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Reduced Switching in SCID B Cells Is Associated with Altered Somatic Mutation of Recombined S Regions

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Deoxyribonucleic acid double-stranded breaks act as intermediates in Ig V(D)J recombination and probably perform a similar function in class switch recombination between IgH C genes. In SCID mice, V(D)J recombination is blocked because the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) protein is defective. We show in this study that switching to all isotypes examined was detectable when the SCID mutation was introduced into anti-hen egg lysozyme transgenic B cells capable of undergoing class switch recombination, but switching was significantly reduced in comparison with control B cells of the same specificity lacking the RAG1 gene. Thus, DNA-PKcs is involved in switching to all isotypes, but plays a lesser role in the switching process than it does in V(D)J-coding joint formation. The higher level of switching observed by us in SCID B cells compared with that observed by others in DNA-PKcs null cells raises the possibility that kinase-deficient DNA-PKcs can function in switching. Point mutation of G:C base pairs with cytidines on the sense strand was greatly reduced in recombined switch regions from SCID cells compared with control RAG1-/- B cells. The preferential loss of sense strand cytidine mutations from hybrid S regions in SCID cells suggests the possibility that nicks might form in S regions of activated B cells on the template strand independently of activation-induced cytidine deaminase and are converted to double-strand breaks when activation-induced cytidine deaminase deaminates the non-template strand. The Journal of Immunology, 2003, 171: 6556–6564.

The ability of Ab-mediated immune responses to deal appropriately with a vast repertoire of Ags with a high degree of specificity depends on three Ig gene mutation events. V(D)J recombination produces the primary repertoire, while somatic mutation and isotype (or class) switching reshape it (1). Isotype switching involves recombination between Ig switch (S) regions to form a new hybrid S region, which requires the deletion of kilobase segments of DNA, and is associated with S region point mutation. S regions are ~1–10 kb in length and G:C rich, contain many semipalindromic and partially conserved repeats from 25 to 80 bp in length, and are located upstream of each H chain C region gene. Ig somatic mutation and class switching are mechanistically related because they both depend absolutely on the cytidine deaminase encoded by the activation-induced cytidine deaminase (AID) gene (2). AID initiates mutation by directly deaminating cytidine bases (C), causing transitions to uracil (U). Replication of uracils causes G to A transitions in the first round and C to T transitions in the second round (3). In addition, repair mediated by uracil-DNA glycosylases (mainly UNG) creates abasic sites, which lead to transitions and transversions at G:C base pairs. Deamination by AID also appears to stimulate mismatch repair using error-prone DNA polymerases, which introduce flanking mutations preferentially targeted to A/T base pairs (4–8). In the case of switching, direct deamination of S region DNA by AID and subsequent base excision by UNG are clearly involved (5), but it is not yet apparent exactly how this leads to recombination rather than merely point mutation.

One possible difference between switching and somatic mutation is the role of DNA double-strand breaks (DSBs). DSBs have been detected in the V regions of mutating cells, but only in S and G2 phases of the cell cycle, so they may represent the stalling of replication forks at single-strand nicks. In contrast, DSBs are detectable in S regions of switching cells in G1 phase (L.O., A.J.L.C., and C.J.J., manuscript in preparation) (9–11). The cause-effect relationship between DSBs and AID activity is not clear (11–13), but the circularization of DNA excised by switching (14–16) strongly implicates the involvement of DSBs without proving the case.

Repair of DSBs in eukaryotic cells occurs by homology-directed repair or by nonhomologous end joining (NHEJ) (for reviews, see Refs. 17 and 18). NHEJ joins dsDNA ends by direct abutment (e.g., V(D)J recombination signal joints), or by very small (typically 1 to 3 bp) sequence overlaps (e.g., V(D)J coding joints), thus rendering the process error prone. The NHEJ pathway is initiated by the binding of Ku70/Ku80 as a heterodimer (hereafter referred to as Ku) to broken DNA ends. Analysis of the role of NHEJ in switching is complicated by the fact that NHEJ is essential for V(D)J recombination, but the block in B cell development in NHEJ-deficient mice can be overcome by the introduction of homologously integrated Ig transgenes encoding rearranged Ab sequences. Mature B cells rescued in this manner in Ku70-/- and Ku80-/- mice failed to undergo any detectable switching when stimulated in vivo or in vitro, implying the complete dependence of switching to all isotypes on NHEJ (19–21).
In vertebrates (but not yeast), NHEJ involves the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) in addition to Ku. DNA-PKcs is an extremely large (~470-kDa) serine kinase that is spontaneously mutated in SCID mice (22). Recruitment of DNA-PKcs to DNA-bound Ku leads to the formation of the DNA-PK holoenzyme and induces the kinase activity of DNA-PKcs (23). DNA-PKcs increases both the rate and fidelity of NHEJ (24, 25), possibly by increasing the recruitment of XRCC4 and DNA ligase IV to the broken DNA ends (26), although its role in NHEJ is certainly not limited to this function (23). Alt and colleagues (27) have reported that DNA-PKcs is absolutely required for switching to all isotypes other than IgG1, which surprisingly was still readily detectable. In contrast, Bosma et al. (28) found that switching to all isotypes was affected only a little, or not at all, in DNA-PKcs mutant (SCID) B cells. A confounding factor in the experiments of both Manis et al. and Bosma et al. (27, 28) was that B cells from V(D)J recombination-proficient Ig-transgenic mice were used as controls. Extensive V gene replacement in both the H and L chain loci of the control mice would almost certainly have occurred, producing polyclonal populations of recirculating B cells as a result of receptor editing. Such editing cannot occur in DNA-PKcs−/− mice, which would have thus maintained monoclonal B cell populations with a specificity conferred by the Ig transgenes used. Thus, the test cells expressing monospecific B cell receptors (BCRs) were compared with polyclonal control populations, which may have obscured the influence of DNA-PKcs deficiency on switching. The need to take the specificity of the BCR into account is illustrated by the fact that it greatly alters the kinetics of both B cell proliferation and switching, independently of exposure to cognate Ag (29). Thus, the exact role of DNA-PKcs in switching remains uncertain.

To examine the role of the SCID mutation in switching in a more definitive way, we elected to use RAG1−/− B cells as controls because they expressed a monoclonal BCR specificity identical with that of the DNA-PKcs-deficient test B cells. Thus, the B cell compartments of both SCID and RAG1−/− mice were rescued by crossing them with Ig transgenic (SW HEL) mice carrying B cells specific for hen egg lysozyme (HEL) (29). As expected, the Ig constructs used to create these mice did not undergo any receptor editing in SCID or RAG1−/− mice. When cells expressing identical BCRs were tested, DNA-PKcs was shown to be involved in the mechanism of switching to all isotypes, and conversely, the SCID mutation did not completely block switching to any isotype. Strikingly, mutation in recombined S regions of base pairs with A, G, or T bases on the sense strand was unaffected by the SCID mutation, whereas mutation of base pairs with cytidines on the sense strand was reduced 7-fold. In addition, the frequency of junction-proximal mutations was reduced, and the degree of identity between S region donor and acceptor DNA ends was slightly increased, relative to control RAG1−/− cells. This suggests that the processing of DNA ends involved in class switching is altered by the SCID defect.

Materials and Methods

Mice

All mice were maintained on a pure C57BL/6 background at the Centenary Institute Animal Facility. SCID (Prkdc<sup>−<sup>−</sup></sup>) and RAG1<sup>−<sup>−</sup></sup> mice on a C57BL/6 background were kindly provided by B. Fazekas (Centenary Institute, Newtown, Australia) (30). The production of SW<sub>HEL</sub> mice has been described elsewhere (29). SW<sub>HEL</sub> mice carry rearranged IgH and Igk transgenes cloned from the HyHEL10 hybridoma specific for HEL. The IgH rearrangement (V<sub>H</sub>L<sub>10</sub>) was targeted to replace the most 3’ D element and all of the J elements present in C57BL/6 embryonic stem cells, allowing the transgene to undergo efficient switch recombination to all Ig isotypes (29). The Igk transgene (LC2) was randomly integrated into the genome in low copy number. Crossing of V<sub>H</sub>L<sub>10</sub> and LC2 transgenic mice produced SW<sub>HEL</sub> mice carrying a large population of B cells specific for HEL. All SW<sub>HEL</sub> mice used in our experiments were heterogeneous for V<sub>H</sub>L<sub>10</sub> and either heterozygous or homozygous for LC2.

Abs and other reagents

The following mAbs to murine Ags were all purchased from BD PharMingen (San Diego, CA): anti-CD40 (1B441), anti-γδ T cell (R6-60.2), anti-IgG1 biotin (A85-1), anti-IgG2a biotin (R19-15), anti-IgG2b biotin (R12-3), anti-IgG3 biotin (R40-82), anti-IgE biotin (R35-118), and anti-IgA biotin (C10-1). Streptavidin-PerCP and annexin V Cy5 were also purchased from BD PharMingen. Anti-mouse IgM PE (1B441), anti-mouse IgD FITC (11–26), and biotin-conjugated goat polyclonal Abs to mouse IgG1, IgG2a, IgG2b, IgG3, and IgA were purchased from Southern Biotechnology Associates (Birmingham, AL); PE- and alkaline phosphocytocin-conjugated anti-mouse B220 (CD45R) (RA3-6B2) and anti-CD4 PE (CT-CD4) were purchased from Caltag Laboratories (Burlingame, CA). Anti-mouse CD80 (16-10-H1 (31)) and anti-mouse CD38 (NIMR-R5 (32)) mAbs were used as hybridoma supernatants. HEL was purchased from Sigma-Aldrich (St. Louis, MO) and conjugated to FITC, as previously described (33). Mouse IFN-γ was purchased from Genzyme (Cambridge, MA), and human TGF-β1 from Boehringer Mannheim (Mannheim, Germany). Cell membranes expressing murine CD40L (a generous gift of A. Grech, Centenary Institute, Sydney) or mouse IL-4 was kindly provided by P. Hodgkin (Walter and Eliza Hall Institute, Melbourne, Australia). Salmonella typhosa LPS was purchased from Sigma-Aldrich.

Serum ELISAs

Anti-HEL Ab in serum samples was detected by direct ELISA, essentially as described (33). Briefly, 96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with HEL at 10 μg/ml, following which wells were blocked with 100 μl of 1% BSA/PBS and serial dilutions of sera were added. Biotinylated isotype-specific mAbs in 0.1% BSA/1% skim milk powder/PBS were used to detect bound Ab. Streptavidin-alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) was then added and visualized with the substrate p-nitrophenyl phosphate (ICN BioMedicals, Aurora, OH). Absorbance at 405 nm was read.

Isolation and culture of B cells

B cells were purified from RBC-depleted single-cell suspensions of splenocytes using anti-CD19-conjugated MACS beads (Miltenyi Biotec, Gladbach, Germany). If cells were to be labeled with CFSE (Molecular Probes, Eugene, OR), it was done as originally described by Lyons and Parish (35). Cells were cultured with various combinations of cytokines and mitogens in 48- or 24-well flat-bottom plates (Falcon; BD Biosciences, San Jose, CA) at a starting density of 2 × 10<sup>5</sup> to 10<sup>6</sup> cells/ml and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 100% relative humidity. RPMI 1640 culture medium supplemented with 2 mM l-glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES (pH 7.4), 100 μg/ml streptomycin, 100 U/ml penicillin, 5 × 10<sup>−3</sup> M 2-ME (all from Sigma-Aldrich), 1 mM sodium pyruvate, and 10% heat-inactivated FCS (both from Life Technologies). The concentrations of mitogens and cytokines added at the start of culture were: LPS (10 μg/ml), CD40L (1 μg/ml), anti-CD40 (5 μg/ml), IL-4 (500 or 100 U/ml), anti-CD38 supernatant (1:20), and anti-CD80 supernatant (1:20). IFN-γ (500 ng/ml) and TGF-β1 (1 ng/ml) were added to cultures after 24–36 h.

Flow cytometry

For phenotyping, PBLs or purified B cells were incubated with allophycocyanin-, PE-, and FITC-conjugated Abs or FITC-conjugated HEL in PBS containing 0.2% BSA and 0.02% NaN<sub>3</sub>, BSA) for 30 min on ice, then washed. Propidium iodide (PI) was added immediately before flow cytometry to enable gating out of dead cells. To enumerate cells present at the end of culture periods, standard numbers of Calibrite beads (BD Biosciences) were added to culture wells immediately before harvest. Cells and beads were then collected into 1-ml tubes in arrays of 96 (Bio-Rad, Hercules, CA); PI was added to 1:100 to label dead cells; and flow cytometry was performed immediately. For switched isotype staining, cells were washed with annexin V buffer (0.1 M HEPES, 0.14 M NaCl, 2.5 mM CaCl<sub>2</sub>, pH 7.4), then stained with annexin V Cy5 (1:100 in same) for 15 min at room temperature. This step labeled dead and apoptotic cells. After washing with annexin V buffer, cells were fixed in 0.1 ml 2% paraformaldehyde in PBS for 10 min at room temperature. They were then washed twice in PBS and incubated at room temperature in PBA + 1% saponin (Sigma-Aldrich) for...
40 min with HEL-FITC (if not previously labeled with CFSE), plus anti-IgM PE, and biotinylated goat anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, or anti-IgA, or monoclonal anti-IgE biotin. Biotinylated Ab binding was revealed by subsequent incubation with streptavidin PerCP. After each incubation, cells were washed twice with 0.5 ml of PBA. Four-color flow cytometry was performed on a dual-laser FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo version 4.2 software (Tree Star, Stanford, CA). Calibrate beads were counted in the appropriate forward and side scatter gate. Cell division number was determined from the CFSE peaks, and gates were drawn around each peak to allow division slicing (36).

PCR amplification and sequencing of hybrid S regions

DNA was extracted from cultured cells using Wizard genomic DNA purification kits (Promega, Madison, WI). To amplify hybrid S regions, aliquots of DNA were used to prime TripleMaster (Eppendorf, Westbury, NY) long-range hot start PCR consisting of 35 cycles of 93°C for 1 min, 65°C for 0.5 min, and 68°C for 10 min. All reactions contained a sense primer positioned 5’ to Sp6 (CTTCT AGAAT TCGCT AAAT GAGGT GATTA CTTCT AGG) and an antisense primer positioned 3’ to one of the other S regions (S5y, TGTTG ATGTC CCATG TACTC ATGC TTGTC; S5y, CTTTG TTAGG GTCCT CATAG AGTAG CCACG AGC; S5y, TCGAG ATCGG AGGTT GATCA CTGTA CA; S5y2, CCCAG AAGGT TGACA TCAGC AACTC CCATG TGTTCA TC; S5y2, AAAAG ACGAT GGTGG CCGCA ATCGG GACC). Primer sequences were based on GenBank accessions AC079180, AC079272, and mCG127237. Re-sequences were based on GenBank accessions AC079180, AC079272, and mCG127237. Reaction products were resolved on 0.8% agarose gels and blotted onto Hybond-N\(^{-}\) membranes (Amersham Biosciences, Little Chalfont, U.K.) by alkaline capillary transfer. Specific PCR products were detected by hybridization with a \(^{32}\)P-labeled oligonucleotide (GCCTA CACTG GACTG TGCTA CC; S for HEL). For the L chain, the hyHEL10 hybridoma-specific primer TTTCT was positioned immediately downstream of the S region (CGTCG TACGG GACCT CTCTG) positioned immediately downstream of the Sp6 PCR primer. For sequencing, hybrid S region PCR products were purified using High Pure PCR Product Purification kits (Roche Diagnostics) and digested with EcoRI plus BamHI (Sp6/y), EcoRI, plus KpnI (Sp6/y2b), or EcoRI plus NlaIV (S/y3). Digested PCR products were cloned into pBluescript KS\(^{-}\)-Strangene, La Jolla, CA), and inserts in plasmids isolated from individual colonies were sequenced by the Australian Genome Research Facility (Brisbane, Australia).

PCR amplification of mRNA transcripts

mRNA was purified from cultured cells using an RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed (Enhanced Avian RT-PCR kit; Sigma-Aldrich). Aliquots of reverse-transcription reactions (± reverse transcriptase) equivalent to 10 ng of mRNA were subjected to 30 cycles of PCR using either β-actin primers (ACG GCC TCA TCA CTA T and CTG CTT GCT GAT CCA CAT C) or β-globin primers (CAA CAA TGG CTG CTA CCA C and CTT ACA AGG GCC AAA GCA TGC).

Results

Production of isotype switching anti-HEL transgenic mice with DNA-PKcs and RAG1 deficiencies

SCID mice (Prkdc\(^{-}\)/SCID) carry a recessive spontaneous mutation that results in the loss of the last 83 aa of DNA-PKcs (22, 37, 38). This decreases the levels of DNA-PKcs protein in the nucleus to below the limit of detection, reduces cyttoplasmic levels ~10-fold, and removes all detectable DNA-dependent serine kinase activity (22). To allow analysis of switch recombination in mature B cells from mice in which V(D)J recombination was blocked by the SCID mutation, the B cell compartment was rescued using V(D)J-rearranged H and L chain Ig transgenes. Conventional IgH transgenes cannot undergo normal switching, necessitating the use of a transgene, V\(\text{H}10/\text{mar}\), previously described by Phan et al. (29), which was targeted to the endogenous IgH locus by means of homologous recombination in C57BL/6 embryonic stem cells. The V\(\text{H}10/\text{mar}\) transgene encodes the V\(\text{H}10/J\text{H}3\) rearrangement from the HyHEL10 hybridoma specific for HEL. For the L chain, the LC2 transgene was used (29), which carries a V\(\text{L}10/\text{J}k2\) rearrangement also cloned previously from the HyHEL10 hybridoma (33), and was randomly integrated into the C57BL/6 genome in low copy number. To create a functional B cell Ag receptor, double-transgenic (SW\(\text{HEL}\)) mice expressing both the targeted V\(\text{H}10/\text{mar}\) gene and the randomly integrated LC2 transgene were produced. SW\(\text{HEL}\) mice used in our experiments were always heterozygous for the targeted IgH transgene (V\(\text{H}10/\text{mar}\)\(^{+/+}\)) and either heterozygous or homozygous for the LC2 transgene. A prominent population of B cells in these mice is specific for HEL and can switch efficiently to all Ig isotypes while retaining the ability to bind HEL (29), thereby demonstrating that the V\(\text{H}10/\text{mar}\) transgene undergo normal switch recombination. The remaining B cells in SW\(\text{HEL}\) mice fail to bind HEL due to V gene replacement in the bone marrow (29).

The role of DNA-PKcs in switching was investigated by crossing SW\(\text{HEL}\) mice with C57BL/6 SCID (Prkdc\(^{-}\)/SCID) mice. Because neither the RAG1 nor RAG2 protein is involved in Ig switch recombination (39, 40), we also bred the SW\(\text{HEL}\) transgenes into congenic RAG1-deficient mice as controls. Given that both V(D)J recombinase of TCR and Ig genes and subsequent receptor editing of rearranged IgH genes depend on wild-type RAG1 and SCID proteins (41-43), SW\(\text{HEL}\)/SCID and SW\(\text{HEL}\)/RAG1\(^{−/−}\) mice lacked mature T cells, as expected. The B cells present in these lines expressed high levels of IgM and displayed uniform (96%) specificity for HEL (data not shown). This phenotype contrasted with that of wild-type SW\(\text{HEL}\) mice, in which only one-half of the B cells are HEL specific (as a consequence of V gene replacement; see Ref. 29), and which expressed variable levels of IgM (data not shown).

Anti-HEL-specific isotypes present in vivo vary between SW\(\text{HEL}\) mice on wild-type, RAG1\(^{−/−}\), and SCID backgrounds

As a prelude to screening for isotype switching, ELISAs were performed to detect spontaneous production of HEL-binding Abs in the sera of age- and sex-matched SW\(\text{HEL}\), SW\(\text{HEL}\)/SCID, and SW\(\text{HEL}\)/RAG1\(^{−/−}\) mice (Fig. 1). HEL-specific IgM was much more abundant in the serum of SW\(\text{HEL}\) mice bred on either the SCID or RAG1\(^{−/−}\) background than it was in wild-type SW\(\text{HEL}\) mice. Such a difference may have been due to the fact that the HyHEL10 Igd is poorly selected into the B1 compartment of V(D)J recombination-proficient (SW\(\text{HEL}\)) mice (29), and thus makes only a minor contribution to the total IgM Ab pool. In contrast, the HyHEL10 Igd was the only specificity available for natural Ab production in mice deficient for V(D)J recombination.

![FIGURE 1.](http://www.jimmunol.org/DownloadedFrom/) HEL-binding Abs present in the serum of SW\(\text{HEL}\) mice on wild-type ( ), RAG1\(^{−/−}\) ( ), and SCID ( ) backgrounds. Serum samples were incubated with HEL-coated ELISA plates at a dilution of 1/40 (1/200 for IgM), and bound Ab were detected using biotinylated isotype-specific mAbs and avidin-conjugated HRP. The mean absorbance of triplicates (± SEM) is shown.
Analysis of spontaneously secreted class-switched Abs revealed that HEL-binding IgG3 and IgA levels were elevated in sera from SW\textsubscript{HEL}RAG1\textsuperscript{−−} mice compared with SW\textsubscript{HEL} mice, while the levels of IgG1 and IgG2a were lower (Fig. 1). These differences showed clearly that the presence of T cells, probably in conjunction with the availability of non-HyHEL10 idiotypes in V(D)J recombination-proficient mice, led to marked alterations in isotype switching in vivo, and confirmed that SW\textsubscript{HEL}RAG1\textsuperscript{−−} mice were a more appropriate control for SW\textsubscript{HEL}SCID mice than were SW\textsubscript{HEL} mice proficient for the RAG proteins. The only switched isotypes readily detectable in SW\textsubscript{HEL}SCID mice were IgG3 and IgG2b (Fig. 1), a finding that differed from that of Manis et al. (27), who reported IgG1 (and IgM) to the exclusion of all other isotypes in Ig-knockin DNA-PKcs\textsuperscript{−−} mice. The low levels of IgG3 and IgG2b, and undetectable level of IgA, in SW\textsubscript{HEL}SCID mice compared with SW\textsubscript{HEL}RAG1\textsuperscript{−−} controls suggested that switching was greatly reduced in SCID B cells. However, the number of mature B cells present in SW\textsubscript{HEL}SCID mice (as reflected by their occurrence in the spleen) was always 2- to 3-fold lower than in SW\textsubscript{HEL}RAG1\textsuperscript{−−} mice (data not shown). The most likely explanation for this observation was death in the bone marrow of immature SW\textsubscript{HEL}SCID B cells, in which attempted V gene replacement had been unsuccessful, an event precluded in SW\textsubscript{HEL}RAG1\textsuperscript{−−} mice. The lower serum levels of switched isotypes in SW\textsubscript{HEL}SCID mice relative to SW\textsubscript{HEL}RAG1\textsuperscript{−−} mice could thus have been due to a reduction either in switching per se or B cell survival. To distinguish between these possibilities, the role of DNA-PKcs in switching was assessed in in vitro experiments in which switching, proliferation, and cell survival could be monitored following activation with the appropriate stimuli.

Switching to all isotypes (except IgA) occurred in in vitro stimulated SW\textsubscript{HEL}SCID B cells

To quantify isotype switching in vitro, B cells were purified from spleens of SW\textsubscript{HEL}SCID and SW\textsubscript{HEL}RAG1\textsuperscript{−−} mice using anti-CD19 MACS beads. Cells from both lines of mice when purified on anti-CD19 beads were 95–98% B220\textsuperscript{−−}, of which 96–99% bound HEL (data not shown). Equal numbers of purified cells were grown separately for 4–5 days in triplicate or quadruplicate cultures supplemented with the agonists appropriate to induce switching (Fig. 2). At the end of the culture period, cells were harvested, stained with annexin V (to improve exclusion of cells apoptotic or...
dead at the time of harvest), and were then stained for IgM and switched Ig isotypes. Fixing and permeabilization were used to ensure that all switched cells could be detected, regardless of surface Ig expression. A number of independent experiments were conducted on cells from different pairs of age-matched SW HEL/SCID and SW HEL/RAG1-/- mice, although switching to all isotypes was not assessed in every experiment. As shown by the data presented in Fig. 2A, in vitro switching to all isotypes, except IgA, was detected by FACS analysis of SW HEL/SCID B cells. The specificity of isotype detection was confirmed by staining cells from cultures in which the isotype of interest was likely to be absent or reduced (Fig. 2B). For instance, IgG1 was detected in B cells from cultures supplemented with CD40L + IL-4 (±IFN-γ), but not in cells from cultures supplemented with LPS or LPS + TGF-β.

We concluded from these experiments that B cells from DNA-PKcs-deficient mice can switch to most, if not all, isotypes, which was in contrast to the recent findings of Manis et al. (27), but in agreement with results published by another group while this manuscript was in preparation (28). The failure to detect in vitro switching to IgA in B cells with either RAG1-/- or SCID defects made it necessary to confirm that the culture system was capable of generating IgA-switched cells. Therefore, B cells from wild-type SW HEL mice were cultured with LPS + TGF-β for 5 days, then stained for HEL specificity in addition to IgM and IgA expression. As shown in Fig. 2C, switching to IgA could be induced under these culture conditions, but was only detectable in cells that had altered their BCR specificity by V gene replacement and thus no longer bound HEL. This result clearly underlined the need to exclude receptor-edited cells from experiments and to compare switching only in cells with identical BCRs. The reason for the failure of HEL-binding cells to undergo significant switching to IgA in response to LPS + TGF-β in vitro may have been due to the lack of these cells in the splenic B cell subset most amenable to IgA switching. For instance, the IgM<sup>high</sup>/IgD<sup>high</sup> subset of splenic B cells, which represents the most mature B cells, does not include anti-HEL B cells (see Ref. 29).

Confirmation of the occurrence of switching events in SCID B cells was sought by identifying recombination junctions between Sμ and downstream S regions. This involved PCR amplification of hybrid Sμ/γ1, Sμ/γ2b, Sμ/γ3, Sμ/γ2a, and Sμ/e junctions using DNA extracted from cultured B cells obtained from SW HEL/SCID vs SW HEL/RAG1-/- donors. Junctions confirming switching to each isotype were only detected in DNA samples when they had been extracted from cells cultured under the appropriate conditions (vide supra). For instance, Sμ/γ1 junctions were detected in cells cultured with CD40L + IL-4, but not in cells cultured with LPS or LPS + TGF-β (Fig. 3). Similarly, Sμ/γ2a junctions were detectable in cells cultured with CD40L + IFN-γ + IL-4 (Fig. 3), but not in cells cultured with either LPS (Fig. 3), or CD40L + IL-4 (data not shown). Hybrid S region PCR amplification products from both SCID and control cells ranged in size from ~300 bp to >8 kbp, with a majority lying between 1 and 4 kbp (Fig. 3). These sizes concur with the potential size range of hybrid S regions in mouse B cells. In agreement with the flow cytometric data, hybrid Sμ/δ junctions could not be consistently amplified from cultures of either SW HEL/RAG1-/- or SW HEL/SCID splenic B cells (data not shown).

Switching to all isotypes is significantly reduced by the SCID mutation

The FACS data presented in Fig. 2 not only indicated that switching to most isotypes occurred in SW HEL/SCID B cells, but suggested that when compared with the level in control SW HEL/RAG1-/- B cells it was always reduced. This reduction was reproducible in multiple independent experiments (Fig. 4), the dif-

![FIGURE 3. Recombination between Sμ and all downstream S regions (except Sκ) is detectable by PCR in DNA isolated from SW HEL/SCID B cells cultured under appropriate conditions. B cells from SW HEL/SCID mice (s) or from SW HEL/RAG1-/- mice (r) were harvested after 4-day culture. Genomic DNA was extracted, and aliquots were subjected to PCR amplification using a sense primer complementary to sequences 5′ to Sμ in combination with individual antisense primers complementary to sequences 3′ to each of the different S regions indicated. PCR conducted as negative controls are indicated by the letters shown in outline. Specific PCR products were detected by Southern blotting. Molecular weight markers (kb) are shown to the right of each panel.](image-url)
ference in switching being significant for IgG1, IgG3, IgG2b, and IgE (p < 0.005, 0.025, 0.05, and 0.005, respectively; paired Student’s t test).

Reduced switching in SCID B cells is independent of reduced B cell viability

Although switching at low levels does occur during the first cell division after activation, efficient switching to all isotypes requires at least three cell divisions in normal B cells (44–46). The reduction in switching observed in SWHEL/SCID B cells could therefore have been due to a decrease in proliferation. This possibility was tested by culturing SWHEL/SCID and SWHEL/RAG1−/− B cells under switching conditions (CD40L + IL-4) and nonswitching conditions (CD40L + anti-CD38 + anti-CD80). AID message was undetectable by RT-PCR amplification of mRNA extracted from cells cultured under the nonswitching conditions (Fig. 5). The number of viable SWHEL/SCID B cells remaining at the end of the culture period was always less than the number of viable SWHEL/RAG1−/− B cells (Fig. 5A), whereas the number of dead and apoptotic (annexin V-staining) cells was always greater (data not shown). Much of the decreased viability of SCID B cells was unrelated to switching because it occurred even in cultures in which proliferation was induced in the absence of switching (Fig. 5, A and C). The decreased viability of cultured SCID B cells raised the possibility that the SCID mutation did in fact reduce switching by reducing the number of cell divisions that B cells underwent in culture. However, CFSE labeling showed that SWHEL/SCID B cells in both switching (e.g., CD40L + IL-4) and nonswitching (CD40L + anti-CD80 + anti-CD86) cultures progressed through almost identical numbers of cell divisions as did control SWHEL/RAG1−/− cells (Fig. 5B). Thus, the SCID mutation caused more cells to die per cell division (see supplemental Fig. 1), but had little effect on the number of times that cells divided in culture.

When switching was analyzed in terms of division number, the SCID mutation was associated with reduced switching in all cell divisions (Fig. 5C). Taken together, the data showed that reduced switching in SCID B cells is not an artifact of reduced proliferation and that DNA-PKcs is likely to be involved mechanistically in switching.

Increased donor/acceptor microhomology in hybrid switch junctions from SCID B cells

To determine whether the SCID mutation qualitatively altered the mechanism by which switching occurred, hybrid S regions were PCR amplified from cultured B cells and cloned into plasmids, and individual recombination junctions were sequenced. PCR products were cloned with EcoRI (which cleaved in the 5′ Sμ primer) plus a restriction enzyme that cut the hybrid S regions at multiple sites in the 3′ S region (conferring by the repetitive nature of S regions). This strategy trimmed much of the 3′ S region sequence from the hybrid S region PCR products before cloning, but left the recombination junction intact and was devised to minimize preferential sequencing of small PCR products. The precise sites of sequenced recombination junctions were determined by BLAST (47) alignment with the National Center for Biotechnology Information and Celera C57BL/6 mouse genome databases. Sμ/γ1, Sμ/γ2h, Sμ/γ3, and Sμ/ε recombination junctions (and two Sγε recombination junctions) that were nonredundant (30 from SCID B cells and 28 from control B cells) are illustrated in supplemental Fig. 2. A small increase in base identity in the 8–9 bp centered around the switch recombination junctions was evident in the SCID cells (mean = 3.4) compared with the control cells (mean = 2.3), but this increase was much less than that previously observed in PMS2, MLH1, or ATM mutants (48–50), and was not significant if only junctions of the same isotype were analyzed.

**FIGURE 5.** Decreased switching in SWHEL/SCID B cells is independent of increased death. B cells from SWHEL/SCID and SWHEL/RAG1−/− mice were labeled with CFSE and cultured for 4 days with CD40L + IL-4 to induce proliferation and switching, or with CD40L + anti-CD38 + anti-CD80 to induce proliferation with negligible switching. Calibrite beads were added to the cultures immediately before harvesting the cells. A. Viable cell numbers. Calibrite bead counts were used to calculate the absolute number of PI-excluding cells present after 4-day culture. The ratio of SCID cell numbers to RAG1−/− cell numbers was then calculated, and the means of the ratio from four independent experiments (±SEM) are shown in the histograms. B. CFSE-fluorescence profiles after 4-day culture. The summed profiles of triplicate cultures are shown, and are typical of four experiments. The frequency distribution of cells across sequential divisions is illustrated, not the absolute cell numbers. C. The dependence of switching on cell division number. B cells from SWHEL/RAG1−/− (○) and SWHEL/SCID (□) mice were cultured with (−) CD40L + IL-4 or (−−) CD40L + anti-CD38 + anti-CD80. The fractions of cells in each division that had undergone switching (identified as IgM+) cells) are plotted (large symbols). Division cohorts were identified using CFSE fluorescence, as shown in B. Data are for triplicate cultures from one typical experiment. D. AID expression in cultured cells. Reverse transcription and PCR were used to detect AID and β-actin transcripts in mRNA extracted from C57BL/6 B cells cultured for 3 days under the conditions indicated.
**Table I. Mutations detected in hybrid S regions cloned from control (SWHEL/RAG-/-, 10,718 bases) and SWHEL/SCID (9,588 bases) B cells**

<table>
<thead>
<tr>
<th>Mutation from:</th>
<th>A</th>
<th>G</th>
<th>C</th>
<th>T</th>
<th>Total</th>
<th>Bases Mutated (%)</th>
<th>Transversions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>SCID</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>G Control</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>16</td>
<td>16</td>
<td>0.45</td>
<td>25</td>
</tr>
<tr>
<td>SCID</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>16</td>
<td>16</td>
<td>0.44</td>
<td>56</td>
</tr>
<tr>
<td>C Control</td>
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<td>4</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>1.26</td>
<td>65</td>
</tr>
<tr>
<td>SCID</td>
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<td>3</td>
<td>4</td>
<td>4</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>T Control</td>
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<td>4</td>
<td>0</td>
<td>4</td>
<td>4</td>
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<td></td>
</tr>
<tr>
<td>SCID</td>
<td>1</td>
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<td>3</td>
<td>4</td>
<td>4</td>
<td>0.18</td>
<td></td>
</tr>
</tbody>
</table>

*Mutations are shown in the context of the sense (non-template) DNA strand. The table lists mutations found in the complete data set of hybrid S region sequences (including, but not limited to, the junction regions shown in supplemental Fig. 2). However, only mutations in which the germline form could be positively identified by clone overlaps are included. Bold indicates transitions and underlining indicates transversions at G:C base pairs. Bases mutated = mutations in base N occurrence of base N in database × 100.*

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**Decreased mutation in hybrid switch junctions from SCID B cells**

Somatic mutations were detected in hybrid S regions (Table I) at a frequency above that predicted to occur by PCR error alone (~1 mutation/1000 bp). Like V region mutation, S region mutation is known to be AID dependent and probably occurs by a similar mechanism (11, 51), although the role of mismatch repair in S region mutation appears to be quite different. In Ig V regions, mismatch repair functions to increase mutation, probably because it introduces mutations (preferentially at A:T base pairs) while removing AID-induced G:U mismatches by short patch repair (5, 52–54), whereas in hybrid S regions mismatch repair instead appears to remove mutations generated by the recombinase process (55). The much stronger preference for mutation at A:T base pairs over A:T base pairs in hybrid S regions from control cells observed by us (Table I) and by Honjo and colleagues (51) is consistent with a minor role for mismatch repair in introducing S region mutations. The frequencies of mutation of base pairs in which A, G, or T was on the sense strand were very comparable between hybrid S regions from SCID and RAG1−/− control cells, whereas the frequency of mutation of base pairs in which C was on the sense strand was reduced 7-fold in SCID cells (Table I, after correction for the difference in total number of base pairs in the two databases $\chi^2 = 15.1, p = 0.002$). It seems unlikely that the reduced mutation of C in SCID cells shown in Table I is a PCR artifact, because the most common error generated by PCR is A:T to G:C transition (56), which is not overrepresented in the SCID database.

In addition to the skewed mutation profile, a striking lack of mutations immediately proximal to switch recombination junctions from SCID B cells was observed (mutations within 12 bp of the recombination junction in 30 SCID clones vs 13 mutations in 28 control RAG1−/− clones; see supplemental Fig. 2, $\chi^2 = 19.1, p = 0.00001$). This difference is highly unlikely to be a PCR artifact and suggests that the processing of DNA ends created during switching was quite different in SCID cells compared with control RAG1−/− cells. One explanation for the altered processing is that recruitment of DNA ligase IV by DNA-PKcs protects the DNA ends from nucleases (26), DNA ends are also more processed in SCID cells during VDJ-signal joint formation, and this increased processing is associated with an increase in donor-acceptor microhomology at the joints formed (25).

**Discussion**

The reduction in switching to all isotypes (excluding IgA) in SCID B cells observed by us contrasts both with the absence of switching to all isotypes, other than IgG1, reported for DNA-PKcs-knockout B cells (27), and with reduced switching to IgG3, IgG1, and IgE, but not IgG2b, IgA, and IgG2a reported for SCID B cells by Bosma et al. (28). The difference between our data and that of Bosma et al. (28) probably lies in the experimental controls used. In our case, V(D)J recombinase-deficient (SWHEL/RAG1−/−) cells served as controls to ensure uniformity in both BCR specificity and the maturation stage of the B cells being compared, whereas controls of Bosma et al. (28) consisted of V(D)J recombination-proficient B cells, which have the potential to undergo V gene replacement. The importance of BCR specificity is illustrated by reference to previous observations made in the SWHEL model. When B cells from V(D)J recombination-proficient (i.e., wild type at the RAG1 and DNA-PKcs loci) SWHEL mice were stimulated to switch in vitro, the proliferative response and switching kinetics of those cells capable of binding HEL were quite different from those in which HEL binding had been lost as a consequence of V gene replacement, a difference that occurred regardless of prior exposure to cognate (HEL) Ag (29). In other words, the nature of the BCR can greatly affect switching frequency independently of Ag binding. This is probably at least partly due to preferential partitioning of cells with different BCRs into different B cell subpopulations (see Ref. 29), which, in RAG- or DNA-PKcs-deficient mice, may be exacerbated by alterations in splenic architecture and thus in B cell maturation resulting from T cell deficiency. Because extensive V gene replacement almost certainly occurred in the control mice used by Bosma et al. (28), the level of switching in the control B cells was not directly comparable to that of the DNA-PKcs-deficient cells. Thus, the enhanced switching to IgG2b reported for SCID B cells by Bosma et al. (28) could have been due to subtle alterations in B cell maturation caused by the 3H9dsVve8sd BCR, whereas these variations were eliminated from our experiments by the choice of RAG1−/− mice as controls.

Receptor editing may also explain the differences between our data and that of Manis et al. (27), but much less plausibly. Thus, the lack of detectable switching to isotypes other than IgG1 in DNA-PKcs−/− B cells (27) could have been due to a combination of the effects of DNA-PKcs deficiency per se and further suppression of switching inherent in the particular transgenic BCR used.
V(D)J recombination, including the nature of the signal joints, is undetectable in all SCID cells examined to date (22, 28, 37, 57–59), the SCID mutation does not remove every trace of DNA-PKcs polypeptide (22, 37, 57–59). In contrast, most evidence indicates that this residual SCID polypeptide is nonfunctional. For instance, V(DJ) recombination, including the nature of the signal joints, is indistinguishable in DNA-PKcs−/− and SCID cells (25, 60–62), and overexpression of the SCID form of DNA-PKcs in DNA-PKcs−/− cells does not significantly alter this phenotype (25). If the SCID protein does participate in the switch recombination process, isotype switching is the first form of DNA repair involving DNA-PKcs in which its kinase activity is dispensable.

It is improbable that DNA-PKcs plays a direct role in S region point mutation because the SCID defect does not affect mutation of Ig V regions (63). This contrasts with the 7-fold reduction in mutation at base pairs in which C was on the sense strand evident in recombinant S regions from SCID cells (Table I). Recombination of S regions depends on both AID and UNG (5), implying that deamination of cytidines in S regions and their subsequent removal by base excision repair are critical events in class switch recombination. Thus, a simple explanation for the reduced frequency of deamination of cytidines on the sense strand is insufficiently lethal in SCID B cells, while deamination of cytidines on the antisense strand is usually benign (reflected by the preservation in SCID cells of mutation at G in Table I). This conclusion is consistent with the higher death rate of cultured SCID B cells (Fig. 5), although it has not yet been possible to establish that switching per se contributes to this death rate (see supplemental Fig. 1). Because the most likely trigger of cell death in SCID cells is an unresolved DSB, we speculate that deamination of the sense strand of S regions may usually cause the formation of a DSB, whereas deamination of the antisense strand usually does not. This raises the question of how DSBs arise during switching. Staggered DSBs certainly appear to be involved in switching (10), and although AID prefers to deaminate the non-template (sense) strand, it does deaminate the template strand as well. In concert with UNG and apurinic/apyrimidinic endonuclease 1, AID could thus generate staggered nicks (64–66), but why should AID-initiated nicking of the nontemplate strand produce a DSB more frequently than nicking of the template strand, as we speculate? One possibility is that nicks have already occurred on the template strand independently of AID and are only converted to DSBs that involve DNA-PKcs in the repair process when AID-induced nicking takes place on the nontemplate strand. Perhaps the recruitment of nucleases to S region R loops (64) produces template strand nicking that is independent of AID.

We do not yet know what fraction of G:C mutations in S regions represents direct cytidine deamination sites. Thus, another explanation for the effect of the SCID defect on mutation of hybrid S regions is that absence of DNA-dependent kinase activity alters the error bias of the DNA polymerases involved in repairing S region DSBs. However, it is difficult to envisage how such an alteration in the error bias could produce the strand bias evident in Table I. Clearly, the skewed loss of mutations from hybrid S regions in SCID cells warrants further investigation.

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References


