain them for repeat FACS analysis and

individual cell cloning. With this approach, it is possible to detect 1 in 10^7 cells after one variant sort using the FACS.

This approach has several advantages. First, the number of variants within a given culture can be directly assessed with one round of variant sorting. The average number of true switch variants sorted per well can be calculated by applying limiting dilution analysis based upon the Poisson distribution [50] to the data obtained from assaying the cultured plates. Dividing this number by the number of actual cells sorted into each well gives the sort fidelity coefficient, or percentage of cells sorted that were indeed switch variants. The calculated proportion of variants within the parent population is provided by multiplying this coefficient by the percentage of cells of the stained parent population that were detected within the fluorescence sort gate during the variant sort. Should only a fraction of the stained cells from the subsequently cloned switch variant population fall within this gate, the true percentage of switch variants within the population is then actually higher than this calculated percentage. In this case, the true percentage of variants within the culture is the calculated proportion of variants divided by the fraction of switch variants detected within the original sort gate after they have been cloned and subsequently restained for the variant isotype. This technique provides variant frequencies without extrapolating sort data from repeated rounds of variant sorting. Also, it is less dependent on the assumption that variant cells have growth characteristics identical to those of the parent population.

A second advantage is the speed at which variants can be isolated. Sorted populations of cells that are highly enriched for switch variants may be identified 1 week after the first round of FACS selection. As the FACS can easily sort with high fidelity cells present at percentages greater than 0.1%, individual switch variant cells can be directly cloned into separate wells of a microtitre plate during the second round of FACS selection. Switch variant cells can be cloned within 2 weeks of the initial sorting of cells from a given cell population.

Kinetics of isotype switching

To appreciate the kinetics with which spontaneously arising switch variants emerge within a culture of monoclonal producing hybridomas, it is important to reflect upon several principles of mutation theory developed in the study of microbial genetics. In 1943, Luria & Delbruck published a theoretical analysis of the rate of increase of the proportion of mutant-type cells in a culture maintained in logarithmic growth [51]. Their analysis applied to cases in which the growth rates of normal and mutant cells are equal and back-mutation is negligible. This analysis also required a low mutation rate so that the number of wild type cells available to generate new mutants would remain virtually the same as the total number of cells. They formulated a theoretical argument that, under these conditions, the proportion of mutant-type cells will increase linearly with the number of generations through which the culture is maintained.

These same conditions apply to many of the switch variants that the authors have analysed. In the several different variant families analysed, the authors found that growth rates for parent hybridomas and their selected switch variant progeny are identical. Furthermore, the rate of isotype switching is very low, ranging from 10^{-5} to 10^{-7} per cell division. The number of parental hybridoma cells available to generate new switch variants will remain identical to the total number of cells in culture, provided the parent cell line is not rapidly losing its ability to produce immunoglobulin. Lastly, the authors have noted that many switch variant cell lines do not switch back to the parental isotype at a detectable rate. Exceptions to this, however, have been noted [43].

Catcheside derived a formula to calculate the actual mutation rate from the increase in the proportion of mutants noted with time in a logarithmically growing bulk culture assayed prior to achieving mutational equilibrium [53]. The mutation rate, m, can be calculated from the following formula:

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

where 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. The natural logarithm of 2 appears in the formula because the mutation rate is per cell per generation rather than the rate per cell division. Since we are dealing with unsynchronized, exponentially growing populations, an event observed to occur within one generation to produce n number of cells will actually be the result of $n(1/2)/(\ln 2)$ divisions. Therefore, for a mutation rate of m, the proportion of mutant cells within a culture will increase by a fixed increment, $m/2(\ln 2)$ per generation.

In order to avoid large fluctuations in the proportion of mutants detected within a newly cloned population of cells, it is important to analyse populations that are large relative to the mutation frequency. Luria & Delbruck analysed a large series of bacterial cultures established from small initial inocula and grown for a small number of generations [51]. They

noted that a few cultures had a high proportion of mutant cells while others lacked any detectable mutants. Such fluctuation was reasoned to be secondary to the random occurrence of mutations. Thus, while the population is still small in some cultures, a mutation will occur by chance early. This will result in a culture with a high proportion of mutants relative to most other cultures, in which no mutation will occur until the population is as large as the reciprocal of the mutation rate.

To test whether or not similar population genetic considerations applied to cultured hybridomas producing antibody of selected specificity, the authors conducted a series of experiments on an anti-dansyl IgG_1-producing hybrid cell line maintained in tissue culture for many weeks [54,55]. This cell line was initially cloned and maintained in logarithmic growth in large bulk culture of at least 10^7 cells. At varying time points, samples from the cell culture were subjected to IgG_2 isotype switch variant selection by the multiple cell cloning technique described above. The authors calculated the proportion of variants within the culture by limiting dilution analysis of data obtained with a solid-phase radioimmunoassay for variant isotype as described above. To provide the number of generations between each sort the authors divided the time elapsed between each variant selection by the cell cycle time of 18 h. The data are presented in Table 109.3. The number of variants within a given culture increased with time after the cell line was established. From the formula described above, the authors calculated the switch frequency of IgG_1 to IgG_2 to be 6×10^{-6}.

These results have important implications for isolation of switch variants. Obviously, the selective pressure applied by the FACS cannot induce isotype switching. It can only isolate the variants after they have occurred randomly. Applying selective pressure on mutational equilibrium will optimize the chance of selecting a switch variant cell. Thus, prior to attempting switch variant selection on a particular cell line, it is recommended that the line be maintained in large bulk culture for several weeks to increase the proportion of variants found within that culture. If the proportion of variants found within the culture drifts upward to 10^{-4}, these cells may be directly cloned from the cell line using one round of FACS sorting.

In conclusion, advances in technology facilitate rapid isolation of switch variant clones from existing hybridoma cell lines. Because of the differences noted between the biologic activity of murine immunoglobulin isotypes, as discussed above, switch variant selection may greatly enhance the application of monoclonal antibodies. Through switch variant selection, a hybridoma producing a monoclonal antibody with a desired specificity may be modified to secrete antigen-specific immunoglobulin with an isotype of desired biologic activity. Through this technology, particular monoclonal antibodies may be fashioned into reagents better suited for a particular task in immunodiagnostics or immunotherapy.

Table 109.3. Analyses of variant proportion within continuously growing culture

Culture generations	Time (days)	Proportion of variants within the IgG_1 hybridoma population $\gamma 2$	$\gamma 2b$	$\gamma 2a$
Newly cloned (G_0)	0	1.0×10^{-5}	0.8×10^{-5}	0.2×10^{-5}
G_{11}	8	1.6×10^{-5}	1.3×10^{-5}	0.3×10^{-5}
G_{25}	19	1.2×10^{-4}	3.0×10^{-5}	9.0×10^{-5}
G_{100}	75	4.0×10^{-4}	5.0×10^{-5}	3.5×10^{-4}

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References

1 LIESEGANG B., RADBRUCH A. & RAJEWSKY K. (1978) Isolation of myeloma variants with predefined variant surface immunoglobulin by cell sorting. *Proc. natn. Acad. Sci. U.S.A.* **75,** 3901–3905.

2 RADBRUCH A., LIESEGANG B. & RAJEWSKY K. (1980) Isolation of variants of mouse myeloma X63 that express changed immunoglobulin class. *Proc. natn. Acad. Sci. U.S.A.* **77,** 2909–2913.

3 PREUD'HOMME J.L., BIRSHTEIN B.K. & SCHARFF M.D. (1975) Variants of a mouse myeloma cell line that synthesize immunoglobulin heavy chains having an altered serotype. *Proc. natn. Acad. Sci. U.S.A.* **72,** 1427–1430.

4 FRANCUS T., DHARMGRONGARTAMA B., CAMPBELL R., SCHARFF M.D. & BIRSHTEIN B.K. (1978) IgG2a-producing variants of an IgG2b producing mouse myeloma cell line. *J. exp. Med.* **147,** 1535–1550.

5 DANGL J.L., PARKS D.R., OI V.T. & HERZENBERG L.A. (1982) Rapid isolation of cloned isotype switch variants using fluorescence activated cell sorting. *Cytometry*, **2,** 395–401.

6 BAUMHAECKEL H., LIESEGANG B., RADBRUCH B., RAJEWSKY K. & SABLITZKY F. (1982) Switch from NIP specific IgG3 to IgG1 in the mouse hybridoma cell line S24/63/63. *J. Immunol.* **128,** 1217–1220.

7 PARHAM P., KIPPS T.J., WARD F.E. & HERZENBERG L.A. (1983) Isolation of heavy chain class switch variants of a

monoclonal anti-DC1 hybridoma cell line: Effective conversion of non-cytotoxic IgG1 antibodies to cytotoxic IgG2 antibodies. *Human Immunol.* **8,** 141–151.

8 Reidler J., Oi V.T., Carlsen W., Vuong T.M., Pecht I., Herzenberg L.A. & Stryer L. (1982) Rotational dynamics of monoclonal anti-dansyl immunoglobulins. *J. Mol. Biol.* **158,** 739–746.

9 Oi V.T., Vuong T.M., Hardy R., Reidler J., Dangl J., Herzenberg L.A. & Stryer L. (1984) Segmental flexibility and effector function of monoclonal antibodies containing identical combining sites joined to different heavy chain C-regions. *Nature*, **307,** 136–140.

10 Grey H.M., Hirst J. & Cohn M. (1971) A new mouse immunoglobulin: IgG3. *J. exp. Med.* **133,** 289–304.

11 Nussenzweig R.S., Merryman P. & Benacerraf B. (1964) Electrophoretic separation and properties of mouse antihapten antibodies involved in passive cutaneous anaphylaxis and passive hemolysis. *J. exp. Med.* **120,** 315–328.

12 Ovary Z. (1966) The structure of various immunoglobulins and their biological activities. *Ann. N.Y. Acad. Sci.* **129,** 776–780.

13 Ovary Z. (1958) Immediate reactions in the skin of experimental animals produced by antibody–antigen interaction. *Prog. Allergy*, **5,** 459–508.

14 Ovary Z. (1964) Passive cutaneous anaphylaxis. In *Immunological Methods*, C.I.O.M.S. Symposium, (ed. Ackroyd J.F.), p. 259. Blackwell Scientific Publications, Oxford.

15 Ovary Z. (1960) Reverse passive cutaneous anaphylaxis in the guinea pig with horse, sheep or hen antibodies. *Immunology*, **3,** 19–27.

16 Barth W.S. & Fahey J. (1965) Heterologous and homologous skin-sensitizing activity of mouse 7S gamma-1 and 7S gamma-2-globulins. *Nature*, **206,** 730–731.

17 Warner N.L., Vaz N.M. & Ovary Z. (1965) Immunoglobulin classes in antibody responses in mice. (I) Analysis by biological properties. *Immunology*, **14,** 410–415.

18 Ovary Z., Fahey J.L. & Barth W.F. (1965) The immunoglobulins of mice. (III) Skin sensitizing activity of mouse immunoglobulins. *J. Immunol.* **94,** 410–415.

19 Kipps T.J., Parham P., Punt P. & Herzenberg L.A. (1985) Importance of isotype in directing human antibody dependent cell-mediated cytotoxicity by murine monoclonal antibodies. *J. exp. Med.*, **16,** 1–17.

20 Wright P.W. & Bernstein I.D. (1980) Serotherapy of malignant disease. *Prog. exp. Tumor Res.* **25,** 140–162.

21 Chard T. (1968) Immunological enhancement by mouse isoantibodies: The importance of complement fixation. *Immunology*, **14,** 583–589.

22 Takasugi M. & Hildemann W.H. (1969) Regulation of immunity toward allogeneic tumors in mice. (I) Effect of antiserum fraction on tumor growth. *J. natn. Cancer Inst.* **43,** 843–856.

23 Fuller P.C. & Winn H.J. (1973) Immunochemical and biological characterization of alloantibody activity in immunological enhancement. *Transplant. Proc.* **5,** 585–587.

24 Bubenik J., Invanyi J. & Koldovsky P. (1965) Participation of 7S and 19S antibodies in enhancement and resistance to methylcholanthrene-induced tumors. *Folia Biologica*, **1,** 258–265.

25 Matthews T.J., Collins J.J., Roloson G., Thiel H.-J. & Bolognesi D.P. (1981) Immunologic control of the ascities form of murine adenocarcinoma 755. (IV) Characterization of the protective antibody in hyperimmune serum. *J. Immunol.* **126,** 2332–2336.

26 Langlois A.J., Matthews T., Roloson G.J., Thiel H.-J., Collins J.J. & Bolognesis D.P. (1980) Immunologic control of the ascites form of murine adenocarcinoma 755. (V) Antibody-directed macrophages mediate tumor cell destruction. *J. Immunol.* **126,** 2337–2340.

27 Bernstein I.D., Nowinski R.C., Tam M.R., McMaster B., Houston L.L. & Clark E.A. (1980) Monoclonal antibody therapy of mouse leukemia. In *Monoclonal Antibodies*, (eds. Kennett R.H., McKearn T.J. & Bechtol K.B.), pp. 275–291. Plenum Press, New York.

28 Honjo T. & Kataoka T. (1978) Organization of immunoglobulin heavy chain genes and allelic deletion model. *Proc. natn. Acad. Sci. U.S.A.* **75,** 2140–2145.

29 Adams J.M., Kemp D.J., Bernard O., Gough N., Webb E., Tyler B., Gerondakis S. & Cory S. (1981) Organization and expression of murine immunoglobulin genes. *Immunol. Revs.* **59,** 5–32.

30 Shimizu A., Takahashi N., Yaoita Y. & Honjo T. (1982) Organization of the constant region gene family of mouse immunoglobulin heavy chain. *Cell*, **28,** 499–506.

31 Davis M., Calame K., Early P., Livant D., Joho R., Weissman I. & Hood L. (1980) An immunoglobulin heavy chain gene is formed by at least two recombination events. *Nature*, **283,** 733–739.

32 Early P., Rogers J., Davis M., Calame K. & Hood L. (1980) An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. *Cell*, **19,** 981–992.

33 Wall R., Choi E., Kuehl M. & Rodgers J. (1981) RNA processing in immunoglobulin gene expression. *Cold Spring Harb. Symp. quant. Biol.* **45,** 879–885.

34 Yaoita Y., Kumagai Y., Okumura K. & Honjo T. (1982) Expression of lymphocyte surface IgE does not require switch recombination. *Nature*, **297,** 697–699.

35 Early P., Rogers J., Davis M., Calame K., Bond M., Wall R. & Hood L. (1980) Two mRNAs produced from a single immunoglobulin mu gene by alternative RNA processing pathways. *Cell*, **20,** 313–319.

36 Dunnick W., Rabbitts T.H. & Milstein C. (1980) An immunoglobulin deletion mutant with implications for the heavy-chain switch and RNA splicing. *Nature*, **286,** 669–675.

37 Kataoka T., Kawakam T., Takahashi N. & Honjo T. (1980) Rearrangement of immunoglobulin gamma-1-chain gene and mechanism for heavy-chain class switch. *Proc. natn. Acad. Sci. U.S.A.* **77,** 919–923.

38 Sakano H., Maki R., Kurusawa Y., Roeder W. & Tonegawa S. (1980) Two types of somatic recombination are necessary for the generation complete immunoglobulin heavy-chain genes. *Nature*, **286,** 676–679.

39 Honjo T., Nakai S., Nishida Y., Kataoka T., Yamawaki-Kataoka Y., Takahashi N., Obata M., Shimizu A., Yaoita Y., Nikaido T. & Ishida N. (1981) Rearrange-

ments of immunoglobulin genes during differentiation and evolution. *Immunol. Revs.* **59,** 33–68.

40 CORY S., WEBB E., GOUGH J. & ADAMS J. (1981) Recombination events near the immunoglobulin C-mu gene join variable and constant region genes, switch heavy chain expression, or inactivate the locus. *Biochemistry*, **20,** 2662–2671.

41 CORY S. & ADAMS J.M. (1980) Deletions are associated with somatic rearrangement or immunoglobulin heavy chain genes. *Cell*, **19,** 37–43.

42 KIPPS T.J. & HERZENBERG L.A. (1985) Allotype switch variants: Demonstration of interchromosomal recombination in isotype switch of cultured monoclonal antibody producing hybridomas. EMBO J. (in press).

43 SABLITZKY F., RADBRUCH A. & RAJEWSKY K. (1982) Spontaneous immunoglobulin class switching in myeloma and hybridoma cell lines differs from physiological class switching. *Immunol. Revs.* **67,** 59–72.

44 PARSONS M., OI V.T. & HERZENBERG L.A. (1981) Monoclonal antibodies as probes of immunoglobulin structure. In *Monoclonal Antibodies and T Cell Hybridomas*, (eds. Hammerling G.J., Hammerling U. & Kearney J.). Elsevier/North–Holland, New York.

45 OI V.T. & HERZENBERG L.A. (1979) Localization of murine Ig-1b and Ig-1a (IgG2a) allotypic determinants defined with monoclonal antibodies. *Mol. Immunol.* **16,** 1005–1018.

46 HUANG C., PARSONS M., OI V.T., HUANG H.S. & HERZENBERG L.A. (1983) Genetic characterization of mouse immunoglobulin allotypic determinants (allotypes) defined by monoclonal antibodies. *Immunogenetics*, **18,** 311–321.

47 OI V.T., HERZENBERG L.A. & BIRSHTEIN B.K. (1983) Localization of murine Igh-1a allotypic determinants by using a panel of mouse myeloma variant immunoglobulins. *J. Immunol.* **130,** 1967–1969.

48 GODING J.W. (1976) Conjugation of antibodies with fluorochromes: Modification of the standard methods. *J. immunol. Meth.* **13,** 215–236.

49 DANGL J.L. & HERZENBERG L.A. (1982) Selection of hybridomas and hybridoma variants using the fluorescence activated cell sorter. *J. immunol. Meth.* **52,** 1–14.

50 HENRY C., MARBROOK J., VANN D.C., KODLIN D. & WOFSY C. (1980) Limiting dilution analysis. In *Selected Methods in Cellular Immunology*, (eds. Mishell B.B. & Shiigi S.M.), p. 138–152. W.H. Freeman & Co., San Francisco.

51 LURIA S.E. & DELBRUCK M. (1943) Mutation of bacteria from virus sensitivity to virus resistance. *Genetics*, **28,** 491–511.

52 STOCKER B.A.D. (1949) Measurements of rate of mutation of flagellar antigenic phase in *Salmonella typhimurium*. *J. Hyg. Camb.* **47,** 398–413.

53 CATCHESIDE D.G. (1949) In *The Genetics of Microorganisms*, pp. 158–162. Sir Isaac Pitman & Sons, Ltd., London.

54 KIPPS T.J. & HERZENBERG L.A. (1984) The fluorescence activated cell sorter: Complementary tools in immunodiagnosis and immunotherapy. In *Rapid Methods in Immunology and Microbiology*, (ed. Habermeh). Springer-Verlag, Heidelberg.

55 KIPPS T.J. (1985) Immunoglobulin isotype switching of monoclonal antibodies. In *Hybridomas in Biotechnology and Medicine*, (ed. Springer T.). Plenum Press, New York.