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Forced expression of AID facilitates the isolation of class switch variants from hybridoma cells

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Abstract

Monoclonal antibodies are used in the treatment and diagnosis of diseases and to study the protective and adverse functions of antibodies *in vitro* and *in vivo*. Since the isotype determines the effector function, half-life in the serum and distribution throughout the body, it would be useful to have a battery of antibodies with the same binding site associated with different isotypes. However, since hybridomas switch isotypes at very low frequencies in tissue culture, it has been difficult and very labor intensive to isolate panels of class switch variants. We show here that stable transfection of activation-induced cytidine deaminase (AID) in hybridomas increased their frequency of switching to a level that greatly facilitated the isolation of subclones expressing monoclonal antibodies of different isotypes. Although forced expression of AID also increased the frequency of somatic hypermutation in the immunoglobulin variable regions that encode the antigen-binding site, antigen recognition was retained in the isotype switched antibodies.

Keywords

AID; CSR; hybridoma

1. Introduction

In response to antigen, already rearranged and expressed immunoglobulin (Ig) genes are altered by somatic hypermutation (SHM) and class switch recombination (CSR). SHM introduces point mutations into variable (V) regions that encode the antigen-binding site and can lead to antibodies with higher affinity that are more effective in neutralizing viruses or toxins and in eliminating pathogens. SHM can also lead to changes in specificity that enables the organism to protect itself from rapidly changing pathogens (Rajewsky, 1996). CSR replaces the μ constant region (C_μ) in the heavy chain locus with one of the downstream constant regions (C_γ , C_ϵ or C_α), allowing a switch from IgM to IgG, IgE or IgA (Stavnezer, 2000; Kinoshita et al., 2001; Manis et al., 2002). These changes in isotype can be very important, since various isotypes are distributed differently in the body, have different half-lives in the circulation and carry out distinct subsets of effector functions (Stavnezer, 2000). Both SHM and CSR require activation induced cytidine deaminase (AID), which converts deoxycytidines in the V and

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switch regions to uracil and initiates both processes (Muramatsu et al., 1999; Di Noia et al., 2002; Manis et al., 2002; Bransteitter et al., 2003). Patients that are genetically deficient in AID make only low affinity IgM antibodies and die of infections if they are not treated with hyperimmune immunoglobulins (Quartier et al., 2004).

The discovery of the hybridoma technology 30 years ago (Kohler et al., 1975) made it possible to immortalize individual B cells that were participating in the antigen-driven immune response by fusing cells from the spleen of immunized mice to tissue culture-adapted mouse plasmacytoma cells. This technology allowed the production of large quantities of homogeneous antibodies of a single isotype in tissue culture and monoclonal antibodies became powerful agents in the treatment and diagnosis of diseases, as well as important research tools (Casadevall et al., 2004; Stacy, 2005). Since its inception, the major drawback of the hybridoma technology has been that it recovers only a tiny percent of the B cells that are making antibody to a particular antigen at the time of fusion. As a consequence, most mouse monoclonal antibodies are either IgM or IgG₁ and often of low affinity. Since monoclonal antibodies are made by fusing plasmablasts to tissue culture adapted plasmacytomas, both of which represent a late stage in B cell development when AID is no longer expressed, it is not surprising that in most hybridomas CSR and SHM occur at very low frequencies. As a consequence, for many years it has not been possible to routinely affinity mature or isotype switch hybridomas at high frequencies in tissue culture.

Because switch variants of hybridomas usually arise at low frequencies of 10^{-5} – 10^{-6} , protocols were developed to recover the rare hybridoma clones that had switched in culture, using either cell sorting (Dangl et al., 1982; Muller et al., 1983) or sequential subcloning (Spira et al., 1984; Spira et al., 1992). Since most hybridomas have low amounts of surface Ig, sequential subcloning, also called sib selection, is often used. However, sib selection is a painfully slow process that requires many months of intensive tissue culture work to recover a set of isotype switched monoclonal antibodies from a single parental hybridoma (Spira et al., 1992). With the discovery that ectopic expression of AID induces CSR in an artificial switch substrate in fibroblast cells (Okazaki et al., 2002; Yoshikawa et al., 2002), it seemed possible that the low frequency of CSR in hybridomas was due to the lack of AID expression and that the frequency of class switching in hybridoma cells in tissue culture could be increased by forcing the expression of AID.

We show that expression of AID increases the frequency of CSR in two different hybridomas. Although the increase in the frequency of CSR induced by the increased expression of AID was not as high as is seen *in vivo* or in naïve B cells that are stimulated to switch in short term culture (Stavnezer, 2000), it greatly facilitated the isolation of switched variants. Although AID expression also increased SHM, antigen recognition was retained in the isotype switched antibodies.

2. Materials and methods

2.1 Cell lines and cell culture conditions

36-65, an A/J hybridoma that produces an IgG₁ anti p-azophenylarsonate, was grown as previously reported (Spira et al., 1994). 36-65.L derives from the low switching 36-65.12.7.30.7.2 subclone of 36-65 (Lin et al., 1996). Hybridoma Sp6, expressing an IgM antibody against hapten 2,4,6-trinitrophenyl, was obtained from Dr. M. J. Shulman (Ronai et al., 1999). Naïve spleen B cells were obtained from two 6 week old C57BL/6 mice. Splenocytes were isolated, depleted of T cells, grown in RPMI 1640 medium containing 10% FCS and stimulated with 40 µg/ml LPS (Sigma-Aldrich, St. Louis, MO) and 25ng/ml IL-4 (R&D Systems, Minneapolis, MN) for 4 days, as described previously (Li et al., 2004). These animal

experiments were approved by the Albert Einstein College of Medicine Animal Use Committee.

2.2 Soft agar cloning

First, 4 ml of 0.4% SeaPlaque agarose (FMC Bioproduct, Rockland ME) in medium supplemented with 20% FCS was introduced into a 60-mm culture plate (Falcon-Becton Dickinson, Mountain View, CA). After solidification at 4°C for 10 min, 1 ml of the same medium containing 10^3 cells was laid over the top of the soft agar and put at 4°C for 10 min. Cells were grown at 37°C for ~7 days. Clones were randomly picked and placed into a 96-well plate, as described (Zhang et al., 2001).

2.3 ELISA spot assay (ESA)

The assay was performed as previously reported (Greene et al., 1990) with modifications (Spira et al., 1992). Briefly, plates were pre-coated with a 1:500 dilution of the anti-mouse antibody against the corresponding isotype (Southern Biotechnology, Birmingham, AL) and blocked with 2% BSA. 5×10^5 cells were plated, removed 18 hours later and spots developed with biotinylated antibody against the corresponding isotype (Southern Biotechnology, Birmingham, AL) and 5-BCIP substrate (Amresco, Solon, OH). ELISpots were counted with a dissecting microscope and the median frequencies of switching were calculated. If no spots were detected in the number of cells used in the assay one spot was assigned to calculate median frequencies.

2.4 Isolation of isotype switch variants by sib selection

Briefly, 10^3 cells were plated per well and grown for 3 days in 96 well plates. Then, half of the cells were examined for the frequency of ELISpots of the desired isotype. The duplicate of the well with the highest number of spots was plated out in a new 96 well plate at successively lower cell densities and replicate plate screened again for the frequency of switch variants (Spira et al., 1992).

2.5 Determination of antigen binding by ELISA

The hapten recognized by 36-65, p-azophenylarsonate, was conjugated to BSA at high density of hapten as previously described (Nisonof, 1967) and used to coat ELISA plates at 5 µg/ml. Antibody concentration in the supernatant was calculated previously using a known concentration of a control antibody of the corresponding isotype. Serial $\frac{1}{2}$ dilutions of each antibody, starting from 0.05 µg/ml, were used. Alkaline phosphatase-labeled antibody against the corresponding isotype (Fisher Scientific, Pittsburgh, PA) was used and hydrolysis of the substrate p-nitrophenylphosphate (Amresco, Solon, OH) was monitored at 405 nm.

2.6 Extraction of total RNA and RT-PCR

Total RNA was isolated from $\sim 5 \times 10^6$ cells using Tryzol (Invitrogen, Carlsbad, CA), digested with DNase I (Worthington Biochemical, Lakewood, NJ) to remove residual genomic DNA. To check expression of hAID in the stable transfectants, RT-PCR one step kit (Invitrogen, Carlsbad, CA) was used to amplify hAID and glyceraldehyde phosphate dehydrogenase (GAPDH), using primers and conditions previously described (Zhang et al., 2001).

2.7 Transfection conditions

5×10^6 cells were transfected with 10 µg full length human AID expressing vector (pCEP4-hAID) or empty vector control linearized with EcoRV and NruI (Martin et al., 2002a), using a GenePulser electroporator (BioRad, Hercules, CA). Cells were distributed in 96-well plates

at 10^4 cells/well, selected with hygromycin B (Calbiochem, San Diego, CA) and stable transfectants were detected ~2 weeks later.

2.8 Sequencing

Genomic DNA was prepared using Wizard purification kit (Promega, Madison WI). 36-65 heavy-chain variable region was amplified using *Pfu* turbo (Stratagene, Cedar Creek, Texas) and primers 5' 36-65VH (5'-CAACCTATGATCAGTGCCTC-3') and 3' 36-65VH (5'-GTGTCCCTAGTCCTTCATGACC-3') for heavy chain and primers 5' 36-65VL 5'-GATATCCAGATGACACAGACTACATCCT-3') and 3' 36-65VL (5'-GAGGAAGCGTATTACCCTGTTGG-3') for light chain region. AID was amplified using primers and conditions described before (Martin et al., 2002b). PCR products were cloned using the Zero Blunt TOPO cloning kit (Invitrogen, Carlsbad, CA). Minipreps were prepared using the Montage plasmid 96 kit (Millipore, Billerica, MA). The insert was verified by digestion with EcoRI and sequenced at the SeqWright and the Albert Einstein Cancer Center facilities using M13 reverse primer. DNA sequences were aligned using the SeqMan program of DNASTar.

2.9 Western blot

AID was detected with a mouse monoclonal IgG₁ antibody (Cell Signaling, Danvers, MA), followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL). Beta actin was detected with mouse monoclonal IgG_{2a} antibody (#5316, Sigma-Aldrich, St. Louis, MO) followed by HRP-conjugated goat anti-mouse IgG_{2a} antibody (Southern Biotechnology, Birmingham, AL). AID and beta actin signals were visualized with a LAS-3000 imager (FujiFilm) and quantified with ImageQuant 4.0 software.

3. Results

3.1 Forced expression of AID further increases CSR in hybridomas

Although primary murine B cells can be stimulated to switch isotypes at frequencies greater than 10% (Stavnezer, 2000), most hybridomas switch in culture at frequencies of 10^{-5} – 10^{-6} (Spira et al., 1992; Spira et al., 1994). The 36-65 hybridoma, which makes an IgG₁ monoclonal antibody to p-azophenylarsonate (Ars) (Rothstein et al., 1983), switches in culture to IgG_{2b} and IgG_{2a} at frequencies of 10^{-5} – 10^{-6} .

We first obtained fresh subclones from the 36-65 hybridoma by agar subcloning and the frequency of switching was determined using the ELISA spot assay (ESA) on multiple independent subclones, as described previously (Spira et al., 1992). Confirming the previous data (Spira et al., 1994; Lin et al., 1996), subclones of the parental 36-65 hybridoma (36-65.L t=0, Fig. 1) switched from IgG₁ to IgG_{2b} at a median frequency of 10^{-5} and to IgG_{2a} at 4×10^{-6} . Two fresh subclones (L25 and L27) that were grown for 2 months retained their frequencies of switching (Fig. 1, 2m).

Since AID is required for a high rate of CSR *in vivo* (Muramatsu et al., 1999) and for the switching of reporter substrates in cultured cells (Okazaki et al., 2002; Yoshikawa et al., 2002), we wanted to know if expression of AID in hybridomas could increase the frequency of switching in tissue culture. L25 and L27 clones were stably transfected with a vector expressing human AID (hAID) (pCEP4-AID), as well as with the empty vector (pCEP4), as previously described (Martin et al., 2002a). RT-PCR was performed to screen for expression of hAID in the transfectants (data not shown) and only those subclones expressing hAID mRNA were used in subsequent experiments. We performed western blot analysis with anti-AID antibody on the total cell extract, but AID protein was not detectable in the L25 and L27

hybridomas (Fig. 2). We were unable to detect significant amounts of endogenous mouse AID mRNA in untransfected L25 and L27 by RT-PCR (data not shown). After transfection with hAID the levels of AID protein were roughly similar to those in mouse spleen B cells stimulated with LPS and IL-4 for 4 days (Fig. 2).

10–20 independent AID transfectants were assayed for their frequency of switching to $\gamma 2b$ (L25A and L27A, Fig. 3a) and $\gamma 2a$ (Fig. 3b). Another ~10–20 independent subclones obtained from the transfection of each of the subclones with the empty vector were used as control (L25C and L27C in Figs. 3a and 3b). The median frequency of switching of each set of these independent transfectants is shown below their scatter plot. For L25, transfection with AID (L25A) increased the median frequency of switching to IgG_{2b} ~13 fold, compared to L25 transfected with empty vector (L25C). For L27 AID increased the frequency of switching to IgG_{2b} by ~10 fold, compared to L27C (Fig. 3a). The median frequency of switching to IgG_{2a} increased 7 fold in the variants transfected with AID, compared to the same hybridomas transfected with empty vector (Fig. 3b). However, amongst the AID transfected clones, there were occasional jackpots (Luria et al., 1943), where a switch event must have occurred early after transfection (see clone 1 in L25A and clone 8 in L27A) and the frequency of switched cells was 10–100 times higher than in the parent cell line, greatly facilitating the recovery of switch variants from such clones.

To examine whether AID would increase switching in other hybridomas, we stably transfected hAID in the Sp6 hybridoma, which produces an IgM antibody specific for the hapten 2,4,6-trinitrophenyl (Ronai et al., 1999). Forced expression of AID induced switching to all the IgG subtypes and increased the median frequency of switching by 6, 17, 40, and 10-fold, to IgG₃, IgG₁, IgG_{2b} and IgG_{2a}, respectively (Fig 4). In addition to this modest increase in switching, there were occasional AID transfected jackpot subclones that switched to each of the IgG subtypes at ~100 times higher frequencies, as was found with 36-65.

3.2 AID induces V region mutations in hybridomas and antigen binding is retained in the isotype switched variants

Because expression of AID induces SHM in hybridomas (Martin et al., 2002a), we sequenced the heavy chain V regions of AID transfected 36-65 subclones carried in culture for two months. In control untransfected 36-65 hybridomas after 2 months in culture, the frequency of mutation was $\sim 1 \times 10^{-4}$ (Table 1), which is slightly higher than the reported error for the *Pfu* polymerase (1.3×10^{-6} mutation frequency/bp/duplication or 4.5×10^{-5} for 35 cycles of amplification) (Cline et al., 1996) and in L27 comes from only one mutation. In two of the AID overexpressing 36-65 subclones (L25A1 and L27A8) the frequency of mutation was much higher (1.7×10^{-3} and 1.6×10^{-3} , respectively (Table 1). These V region mutation levels were comparable to those reported earlier in other hybridomas (Martin et al., 2002a). Most of the mutations were transitions and in hotspots and all were in G:C base pairs (Table 1). In most cultured B cells approximately 80% of the mutations are in G:C base pairs (Martin et al., 2002a), which is higher than is seen *in vivo* in humans or mice (Martin et al., 2003; Li et al., 2004). However, it is unusual to see no mutations in A:T base pairs (Green et al., 1998), although we have seen this in another hybridoma (Martin et al., 2002a). In these AID transfected 36-65 hybridomas one mutation was found to create a premature stop codon, 15 out of 50 mutations (30%) were silent, whereas 34 (68%) caused an amino acid replacement.

Since some of the somatic mutations in the V region result in amino acid replacements, it is possible that such hybridomas could also have changes in antigen binding. Although the frequencies of these two events makes it unlikely that CSR and a mutation that would affect antigen binding could occur in the same clone (see discussion), we isolated ten hybridomas producing IgG_{2a} antibodies from subclone L27A8 (see Fig. 3b) by sib selection and ESA. We sequenced the heavy and light-chain variable regions and found that additional mutations had

not accumulated, which was surprising. However, since AID is able to mutate itself, as previously reported (Martin et al., 2002b; Ronai et al., 2005), we sequenced the AID gene in these class switch variants and found that they all had the same premature stop codon (Y13*) that led to a loss of the AID enzymatic activity.

To determine whether the V-region mutations that had occurred affected binding to antigen (Ars), these sib selected IgG2a switching variants were examined by ELISA with plates coated with Ars coupled to BSA (Fig. 5). We compared the binding of each monoclonal antibody to the parental 36-65 and L27A8 antibodies, to unrelated antibodies of the corresponding isotype and to 36-71, that utilizes the same 36-65 germ-line V region gene but produces a 200 fold higher affinity antibody to Ars (Rothstein et al., 1983). ELISA analysis revealed that the γ 2a switch variants had similar binding affinities to Ars as the parental clones (Fig. 5).

4. Discussion

Since monoclonal antibodies are used both to study how antibodies work *in vitro* and *in vivo* in model systems and for the treatment of diseases, it is important to be able to generate families of monoclonals expressing the same V regions and different isotypes. It would be most convenient to do this by switching isotypes *in vitro*. However, CSR in hybridomas occurs at a very low frequencies in culture. We have attempted in the past to increase the frequency of switching of a number of hybridomas by stimulating with mitogens and cytokines with no success (data not shown). It is possible that the necessary receptors are not expressed or that the signal transduction pathways are not properly wired to activate the expression of all of the molecules and target all of the many processes required for CSR in these highly differentiated cells.

We examined whether forced expression of AID could raise the frequency of switching in hybridoma 36-65. We found that expression of AID increased the frequency of switching in hybridomas 10–40 fold, although CSR did not reach the levels of 10–40% that occurs in primary spleen B cells that are stimulated *in vitro*. This might be due to the lack of germline transcripts (Lin et al., 1996) or because something aside from AID still missing in hybridomas. CSR not only requires AID, but also base excision repair, mismatch repair, AID co-factors and the enzymes involved in non-homologous end joining (Chaudhuri et al., 2004). Forced expression of AID also increased the frequency of switching in another hybridoma, Sp6. Without this increase, the isolation of an isotype switched hybridoma is very labor intensive and normally takes many months to a year, due to the need for many rounds of enrichment (Spira et al., 1992; Lin et al., 1996). One of the major benefits of AID transfection is that in some of the AID transfected subclones, a switch event occurred early in their propagation and that lead to the accumulation of many switched cells as the clone propagated (Luria et al., 1943). The accumulation of ~100 times more switched cells in these jackpot clones (see L25A in Fig. 3 and Sp6A in Fig. 4) greatly facilitated the subsequent isolation of hybridomas expressing the new isotype.

We also examined whether the increases in CSR were associated with SHM. SHM could introduce disabling mutations and be detrimental or increase the affinity of antigen binding, and thus be useful. We found the accumulation of V region mutations after ectopic AID expression (Table 1), as reported previously (Martin et al., 2002a), but the mutations that occurred did not cause a loss of antigen binding (Fig. 5). AID induced V region mutation occurs at a frequency of $\sim 10^{-4}$ and switching at a maximum frequency of $\sim 5 \times 10^{-3}$ in these hybridomas, so it is unlikely that a V region mutation and isotype switching would occur in the same cell at any given time. Nevertheless, we isolated ten hybridomas producing IgG_{2a} antibodies from the jackpot subclone L27A8 (see Fig. 3b) by sib selection and ESA. Additional mutations were not found when the heavy and light-variable regions were sequenced, due to

a nonsense mutation in the AID. While mutations might accumulate with time, AID will eventually get inactivated for mutating itself so the likelihood of a disabling mutation in the V region of switch variants is low. Nevertheless, this low potential for non desired mutations can be prevented by removing the AID gene from switched cells for example by using a Cre-lox strategy (Feng et al., 1999).

Taken together, these studies suggest the following strategy for obtaining individual or families of class switched monoclonal antibodies from hybridomas. Hybridomas making monoclonal antibodies with the optimal binding characteristics should: i) be transfected with AID; ii) subclones should be screened for AID expression by RT-PCR; iii) a few high AID expressing subclones should be examined for the presence of class switch variants using ESA, coating the plates with antibodies to the different classes or subclasses; iv) once a high switching AID expressing clone is been identified, switch variants should be isolated by sib selection or limiting dilution cloning with continuous monitoring of switching to the desired isotype by ESA; v) since the forced expression of AID also results in V region mutation, antigen binding should be checked.

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Abbreviations

AID	activation-induced cytidine deaminase
Ars	p-azophenylarsonate
CSR	class switch recombination
ESA	ELISA spot assay
Hs	hotspots
Ig	immunoglobulin
SHM	somatic hypermutation
Ts	transition mutations

References

- Bransteitter R, Pham P, Scharff MD, Goodman MF. Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase. *Proc Natl Acad Sci U S A* 2003;100:4102. [PubMed: 12651944]
- Casadevall A, Dadachova E, Pirofski LA. Passive antibody therapy for infectious diseases. *Nat Rev Microbiol* 2004;2:695. [PubMed: 15372080]
- Chaudhuri J, Alt FW. Class-switch recombination: interplay of transcription, DNA deamination and DNA repair. *Nat Rev Immunol* 2004;4:541. [PubMed: 15229473]
- Cline J, Braman JC, Hogrefe HH. PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res* 1996;24:3546. [PubMed: 8836181]
- Dangl JL, Herzenberg LA. Selection of hybridomas and hybridoma variants using the fluorescence activated cell sorter. *J Immunol Methods* 1982;52:1. [PubMed: 6811662]
- Di Noia J, Neuberger MS. Altering the pathway of immunoglobulin hypermutation by inhibiting uracil-DNA glycosylase. *Nature* 2002;419:43. [PubMed: 12214226]
- Feng YQ, Seibler J, Alami R, Eisen A, Westerman KA, Leboulch P, Fiering S, Bouhassira EE. Site-specific chromosomal integration in mammalian cells: highly efficient CRE recombinase-mediated cassette exchange. *J Mol Biol* 1999;292:779. [PubMed: 10525404]
- Green NS, Lin MM, Scharff MD. Ig hypermutation in cultured cells. *Immunol Rev* 1998;162:77. [PubMed: 9602354]
- Greene G, Hodous J, Dintzis RZ, Dintzis HM. Modification, optimization and simplification of the spot ELISA technique for the enumeration of cells secreting anti-hapten antibodies. *J Immunol Methods* 1990;129:187. [PubMed: 2191043]
- Kinoshita K, Honjo T. Linking class-switch recombination with somatic hypermutation. *Nat Rev Mol Cell Biol* 2001;2:493. [PubMed: 11433363]
- Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975;256:495. [PubMed: 1172191]
- Li Z, Scherer SJ, Ronai D, Iglesias-Ussel MD, Peled JU, Bardwell PD, Zhuang M, Lee K, Martin A, Edelman W, et al. Examination of Msh6- and Msh3-deficient mice in class switching reveals overlapping and distinct roles of MutS homologues in antibody diversification. *J Exp Med* 2004;200:47. [PubMed: 15238604]
- Lin M, Spira G, Scharff MD. Molecular comparison of cultured hybridoma cells that switch isotypes at high and low rates. *Somat Cell Mol Genet* 1996;22:329. [PubMed: 9000175]
- Luria SE, Delbruck M. Mutation of bacteria from virus sensitivity to virus resistance. *Genetics* 1943;28:491. [PubMed: 17247100]
- Manis JP, Tian M, Alt FW. Mechanism and control of class-switch recombination. *Trends Immunol* 2002;23:31. [PubMed: 11801452]
- Martin A, Bardwell PD, Woo CJ, Fan M, Shulman MJ, Scharff MD. Activation-induced cytidine deaminase turns on somatic hypermutation in hybridomas. *Nature* 2002a;415:802. [PubMed: 11823785]
- Martin A, Li Z, Lin D, Bardwell PD, Iglesias-Ussel MD, Edelman W, Scharff MD. Msh2 ATPase Activity is Essential for Somatic Hypermutation at A-T Basepairs and for Efficient Class Switch Recombination. *J Exp Med* 2003;198:1171. [PubMed: 14568978]
- Martin A, Scharff MD. Somatic hypermutation of the AID transgene in B and non-B cells. *Proc Natl Acad Sci U S A* 2002b;99:12304. [PubMed: 12202747]
- Muller CE, Rajewsky K. Isolation of immunoglobulin class switch variants from hybridoma lines secreting anti-idiotypic antibodies by sequential sublining. *J Immunol* 1983;131:877. [PubMed: 6190943]
- Muramatsu M, Sankaranand VS, Anant S, Sugai M, Kinoshita K, Davidson NO, Honjo T. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem* 1999;274:18470. [PubMed: 10373455]
- Nisonof, A. *Methods in Immunology and Immunochemistry*. Williams, CA.; Chase, AW., editors. 1967. p. 120

- Okazaki IM, Kinoshita K, Muramatsu M, Yoshikawa K, Honjo T. The AID enzyme induces class switch recombination in fibroblasts. *Nature* 2002;416:340. [PubMed: 11875397]
- Quartier P, Bustamante J, Sanal O, Plebani A, Debre M, Deville A, Litzman J, Levy J, Fermand JP, Lane P, et al. Clinical, immunologic and genetic analysis of 29 patients with autosomal recessive hyper-IgM syndrome due to Activation-Induced Cytidine Deaminase deficiency. *Clin Immunol* 2004;110:22. [PubMed: 14962793]
- Rajewsky K. Clonal selection and learning in the antibody system. *Nature* 1996;381:751. [PubMed: 8657279]
- Ronai D, Berru M, Shulman MJ. Variegated expression of the endogenous immunoglobulin heavy-chain gene in the absence of the intronic locus control region. *Mol Cell Biol* 1999;19:7031. [PubMed: 10490640]
- Ronai D, Iglesias-Ussel MD, Fan M, Shulman MJ, Scharff MD. Complex regulation of somatic hypermutation by cis-acting sequences in the endogenous IgH gene in hybridoma cells. *Proc Natl Acad Sci U S A* 2005;102:11829. [PubMed: 16087866]
- Rothstein TL, Geffer ML. Affinity analysis of idiotype-positive and idiotype-negative Ars- binding hybridoma proteins and Ars-immune sera. *Mol Immunol* 1983;20:161. [PubMed: 6843553]
- Spira G, Bargellesi A, Teillaud JL, Scharff MD. The identification of monoclonal class switch variants by sib selection and an ELISA assay. *J Immunol Methods* 1984;74:307. [PubMed: 6438240]
- Spira G, Gregor P, Aguila HL, Scharff MD. Clonal variants of hybridoma cells that switch isotype at a high frequency. *Proc Natl Acad Sci U S A* 1994;91:3423. [PubMed: 8159763]
- Spira G, Scharff MD. Identification of rare immunoglobulin switch variants using the ELISA spot assay. *J Immunol Meth* 1992;148:121.
- Stacy K. Therapeutic MAbs: Saving Lives and Making Billions. *The Scientist* 2005;19:17.
- Stavnezer J. Molecular processes that regulate class switching. *Curr Top Microbiol Immunol* 2000;245:127. [PubMed: 10533321]
- Yoshikawa K, Okazaki IM, Eto T, Kinoshita K, Muramatsu M, Nagaoka H, Honjo T. AID enzyme-induced hypermutation in an actively transcribed gene in fibroblasts. *Science* 2002;296:2033. [PubMed: 12065838]
- Zhang W, Bardwell PD, Woo CJ, Poltoratsky V, Scharff MD, Martin A. Clonal instability of V region hypermutation in the Ramos Burkitt's lymphoma cell line. *Int Immunol* 2001;13:1175. [PubMed: 11526098]

Figure 1.

Frequency of switching was calculated by ELISA spot assay in fresh 36-65.L subclones (t=0). Two subclones (L25, L27) were grown for 2 months (2m) in culture. Each circle represents the frequency of switching from IgG₁ to IgG_{2b} (open circles) or IgG_{2a} (shaded circles) of a subclone. Black circles refer to subclones where no spots were obtained when 0.5×10^6 cells were plated. Median frequencies of switching are represented by horizontal bars and values are shown at the bottom of each column. Subclone L27 was sequenced after 2 months in culture and the result is shown in Table 1.

Figure 2.

AID protein levels in 36-65 hybridomas before (L25, L27) and after AID transfection (L25A1, L27A8) were compared to those in naïve mouse spleen cells stimulated with LPS and IL-4 for 4 days. Western blot: AID signal was normalized to beta actin and resulting values are presented under each lane.

Figure 3.

Forced expression of AID induces CSR in 36-65.L hybridomas. Two fresh subclones (L25, L27) were stably transfected with the hAID construct (A) or empty vector control (C). Frequency of class switch recombination from IgG₁ to IgG_{2b} (a) and IgG_{2a} (b) was determined by ELISA spot assay. Median frequencies of switching are represented by horizontal bars and values are shown on the bottom of each column. Only hAID expressing clones were plotted. Data from the parent clone is labeled t=0 and come from Fig. 1. Subclones L25A1 and L27A8 were used to obtain the V region sequences shown in Table 1.

Figure 4.

Forced expression of AID induces CSR in Sp6 hybridomas. Sp6 hybridomas were stably transfected with the hAID (A) or empty vector (C). The frequency of class switch recombination from IgM to the full set of IgG subclasses was determined by ELISA spot assay. Median frequencies of switching are represented by horizontal bars and values are shown on the bottom of each column. Only hAID expressing clones were plotted. Shaded circles refer to subclones where no spots were obtained when $0.5-1 \times 10^6$ cells were plated.

Figure 5.

Antigen-binding analysis by ELISA for the L27A8 γ 2a switching variants obtained by sib selection. Control hybridomas like parental untransfected (L27) and AID transfected (L27A8), 36-65 (low affinity), 36-71 (that utilizes the same 36-65 germ-line V region gene but produces a 200 fold higher affinity antibody to Ars), as well as unrelated controls of each isotype (MOPC21 for IgG1 and RPC5 for IgG2a) were used. Antibody concentration in the supernatant was determined using a control antibody of the corresponding isotype. Plates were coated with 5 μ g/ml of Ars-BSA and developed with alkaline phosphatase-labeled anti mouse IgG1 or IgG2a antibody. Each point is the average of triplicate wells.

Table 1SHM in V₃₆₋₆₅ region in hybridomas^a

	Control	AID transfected (A)	
	L27	L25A1	L27A8
Mutated sequences/total sequences (%)	1/24 (4.2%)	12/26 (46.1%)	12/23 (52.2%)
Mutation frequency^b	1.1×10 ⁻⁴	1.7×10 ⁻³	1.6×10 ⁻³
G:C mutations/total mutations	1/1 (100%)	16/16 (100%)	14/14 (100%)
Ts^c/total mutations	1/1 (100%)	13/16 (81.2%)	10/14 (71.4%)
Hs^d/total mutations	1/1 (100%)	13/16 (81.2%)	9/14 (64.3%)

^aHybridomas transfected with AID were grown for 2 months. Heavy-chain V regions were amplified with Pfu polymerase, cloned in pCR blunt TOPO and sequenced. The region sequenced corresponds to 36-65 and is 363 bp long. The consensus sequence is available from Genbank accession no. M19292. Duplicated mutations were scored only once. V regions that shared the same mutation were scored as only one mutated sequence.

^bFrequency corresponds to mutations per base pair sequenced.

^cTs: transition mutations

^dHs: hotspots (mutations at G or C nucleotides within RGYW and WRCY).