Homologous chromosome recombination generating immunoglobulin allotype and isotype switch variants

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We investigated whether spontaneous isotype switching in monoclonal antibody-producing hybridomas always occurs with genes on the same chromosome. Spleen cells of (BAB/25 × AKR/J) F1 mice, immunized with dansyl-keyhole limpet hemocyanin (DNS-KLH), were hybridized with NS-1 to generate hybridomas producing monoclonal anti-DNS antibodies of either the b or d haplotype of the BAB/25 or AKR/J parent, respectively. We selected isotype switch variants of such hybridomas using the fluorescence-activated cell sorter (FACS). Although in most cases the allotypic haplotype expressed by the parent and switch-variant hybridomas are the same, in one family of variants we noted a switch in haplotype along with the switch in isotype. This was noted in the selection of IgG_{2a} switch variants from an IgG₁ switch variant originally derived from an IgG₃-producing parent. Biochemical and molecular studies confirm that the allotype switch variant expresses the same heavy-chain variable region gene complex as its parent hybridomas. As such, the allotype switch represents an example of spontaneous mitotic recombination between immunoglobulin heavy-chain genes, generating a single actively transcribed gene from loci previously positioned on different chromosomes.

Key words: isotype switching/hybridomas/monoclonal antibodies/chromosome recombination/allotype

Introduction

Immunoglobulin (Ig) heavy-chain class switching is a process of normal B-cell differentiation that occurs spontaneously in cultured murine myelomas and monoclonal antibody-producing hybridomas at frequencies ranging from 10^{-4} to 10^{-7} /cell/generation (Preud'homme *et al.*, 1975; Liesegang *et al.*, 1978; Radbruch *et al.*, 1980; Neuberger and Rajewsky, 1981; Beyreuther *et al.*, 1981; Dengl *et al.*, 1982; Kipps and Herzenberg, 1984). Using the fluorescence-activated cell sorter (FACS), isotype switch variants can be isolated by sorting for cells expressing variant surface immunoglobulin, allowing one to study immunoglobulin gene expression in both parent and switch-variant progeny (Liesegang *et al.*, 1978). *In vitro* isotype switching in cultured cells apparently results from DNA rearrangement(s) in the heavy-chain immunoglobulin gene complex similar to those found in plasmacytomas. The newly expressed heavy-chain gene is found 3' of the expressed VDJ gene, the exons coding for the formerly expressed isotype having been deleted (Sablitzky *et al.*, 1982). Although the sites at which such rearrangement(s) occur are found outside the physiologic switch regions (S), isotype switch variants generally produce antibodies that are the same

as physiologically switched antibodies of the same isotype in mol. wt. glycosylation and primary protein sequence.

One unusual aspect of *in vitro* isotype switching is that some isotype switch variant hybridomas are noted to revert to expressing the original parental isotype at frequencies comparable with or greater than that of the original 'forward' switch (Beyreuther *et al.*, 1981; Dengl *et al.*, 1982). Because upstream isotype genes are often deleted in switch variant hybridomas, such 'backswitching' is difficult to explain. One hypothesis proposes that unequal mitotic recombination between the immunoglobulin isotype genes on homologous chromosomes may permit the re-expression of an upstream antibody isotype gene previously deleted from the expressed heavy-chain immunoglobulin gene complex. Some evidence for this has been obtained from restriction enzyme analyses of genomic DNA of these hybridoma revertants (Sablitzky *et al.*, 1982).

To investigate whether antibody isotype switching in hybridomas may involve mitotic recombination between the immunoglobulin genes, we constructed murine hybridomas from an F1 animal derived from parents with different immunoglobulin haplotypes (Herzenberg and Warner, 1967; Herzenberg *et al.*, 1968; Herzenberg and Herzenberg, 1978). In so doing, we could monitor the expression of the immunoglobulin alleles by distinguishing the allotype of the expressed antibody using several well-characterized murine monoclonal anti-allotypic antibodies (Oi and Herzenberg, 1979; Parsons *et al.*, 1981; Oi *et al.*, 1983; Huang *et al.*, 1983). This report describes our experience in selecting immunoglobulin isotype switch variants from such hybridomas.

Results

Isolation of the 2.30 switch variant family

Using FACS we selected a large family of switch variant hybridomas from an IgG₃ anti-dansyl (DNS) antibody-producing hybridoma, 2.30 (Figure 1). Most variants were identified by staining cells with fluoroconjugated heterologous antibodies specific for the variant isotype. During the first rounds of FACS sorting, we selected the brightest 0.1% of $1-2 \times 10^7$ labeled hybridoma cells. These cells were then grown to $>5 \times 10^6$ cells, relabeled and sorted again for the variant isotype.

After sufficient enrichment, single cells were cloned into individual wells of a 96-well microtiter plate. Identified switch variant clones were cultured for additional variant selections. The variant V107 was isolated from the original parental 2.30 population using fluorescein isothiocyanate (FITC)-goat anti-mouse IgG₁. The IgG_{2a} and IgG_{2b} variants, V19 and V14, respectively, were derived from the cloned V107 variant by staining with FITC-goat anti-mouse IgG₂. Another IgG_{2a} variant, V17, was derived from V14 by selecting for variants staining with FITC-goat anti-mouse IgG_{2a}. The IgE and IgA variants, V19-7.1 and V19-2.2, respectively, were selected from V19 using two-color immunofluorescence, sorting for hybridomas that had lost surface IgG_{2a} expression but retained DNS binding activity. Subsequently,

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Fig. 2. Two-dimensional gel electrophoresis of the secreted monoclonal antibodies produced by various members of the 2.30 switch-variant family. Reduced and biosynthetically ^{35}S -labeled anti-DNS antibodies were separated in the first dimension via non-equilibrium pH gradient electrophoresis and in the second dimension via SDS-polyacrylamide gel electrophoresis. Listed are pI values and mol. wts of added protein standards (Pharmacia). Samples are: (A) 2.30; (B) V107; (C) V14; and (D) V19.

quences in C_H or J_H , respectively, we specifically primed synthesis of cDNA on purified poly(A)-rich mRNA. The variable region sequences of the parent hybridoma, 2.30, and switch variant progeny V107, V14 and V19 were determined from J to the second hypervariable region (Figure 4). All hybridomas express the same $\text{V}_\text{H}\text{IIb}$, D segment and $\text{J}_\text{H}4$, thus confirming the clonal origin of this switch variant family.

Discussion

To investigate whether antibody isotype switching in hybridomas may involve mitotic recombination between the immunoglobulin genes on homologous chromosomes, we constructed murine hybridomas from an F1 animal derived from parents with different immunoglobulin haplotypes. This allowed us to monitor the expression of the immunoglobulin alleles by distinguishing the allotype of the expressed antibody using several well-characterized murine monoclonal anti-allotypic antibodies. We selected isotype switch variants of such hybridomas using the FACS. Although in most cases the allotypic haplotypes expressed by the parent and switch variant hybridomas are the same, in one family of variants we noted a change to an allotype included in the other parental haplotype along with the switch in isotype. This was noted in the selection of $\text{IgG}_{2\text{a}}$ switch variants from an IgG_1 switch variant originally derived from an IgG_3 -producing parent. Biochemical and molecular studies confirm that the allotype switch variant expressed the same heavy-chain variable-region gene complex as its parental hybridoma. As such, the allotype switch represents an example of spontaneous mitotic recombination between immunoglobulin heavy-chain genes, generating

Fig. 3. Autoradiogram of Southern blot analysis of *Eco*RI-digested genomic DNA probed with $\text{pJ}_\text{H}34$. Arrows indicate the kilobases of detectable bands. Sample lanes represent analyses of genomic DNA extracted from: (A) AKR/J liver; (B) BAB/25 liver; (C) (AKR/J $\times$ BAB/25) F1 liver; (D) NS-1; (E) 2.30; (F) V19; (G) V14.

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switch from Igh-4b to Igh-1d is associated with the loss of a rearranged J segment. This loss may be secondary to non-reciprocal recombination between the immunoglobulin isotype genes with subsequent loss of the abortive variable region rearrangement on the formerly non-expressed chromosome. Rather than non-reciprocal recombination between sister chromatids, the allotype switch may represent a rather unusual pairing and subsequent non-reciprocal exchange between the immunoglobulin heavy-chain genes of different homologous chromosomes, resulting in mitotic recombination.

Spontaneous mitotic recombination has been demonstrated in several eucaryotic organisms (Stern, 1936; Pontecorvo and Kafer, 1958; Vig and Paddock, 1968; Katz and Kao, 1974) and recently in somatic mammalian cells grown in culture (Wasmuth and Hall, 1984). Mitotic recombination has been implicated in isotype 'backswitching' of cultured switch variant hybridomas (Sablitzky *et al.*, 1982), somatic mutation of heavy-chain variable region genes (Dildrop *et al.*, 1982; Clarke and Rudikoff, 1984), and in the generation of hybrid gamma2b-gamma2a heavy-chain producing variants from MPC-11 after mutagenesis with ICR 11.19.3 (Tilley *et al.*, 1983).

Recombination between highly homologous genes within multi-gene families may be an important mechanism at the meiotic level for generating allotypic polymorphisms in both constant region and variable region genes (Herzenberg *et al.*, 1968; Gally and Edelman, 1970; Capra and Kindt, 1975; Baltimore, 1981; Ollo and Rougeon, 1982, 1983; Kvist *et al.*, 1983). The capacity to study such recombination at the somatic level in cultured hybridomas may shed light on the *in vivo* mechanisms of these rearrangements.

Materials and methods

Animals

Mice were raised and maintained in the Herzenberg breeding colony.

Antibodies

BV46 was obtained from Dean Ballard, University of Illinois, Urbana. HY16, developed by M. Potter, was obtained from C. Muller, Stanford University. All other monoclonal antiallotype antibodies (Table I) were generated in our laboratory (Oi and Herzenberg, 1979; Parsons *et al.*, 1981; Huang *et al.*, 1983). Rat anti-mouse kappa, 187.1, developed by D. Yelton and M. D. Scharff (Yelton *et al.*, 1981) was obtained from the American Type Culture Collection (Rockville, MD). Heterologous goat antibodies specific for mouse immunoglobulin isotypes were prepared by solid-state absorption and elution (e.g. Parham *et al.*, 1983).

Hybridomas

Cells were cultured in RPMI-1640 (GIBCO) supplemented with 2 mM L-glutamine and 10% horse serum at 37°C in a 7% CO₂-in-air incubator. The parent hybridoma, 2.30, was isolated from the fusion of NS-1 with spleen cells of (AKR/J × BAB/25) F1 mice primed and boosted with dansyl-keyhole limpet hemocyanin. Hybridization was performed with polyethylene glycol and subsequent selection in culture medium containing hypoxanthine, aminopterin and thymidine (Oi and Herzenberg, 1980).

Dansylation

Protein was solubilized in 0.5 M NaHCO₃ (pH 9.5) at concentrations ranging from 0.5 to 1.5 × 10⁻⁴ M. 5-Dimethylaminonaphthalene-1-sulfonyl chloride (dansyl-chloride, Sigma, MO) was dissolved in dimethylformamide to 10 mg/ml. A 25 M excess of dansyl chloride was added slowly to a rapidly stirring solution of protein and allowed to react at 4°C overnight. Unreacted dansyl chloride was separated from dansylated protein by G25-Sephadex chromatography. The approximate numbers of dansyl molecules bound per molecule of protein was determined spectrophotometrically at 280 and 340 nm.

Fluorescence-activated cell sorting

One- or two-color fluorescence-activated cell sorting was performed as described (Parks and Herzenberg, 1984), on a modified FACS II equipped with an argon laser and a variable-frequency adjustable rhodamine-dye laser. Sorting for hybridomas expressing anti-DNS surface immunoglobulin was achieved by staining cells with a phycobiliprotein, allophycocyanin (Oi *et al.*, 1982) obtained from Dr

D.R. Parks, Stanford University, that had been coupled to dansyl-chloride. Allophycocyanin excitation at 600 nm was monitored at 640 nm.

Two-dimensional gel electrophoresis

For biosynthetic labeling, 5 × 10⁷ hybridoma cells were cultured for 5 h in 2 ml methionine-free culture medium supplemented with 5% fetal calf serum (FCS) and 0.5 mCi [³⁵S]methionine, 1000 Ci/mmol (New England Nuclear). Harvested supernates were added to polystyrene plates previously coated with DNS₂₀-BSA. After 1 h of incubation at room temperature, plates were extensively washed with phosphate-buffered saline (PBS) supplemented with 1% BSA. Specifically bound anti-DNS antibody was washed off the plate using Nonidet P-40 sample buffer for two-dimensional gel electrophoresis, as described (Jones, 1980).

Spectroscopic measurements

Fluorescence spectra were obtained on a Perkin-Elmer model 44B fluorimeter equipped with a DCSCU-2 corrected emission spectra unit. The fluorescence of purified murine monoclonal antibodies with ε-dansyl-L-lysine (Sigma, MO) were compared with the fluorescence of ε-dansyl-L-lysine with non-specific purified chicken gamma globulin (Sigma, MO), at antibody concentrations ranging from 5 × 10⁻⁷ to 10⁻⁵ M in 0.15 M NaCl, 0.05 M Tris pH 8.2.

Radioimmune assays

Polystyrene microtiter plates were coated with DNS₂₀-BSA at 50 µg/ml for 1 h. Radioimmune assays (RIA) were performed as described (Tsu and Herzenberg, 1980).

Analysis of genomic DNA

Genomic DNA of freshly isolated hepatocytes and cell lines were isolated as described (Blin and Stafford, 1976). 10 µg samples of DNA were digested with 50 U of *Eco*RI or *Hind*III (New England Biolabs) size separated on gels of 0.8% agarose (100 mA, 40 V for 12 h) and transferred onto nitrocellulose (Southern, 1975). Hybridization was performed as described (Migone *et al.*, 1983), using pJ34 labeled with ³²P via nick translation to a specific activity of 1–3 × 10⁸ d.p.m./µg (Rigby *et al.*, 1977). pJH34, a 1.6-kb genomic *Eco*RI-*Bam*HI fragment spanning the third and fourth J_H segments subcloned into pBR322 (Sakano *et al.*, 1980), was obtained from J. Dangel, Stanford University.

RNA sequencing

RNA was extracted from each of the cell lines with 6 M guanidium isothiocyanate followed by CsCl density-gradient centrifugation (Glisin *et al.*, 1974). Poly(A)⁺ RNA was purified using oligo(dT)-cellulose (Edmonds *et al.*, 1971). Synthetic oligonucleotide DNA primers were used to initiate cDNA synthesis by reverse transcriptase on poly(A)⁺ RNA. Specific priming of cDNA synthesis was accomplished using either a 15-bp oligonucleotide C-gamma primer (3'-CAGATAGGTGACCG-5') or a mixture of two 14-bp primers (J1 primer: 3'-CAGACCCCGTGTC-5'; J2-4 primer: 3'-ATGACCCCGGTTCC-5'), as described (Kaartinen *et al.*, 1983; Sablitzky and Rajewsky, 1984). These primers were provided by Dr Fred Sablitzky (Universität zu Köln, Köln, FRG). The cDNA synthesis with either [³²P]ATP or [³²P]CTP was specifically terminated using dideoxynucleotides to determine the RNA sequence (Hamlyn *et al.*, 1978, 1981).

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