Immunoglobulin class switch recombination: study through human natural mutants

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Immunoglobulin class switch recombination deficiencies in humans are exquisite models to analyse the mechanisms of class switch recombination (CSR). Besides defects in CD40L/CD40 interaction, others result from an intrinsic B-cell deficiency. The recent elucidation of the molecular basis of some of them has made it possible to delineate the molecular events involved in antibody maturation. Activation-induced (cytidine) deaminase (AID) and uracil-N-glycosylase deficiencies have demonstrated the role of AID as the inducer of DNA lesions in switch and variable regions. However, most of these CSR deficiencies remain molecularly undefined. Their characterization would lead to a better understanding of the complex machinery involved in CSR.

Keywords: class switch recombination; activation-induced (cytidine) deaminase; mismatch repair; non-homologous end-joining repair; double-strand DNA breaks

1. INTRODUCTION

Immunoglobulin class switch recombination (Ig-CSR) deficiencies are rare primary immunodeficiencies whose frequency is estimated as approximately 1 in 100 000 births. These deficiencies are characterized by normal or increased serum IgM levels and a contrasting, marked decrease or absence of IgG, IgA and IgE (Notarangelo et al. 1992). As a result of this molecular defect, defective CSR may be associated with faulty generation of somatic hypermutations (SHM) in the Ig variable (V) region. The molecular identification and analysis of several Ig-CSR deficiencies has made it possible to better describe the mechanisms underlying CSR and SHM, both of which are key elements in the maturation of antibody responses (Durandy et al. 2007).

Maturation of the antibody repertoire produces several antibody isotypes with high antigen affinity, a necessary feature for optimized antibody responses. Antibody maturation occurs mainly in the germinal centres of the secondary lymphoid organs, following antigen and T-cell-driven activation. CSR results in the production of antibodies of different isotypes (IgG, IgA and IgE) with the same V(D)J specificity and therefore the same antigen affinity (Iwasato et al. 1990). SHM primarily introduces stochastic mutations (10⁻³ bp/cell cycle) into the variable (V) region of the Ig, a genetic modification that is followed by the positive selection of B cells harbouring a B-cell receptor (BCR) with high antigen affinity (Storb et al. 1998). CSR and SHM occur together in germinal centres with BCR/CD40 activation; however, neither is a prerequisite for the other because IgMs may be mutated in the absence of any such feature in IgG or IgA isotypes (Kaartinen et al. 1983).

Mutations in the gene encoding the CD40 ligand (CD40L, CD154; Korthauer et al. 1993), a molecule highly expressed in activated follicular helper T cells (Breitfeld et al. 2000), results in an Ig-CSR deficiency. The defect is generally associated with reduced SHM generation. This observation has been corroborated by the description of a similar phenotype caused by mutations in CD40 (Ferrari et al. 2001) and demonstrates the essential role of the CD40 signalling pathway in B cells for both CSR and SHM.

Other Ig-CSR deficiencies are a consequence of an intrinsic defect in the CSR machinery (Durandy et al. 1997). The autosomal recessive Ig-CSR deficiency, caused by mutations in the AICDA gene (encoding the activation-induced (cytidine) deaminase, AID), is characterized by the impairment of both CSR and SHM (Revy et al. 2000). This finding, together with the description of the phenotypic characteristics of AID⁻/⁻ mice (Muramatsu et al. 2000), has demonstrated the master role of AID in antibody maturation. In fact, AID selectively changes cytosine residues into uracils in the switch (S) and V regions (Bransteitter et al. 2003). Uracil-N-glycosylase, which is also mutated in another reported CSR deficiency (Imai et al. 2003b), removes uracil residues (introduced into DNA by AID; Rada et al. 2002) and thus produces an abasic site that is cleaved by a specific endonuclease (Guikema et al. 2007). This event ultimately leads to scattered, single-stranded DNA breaks.

In fact, AID- and UNG-induced DNA lesions are differently repaired in S and V regions, respectively. In S regions, CSR-induced double-strand break (DSB) repair requires phosphorylation of the H2AX histone (γH2AX) (Celeste et al. 2002) and the presence of the...
MRE11/RAD50/NBS1 complex (Theunissen et al. 2003; Reina-San-Martin et al. 2005), 53BP1 (Ward et al. 2003), MDC1 (Lou et al. 2006) and some of the molecules in the non-homologous end-joining (NHEJ) repair pathway, as shown by the phenotype of mice or B cells deficient in each of these molecules (Rolink et al. 1996; Casellas et al. 1998). It is very likely that other DNA repair molecules are required, since a B-cell-intrinsic CSR defect (associated with increased sensitivity to ionizing radiation) has been shown not to be linked to any defects in these molecules (Peron et al. 2007). Mutations in the MRE11 (leading to ataxia-like disease), Nijmegen breakage syndrome (NBS1) and ATM (ataxia-telangiectasia) genes can lead to Ig-CSR deficiencies and thus reveal the role of these molecules in S region DNA repair in humans (Pan et al. 2002; Lahdesmaki et al. 2004). The mismatch repair (MMR) system is known to play a role in CSR in mice, as shown by diminished, switched, isotype serum levels and abnormal switch junctions (Ehrenstein & Neuberger 1999; Schrader et al. 1999): the MSH2/MSH6 complex appears to recognize AID-induced DNA mismatch in the absence of UNG, leading to back-up CSR and a skewed SHM pattern, as shown by the CSR-defective phenotype of a double UNG–MSH2 knockout mutant (Rada et al. 2004). Recently, it has also been reported that MSH5 variants in humans can be associated with common variable immunodeficiency and IgA deficiency (Sekine et al. 2007). The role of the PMS2/MLH1 complex is less clear; it has been recently proposed that it can convert DNA single-strand breaks (SSBs) into DSBs, as suggested by the low occurrence of blunt DSB observed in S regions of murine PMS2-deficient B cells (Schrader et al. 2007).

The error-prone repair of SHM-induced DNA breaks requires the MMR and error-prone DNA polymerases but not NHEJ (Rolink et al. 1996; Casellas et al. 1998), γH2AX (Reina-San-Martin et al. 2003), 53BP1 (Manis et al. 2004), MRE11, NBS1 or ATM (Pan-Hammarstrom et al. 2003). The MSH2/MSH6 complex is essential in SHM for (i) recognizing the U/G residues induced by cytidine deamination by AID and (ii) recruiting EXO1 and polymerase η (Xue et al. 2006). The role of the PMS2/MLH1 complex in SHM remains subject to debate (Winter et al. 1998; Phung et al. 1999).

All steps of antibody maturation are not thus perfectly understood, but their understanding has been greatly improved by the characterization of inherited Ig-CSR deficiencies. We herein describe two new Ig-CSR deficiencies, one is very likely due to a defect in AID targeting on S regions and the other caused by an abnormal repair of S regions.

2. RESULTS

(a) Ig-CSR deficiency possibly due to a defect in AID CSR-specific cofactor?

In our experience, a subgroup of 16 patients are affected by a novel Ig-CSR deficiency, the phenotype of which has been extensively studied.

(i) Patients’ phenotype

This syndrome is probably transmitted as an autosomal recessive disease, according to the M/F ratio (≈1) and the frequency of consanguinity (20%). Patients present with a similar phenotype as AID-deficient patients, i.e. recurrent bacterial infections, auto-immune manifestations and lymphadenopathies. However, lymphadenopathies are less impressive than those observed in AID deficiency and no typical AID-deficient giant germinal centres are observed.

(ii) Ig-CSR deficiency

The in vitro CSR defect, as judged by serum Ig levels, is severe with intense decrease or absence of IgG and complete lack of IgA. No switched IgM–IgD B cells are observed in peripheral blood, although CD27+B cells are normally present.

The in vitro CSR defect is intense, with no recombination towards switched isotypes in CSR-activated B cells. In order to localize the CSR defect, we checked for the expression of germline, circular and functional IgE transcripts in CD40L+IL4-activated B cells. Germline transcripts were normally present, but circular and functional transcripts were missing, in correlation with lack of IgE in supernatants. Moreover, blunt DNA DSBs were not detected by the LM-PCR technique sensitive in Sμ regions. Since these characteristics were reminiscent of those of AID deficiency, we sequenced the AICDA gene: no abnormality was found, AICDA transcripts were normally expressed and AID protein expression was normally found by Western blot in activated B cells.

Thus, the defect is located downstream from the transcription step and upstream from the S region DNA cleavage, and is not caused by AID deficiency.

(iii) Normal uracil excision assay

The uracil excision as shown in mice (Rada et al. 2002) and humans (Imai et al. 2003b) and the APEX-induced abasic site cleavage as shown in mice (Guikema et al. 2007) are the following steps required for repair of the AID-induced DNA lesions. Although UNG, APEX1 and 2 sequences were normal as well as their RNA transcripts’ expression, we checked for their activities on a nucleotidic substrate containing a U:G mismatch. Protein extracts from patients’ EBV-B cell lines did normally cleave the probe, ruling out a defect in this pathway.

(iv) Normal SHM

AID deficiency is known to induce a defect in both CSR and SHM (Revy et al. 2000), and UNG deficiency to lead to a skewed pattern of SHM. We therefore checked for SHM occurring in VH of IgM in patients’ CD27+B cells: normal frequency and pattern of SHM was observed.

(v) Normal radiation sensitivity

Since some Ig-CSR deficiencies are associated with a DNA repair defect (Peron et al. 2007) and see below, we checked for radiosensitivity of patients’ fibroblasts and EBV-B cell lines: radiosensitivity was found in normal ranges.

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This phenotype is very similar to that caused by AID C\textsuperscript{ter} mutations. Indeed, we have previously shown that mutations located in the C\textsuperscript{ter} of AID lead to a profound Ig-CSR deficiency without affecting the SHM process (\textit{Ta et al.} 2003), strongly suggesting that AID, besides its cytidine deaminase activity, exerts another activity in CSR, possibly by recruiting a CSR-specific cofactor. Similar observation was obtained when the activity of an artificial AID mutant with deletion of its C\textsuperscript{ter} part was studied after transduction in AID\textsuperscript{−/−} murine B cells (\textit{Barreto et al.} 2003). This Ig-CSR deficiency could thus be caused by a defect in this AID CSR-specific cofactor. The nature of this cofactor remains unknown.

One hypothesis could be that the cofactor is required for targeting of AID on S regions since the CSR-induced DSBs are shown not to occur in S\textsubscript{i} regions from patients’ B cells, localizing the CSR defect, as in AID deficiency, downstream from the transcription step but upstream from the DNA cleavage. AID targeting remains indeed unelucidated: there are neither consensus nor homologous sequences around the S junctions, in contrast to the RSS regions recognized specifically by Rag enzymes during the V(D)J recombination. The sequence structure of S regions (\textit{Honjo 2002; Yu et al. 2003}) or the DNA conformation during transcription could be required for AID targeting (\textit{Yu et al. 2003; Shen et al. 2005}), but no molecular characterization of the interaction is available, making possible the role of cofactors. An interaction between the 32 Kd subunit of the DNA-binding replication protein A (RPA) complex and AID has been reported as essential for AID binding to DNA in both S and V regions (\textit{Chaudhuri et al. 2004}). However, this interaction is probably unaffected in this Ig-CSR deficiency since SHM are normally found.

Another possibility could be a defect in AID phosphorylation. Indeed, AID phosphorylation by protein kinase A (PKA) on S38 and T27 amino acids has been shown to be required for AID interaction with RPA, binding to double-stranded DNA, and activity in CSR. Artificial mutants carrying alanine substitutions on S38 and T27 amino acids are unable to induce CSR when transduced in AID\textsuperscript{−/−} murine splenic B cells (\textit{Basu et al. 2005}). However, the same requirement of AID phosphorylation for interaction with RPA and SHM has also been recently described (\textit{McBride et al. 2006}).

Other additional post-transcriptional modification of AID, required for CSR and dispensable for SHM, could thus be responsible for this Ig-CSR deficiency if defective.

Conversely, study of mutations occurring in S\textsubscript{i} regions under AID activation has revealed that they normally occur in mice AID\textsuperscript{−/−} B cells when transduced by AID C\textsuperscript{ter} mutants, strongly suggesting that the AID cofactor binding the C\textsuperscript{ter} part of AID is not involved in AID targeting on S regions but in downstream steps (\textit{Barreto et al. 2003}). Several cofactors, DNA-PKcs and murine double minute 2 (MDM2), have been reported as binding the C terminal of AID (\textit{Macdull et al. 2005; Wu et al. 2003}). They could represent CSR-specific AID cofactors since (i) the NHEJ system has been shown to be required for CSR but dispensable for SHM (\textit{Rolink et al. 1996}) and (ii) MDM2 binds the NBS1 molecule (\textit{Alt et al. 2005}), for which a role in CSR-induced DNA repair has been described (\textit{Reina-San-Martin et al. 2005}). MDM2 has been further shown to be required for gene conversion in the chicken DT-40 cell line, but no data on CSR are available. The involvement of both molecules has been excluded as causative of the Ig-CSR deficiency observed in the patients. Its molecular characterization would make it possible to define the CSR-specific cofactor that AID recruits by its C terminus, up to now elucidated.

(b) Ig-CSR deficiency probably due to a defect in DNA repair of S regions

A novel Ig-CSR deficiency, so far found in 36 patients, has been defined, even if its molecular basis remains unknown (\textit{Imai et al. 2003a}).

(i) Patients’ phenotype

This syndrome is probably transmitted as an autosomal recessive disease, according to the M/F ratio (= 1.2) and the frequency of consanguinity (17%). The prognosis is worsened by the severity of infections (four patients died from infections), the occurrence of auto-immune manifestations found in eight patients and of B-cell lymphomas (seven patients). Lymphadenopathies can be present and biopsies reveal disorganized germinal centres.

(ii) Ig-CSR deficiency

The in \textit{vivo} CSR defect, as judged by serum Ig levels, is less severe than AID deficiency with often residual levels of IgG and IgA. No switched IgM–IgD B cells are observed in peripheral blood, and CD27\textsuperscript{+} B cells counts are strongly decreased.

The in \textit{vitro} CSR defect is intense, with no recombination towards switched isotypes in CSR-activated B cells. In order to localize the CSR defect, we checked for the expression of germline, circular and functional IgE transcripts in CD40L\textsuperscript{+} IL4-activated B cells. Germline transcripts were normally present, but circular and functional transcripts were missing, in correlation with lack of IgE in supernatants. In contrast to what is observed in AID deficiency, blunt DNA DSBs are normally detected in S\textsubscript{i} regions of CSR-activated B cells. Thus, the CSR defect is located downstream from DSB.

The residual IgA production allowed us to characterize S\textsubscript{i}–S\textsubscript{a} junctions \textit{ex \textit{vivo}}, from patients’ B cells. The majority of junctions displayed a perfectly matched homology (microhomology) of 1 bp or above and no junction showed precisely joined blunt ends, whereas control junctions were either blunt or use 1 bp homology.

(iii) Skewed pattern of SHM

SHM frequency was found slightly decreased or normal variably within the CD27\textsuperscript{+} B cells. Of note, the count of this population was lower than normal (less than 10%). Nucleotide substitution reveals a skewed pattern with an excess of transitions on G:C residues.

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(iv) Normal uracil excision assay
Since the skewed pattern observed in SHM was very similar to that observed in UNG deficiency (Imai et al. 2003b), we checked for UNG activity using a nucleotidic probe containing a U:G mismatch. Protein extracts from patients’ EBV-B cell lines and fibroblasts did normally cleave the probe, ruling out a defect in this pathway.

(v) Increased radiosensitivity
The occurrence of B lymphomas, the CSR defect located downstream from the DSB and the abnormality of switch junctions were strongly evocative of a DNA repair defect in S regions. Since it is known that AID is sufficient in fibroblasts to induce CSR on an appropriate substrate (Okazaki et al. 2002), we postulate that the defect in the DNA repair factor should be detectable in patients’ fibroblasts. The radiosensitivity of patients’ fibroblasts was thus tested, giving evidence for a reproducibly increased radiosensitivity. Although the increased radiosensitivity was less marked than that of fibroblasts from patients suffering from ataxia-telangiectasia (A-T) or Artemis deficiency used as controls, it was significantly different from that of healthy fibroblasts. These results were confirmed by using EBV-B cell lines. Thus, this Ig-CSR deficiency is associated with a DNA repair defect.

(vi) Characterization of the DNA repair defect
Excessive radiosensitivity could be due to a DNA repair defect caused by an impaired recruitment of repair proteins to DSB. We therefore studied one of the earliest responses to DNA damage, namely the induction of histone H2A.X (γH2AX) phosphorylation (Rogakou et al. 1998; Paul et al. 2000). γH2AX is essential for keeping DNA ends together and for stabilizing the association of DNA repair factors such as the MRE11/RAD50/NBS1 (MRN) complex, 53BP1 (tumour protein p53-binding protein 1) and mediator of DNA damage checkpoint 1 (MDC1) at the site of the damage. DNA repair foci, including γH2AX, MRE11, 53BP1 and MDC1 were equally recruited after irradiation in control and patients’ cells, both in fibroblasts and in EBV-B cell lines.

Another early event occurring rapidly after DNA damage is cell-cycle progression arrest. We thus studied the inhibition of cycle progression in patients’ fibroblasts after irradiation. Both arrests of entry into S phase (G1/S checkpoint) and into mitosis (G2/M checkpoint) were normally observed in patients’ cells in contrast to A-T fibroblasts that exhibited, as checkpoint (G1/S checkpoint) and into mitosis (G2/M checkpoint) were normally observed in patients’ cells in contrast to A-T fibroblasts that exhibited, as checkpoint were normally observed in patients’ cells in contrast to A-T fibroblasts that exhibited, as checkpoint were normally observed in patients’ cells in contrast to A-T fibroblasts that exhibited, as checkpoint were normally observed in patients’ cells in contrast to A-T fibroblasts that exhibited, as checkpoint were normally observed in patients’ cells in contrast to A-T fibroblasts that exhibited, as checkpoint were normally observed in patients’ cells in contrast to A-T fibroblasts that exhibited, as checkpoint were normally observed in patients’ cells in contrast to A-T fibroblasts that exhibited, as checkpoint were normally observed in patients’ 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