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Predominant role for activation-induced cytidine deaminase in generating IgG anti-nucleosomal antibodies of murine SLE

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Abstract

Serum IgG anti-nuclear antibodies (ANA) directed to complexes of DNA and histones are a hallmark of systemic lupus erythematosus (SLE) and reflect a failure in lymphocyte self-tolerance. A prior study utilizing spontaneously autoimmune B6.*Nba2* mice deficient in terminal deoxynucleotidyl transferase (TdT) and with heterozygous deficiencies in *Jh* and *Igk* loci underscored the importance of somatic hypermutation (SHM) as a major generator of SLE-associated ANA. This interpretation had to be qualified because of severely limited opportunities for receptor editing and restricted VHCDR3 diversity. Therefore, we performed the converse study using mice that carried functional *Tdt* genes and wild type *Jh* and *Igk* loci but that could not undergo SHM. Analyses of ANA and ANA-producing hybridomas from B6.*Nba2 Aicda*^{-/-} mice revealed that few animals produced high titers of the prototypical ANA directed to complexes of histones and DNA, that this response was delayed and that those cells that did produce such antibody exhibited limited clonal expansion, unusual *Jk* use and only infrequent dual receptor expression. This, together with the additional finding of an intrinsic propensity for SHM to generate Arg codons selectively in CDRs, reinforce the view that most IgG autoimmune clones producing prototypical anti-nucleosome antibodies in wild type mice are created by SHM.

Keywords

Lupus; Anti-nuclear; Autoantibodies; Somatic hypermutation

1. Introduction

SLE is a systemic autoimmune disease, characterized by high-avidity IgG ANA that are often associated with various end-organ pathologies. Although the term ANA denotes a diverse group of self-reactive antibodies (Abs) with many specificities, those that react with

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Appendix A. Supplementary data

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complexes of double-stranded (ds) DNA and histones (nucleosomes) are by far the most common and are routinely used in the diagnosis of SLE [1]. Autoimmune clones with such specificity are particularly important to the investigation of disease etiology. This is because they represent a clear and egregious breach in immune self-tolerance, as demonstrated through studies involving mice carrying Ig transgenes specifying nucleosome-reactive B cell receptors (BCR) [2–10]. When these ANA clones arise in autoimmune disease, they bear all of the hallmarks characteristic of T cell-dependent immunity, including secretion of IgG autoantibody, evidence of having undergone clonal selection/expansion, and expression of hypermutated Ig V region genes [11–13].

To shed light on how and when nucleosome-reactive B cells breach self-tolerance, several groups have investigated their point of origin [14,15]. Studies with anti-nuclear B cell receptor (BCR) transgene (Tg) mice have shown that B cell tolerance is incomplete in mice with lupus-prone genetic predispositions, suggesting that ANA arise from B cells that are generated in the bone marrow with an autoreactive receptor [8,16–21]. This is in agreement with results of other studies showing that when somatic mutations in ANA were reverted to germline sequence, autoreactivity and/or poly-reactivity was preserved [22–24].

An alternative possibility is that ANA clones arise from nonautoreactive B cells that acquire their autoreactive specificity via the process of SHM. In support of this idea, several investigators have provided examples in which reverting V region somatic mutations to germline sequence in ANA clones eliminated detectable anti-nuclear activity [23,25]. In general, however, studies assessing the importance of germline sequences versus mutated sequences to autoreactivity have suffered from uncertainty regarding the mutational status of VHCDR3, where untemplated nucleotides are frequently added by TdT during B cell development in the bone marrow, long before the induction of SHM [26,27]. Undisclosed somatic mutations in VHCDR3 could account for the inconsistent results regarding preservation or loss of autoreactivity among different mutation reversion studies.

To circumvent this problem, in a prior study we reverted V gene somatic mutations in ANA hybridomas derived from autoimmune B6.*Nba2 Tdt^{-/-} Jh^{+/-} Igk^{+/-}* mice. The *Nba2* interval on chromosome 1 was derived from the NZB genome and predisposes B6 mice to spontaneously develop ANA. The TdT deficiency enabled us to identify all somatic mutations, including those in VHCDR3. And the heterozygous deficiencies in the Ig loci enabled us to determine whether a given autoreactive clone expressed one or two BCR. In this study all detectable anti-nuclear activity was eliminated upon mutation reversion in 9 of 10 clones, and 95% of it was eliminated from the 10th clone, thus implicating SHM as the predominant generator of ANA in murine SLE [28]. This scenario of ANA origin is attractive because it requires the autoreactive clone to escape only the most terminal checkpoints in self-tolerance that take place following immune activation and SHM. However, a caveat to our interpretation is that cells with anti-nuclear specificity might be underrepresented in the *Tdt^{-/-}* repertoire relative to the wild type *Tdt⁺* repertoire [29,30]. In addition, although we found no clones expressing dual BCRs, which might promote an escape from self-tolerance, deficiencies in *Jh* and *Igk* loci had restricted receptor editing to the lambda locus in our model [10,31–43]. Potential editing of the BCR has complicated interpretations regarding the origin of nuclear-reactive clones.

To address both of these limitations, we analyzed anti-nucleosomal responses in mice that could not undergo SHM but that carried functional *Tdt* genes and homozygous wild type alleles at all Ig loci. We also determined the relative frequencies of AGC and AGT serine codons in CDRs and FRs of all mouse and human germline Ig V region genes, as these are prone to mutate toward Arg codons, which frequently confer anti-nuclear specificity upon the BCR [44]. Results of our study reinforce the idea that SHM is the major generator of the most predominant IgG ANA directed against complexes of histones and DNA in AID⁺ autoimmune mice.

2. Materials and methods

2.1. Mice

B6.*Nba2* congenic mice were originally provided by Drs. S. Rozzo and B. Kotzin (University of Colorado Health Sciences Center; Denver, CO) [45]. AID-deficient mice were provided by Dr. T. Honjo [46]. The AID deficiency was crossed into the B6.*Nba2* background to generate B6.*Nba2 Aicda*^{-/-} mice. Genotyping was performed by PCR with primers described in Table S1, and the AID deficiency confirmed with an anti-IgG1 ELISA. Blood was collected by submandibular bleeding. This study was conducted in accordance with an IACUC-approved protocol.

2.2. Immunoassays

Sera from experimental and control mice were tested for the presence of autoAbs or total Igk as described with the following modifications [47]. 96-well trays were coated overnight with calf-chromatin, ssDNA (Sigma–Aldrich), total histones (Roche), dsDNA/total histones or bovine serum albumin (BSA) at 10 µg/ml in PBS-1mM EDTA or goat anti-mouse Ig at 1 µg/ml (Sigma–Aldrich) in PBS. Bound Abs were detected with HRP conjugated to goat-anti mouse Igκ (Southern Biotech). Nonspecific Ab binding to BSA-coated wells was subtracted from binding measured in autoantigen-coated wells. In some experiments, sera were pre-treated with DNase I (10 µg/ml) overnight at 4 °C in the presence of 2 mM MgCl₂. IgMκ Units were defined using pooled sera from wild type B6 mice as a standard.

2.3. HEp-2 immunofluorescence and autoantigen array

Abs were tested for autoreactivity against fixed HEp-2 cells (Bio-Rad Laboratories) by immunofluorescence as previously described [28]. HEp-2 slides were covered with mounting medium containing DAPI (Vector Laboratories), viewed with an Axiovert 200M microscope (Carl Zeiss, Inc.) and analyzed with Slidebook 5 software (Intelligent Imaging Innovations Inc.). In any given experiment, all pictures were taken with the same exposure.

The autoantigen array was performed by the Microarray Core at University of Texas Southwestern Medical Center (Dallas, TX) [48]. Samples were considered positive when the IgM-Cy5 signal-to-noise-ratio (SNR) was greater than the mean plus 2 standard deviations of 3 independent B6 pooled sera (n = 9).

2.4. Generation of B cell hybridomas

Hybridomas were generated from two 15-months-old spontaneously autoimmune B6.*Nba2 Aicda*^{-/-} mice. One mouse with a high-titer of antibodies to dsDNA/histones (#1 in Table 1) and one with a low titer of such antibodies (#2 in Table 1) were chosen. Monoclonal antibodies specific to the hapten p-azophenylarsonate (Ars) were generated by immunization of a B6 *Aicda*^{-/-} mouse with 50 µg of Ars-kehyole limpet hemocyanin (KLH) i.p in alum at day 0, followed by 3 i.p. injections at days 7, 9 and 11 in PBS. At day 14, splenocytes were harvested and fused with SP2/0/mIL-6 cells [49]. Hybridoma culture supernatants were tested for the production of Igκ antibodies against chromatin or Ars-ovalbumin (OVA) as described above. Positive hybridomas were cloned by limiting dilution. Autoreactive specificities of mAbs were defined based on binding to antigens by ELISA combined with the HEP-2 staining pattern after DNase I treatment followed or not by centrifugation with Amicon-Ultra 100 kDa filters (Millipore, Temecula, CA). Hybridoma supernatants were tested for light-chain isotypic exclusion by ELISA using the total-Ig coated 96-well trays and biotin-labeled anti-mouse Vλ1, 2 and 3 (BD-Pharmingen, San Diego CA) or anti-Vλx. Anti-Vλx [50] was a generous gift from Dr. Mark Shlomchik.

2.5. Sequencing hybridoma V region genes

Variable genes of the hybridomas were cloned with RT-PCR and 5' rapid amplification of cDNA ends (RACE) using sets of constant region primers and anchor primers as previously described (Table S1) [28]. The cloned V genes were sequenced at the University of Colorado Cancer Center DNA Sequencing and Analysis Core Facility (Aurora, CO).

2.6. Sequence analysis

Sequences of mAb were analyzed by Ig-Blast (www.ncbi.nlm.nih.gov/projects/igblast). VHDJH and VκJκ gene designations were taken from Johnston et al. [51] and from Brekke and Garrard [52]. The VHCDR3 sequences were defined using IMGT-numbering system [53]. Briefly CDR3 starts after the Y^Y/_FC and ends before ^F/_W G×G motif. The pI was calculated using the function at (http://web.expasy.org/compute_pi/).

2.7. Codon analyses

Germline V genes sequences were extracted from (www.ncbi.nlm.nih.gov/projects/igblast). Pseudogenes were excluded, and functional V genes were aligned against the B6 genome using NCBI-BLAST [54] to confirm sequence accuracy. Frameworks (FR) 1–3 and CDRs 1 & 2 were defined using Ig-BLAST and the KABAT definition. CDR and FR sequences from germline VH, Vκ or Vλ families of mice and humans were grouped into 30 data files, one for each subregion of a given V gene family for the two species. FR or CDR sequences for a given V family were fused to form a continuous sequence, and codon frequencies were calculated by the function provided at (<http://www.kazusa.or.jp/codon/>). This approach was made possible by the fact that CDR and FR definitions begin and end with intact codons.

2.8. Light-chain dual-expression

Allelic exclusion in ANA was determined by Southern blotting [55]. Briefly proteinase K and phenol-extracted genomic DNA was digested with Hind III-HF (New England Biolabs)

at 10 U/μg of DNA and run on 0.8% agarose gels. Light-chain alleles were detected by aqueous hybridization using an ~800 bp Jκ probe amplified from B6 genomic kappa clone with primers listed in Table S1 and radio-labeled with ³²P-dCTP using a Nick Translation Kit (Roche, Nutley NJ).

2.9. Jκ database and statistical analysis

188 independent published IgG -ANA were used to build a Jκ usage distribution among ANA [11,12,28,56–62]. Statistical analyses were performed with GraphPad Prism5.

3. Results

3.1. Predominant cytoplasmic staining pattern by autoantibodies in sera of B6.Nba2 *Aicda*^{-/-} mice

We have reported that a large majority of anti-nucleosomal clones arising in autoimmune B6.Nba2 *Tdt*^{-/-} *Jh*^{+/-} *Igk*^{+/-} mice are derived from nonautoreactive precursors via the process of SHM [28]. However, CDR3 diversity and the potential to escape self-tolerance due to expression of 2 BCRs upon receptor editing are limited in this model. To address these limitations, we analyzed the development of ANA under circumstances in which VHCDR3 diversity and receptor editing were unrestricted. To this end, we bred genetically-prone autoimmune mice that carried wild type copies of *Tdt* and Ig genes, but that could not undergo SHM [46]. We chose the B6.Nba2 model because the autoantibody response in these mice closely resembles that of humans with SLE in terms of age-dependence, gender bias and a predominantly uniform nuclear staining pattern in HEp-2 cells by immunofluorescence [45]. In addition, the *Nba2* interval on chromosome 1 is syntenic with a region in humans that is associated with SLE [63]. As such, this is considered an excellent model of spontaneous ANA development in human SLE [64–67].

Fig. 1A shows that most female B6.Nba2 *Aicda*^{-/-} mice developed detectable titers of IgM anti-chromatin antibodies by 6 months of age. Although the anti-chromatin ELISA is a simple screening method for lupus-associated autoantibodies, chromatin preparations carry nonnucleosomal autoantigens. Therefore, we retested these sera for antibodies that react with complexes of purified dsDNA and histones, an autoantigen to which self-tolerance is normally enforced [2–10]. Fig. 1B shows that sera from B6.Nba2 *Aicda*^{-/-} were largely devoid of such antibodies. Typically, B6.Nba2 female mice develop ANA with nucleosomal specificity by 6 months of age (Fig. 1C).

The absence of anti-nucleosomal Ab in B6.Nba2 *Aicda*^{-/-} sera was confirmed by immunofluorescence, where the staining pattern against fixed HEp-2 cells was predominantly cytoplasmic (Fig. S1). The presence of IgM anti-chromatin Abs and a predominantly cytoplasmic HEp-2 staining pattern together with the absence of antibodies to dsDNA/histones, led us to speculate that B6.Nba2 *Aicda*^{-/-} mice develop Abs against other antigens that are less consistently targeted in systemic autoimmune diseases. Therefore, we tested sera of B6.Nba2 *Aicda*^{-/-} mice in an autoantigen array (Fig. 1D–E). As expected, B6.Nba2 *Aicda*^{-/-} sera contained auto-antibodies directed to several RNA-related antigens and to other relatively common autoantigens when compared to sera of C57BL/ 6J (B6)

mice. Thus, development of autoantibodies to several nonnucleosomal antigens did not require SHM.

3.2. Few B6.Nba2 *Aicda*^{-/-} mice develop anti-nucleosomal Ab even at 12 months of age

To further investigate ANA development in B6.Nba2 *Aicda*^{-/-} mice, we tested sera from a cohort of females that were aged to 12 months. As shown in Fig. 2A-B, only 2 of 8 B6.Nba2 *Aicda*^{-/-} mice developed high titers of the prototypical ANA directed to histone/ DNA complexes, even at this late time point. This was confirmed by a nuclear staining pattern against HEp-2 cells (Fig. S2), and could not be accounted for by nonspecific binding because the total concentration of IgM in these mice was only ~1.5 times higher than that of age-matched B6 controls (Fig. 2C). However, the lack of a strong ANA response by most of the mice was notable because B6.Nba2 female mice develop such ANA well before this age, illustrated again with sera from a second cohort of B6.Nba2 controls that were 8 months old (Fig. 2B). At 12 months, sera of the other 6 B6.Nba2 *Aicda*^{-/-} mice still produced a predominantly cytoplasmic staining pattern against fixed HEp-2 cells (Fig. S2 and Fig. 2D). Thus, while it is clear that antibodies to nucleosomes arose in autoimmune-predisposed mice without functional AID, only a minority of mice produced them, and they arose with delayed kinetics.

3.3. Negligible clonal expansion by anti-nuclear B cells in B6.Nba2 *Aicda*^{-/-} mice

In mouse models of SLE, a common feature of the anti-nucleosome response is its oligoclonal nature, indicating selection and proliferation of cells with this specificity [11–13]. To assess clonality of dsDNA/histone-reactive B cells in B6.Nba2 *Aicda*^{-/-} mice, we generated B cell hybridomas from two 15 months-old mice, one with a high titer of ANA against dsDNA/histone and one with a low titer of such antibody (Fig. S3). To provide a comparative analysis with our previous report in the B6.Nba2 *Tdt*^{-/-} mouse, all monoclonal antibodies (mAb) were initially selected on the basis of binding to whole chromatin. Fig. 3 shows that chromatin-reactive hybridomas were generated from both mice, and that the mAb displayed a relatively wide range of avidities reflecting either differences in intrinsic association constants or in epitope fine specificity and abundance.

Initial binding assays suggested that most of the anti-nuclear mAb bound to purified histones regardless of whether they were complexed with DNA (Fig. 4A, B). However, anti-nuclear mAb are often mistakenly assigned particular autoantigenic specificities due to bridging between immobilized antigen and antigen carried over by the mAb from hybridoma culture supernatants [3]. To test for this, we treated culture supernatants with DNase, and then neutralized the DNase with EDTA prior to assay. Fig. 4C and D show that this treatment reduced binding to purified histones by ~20-fold on average, while increasing it for histone/DNA complexes by ~2-fold. Binding to single-stranded (ss) DNA still occurred, but this was misleading because when the DNase-treated supernatants were filtered at a 100 kDa cutoff, ssDNA binding disappeared (Fig. S4). Therefore, it is likely that DNase treatment of the mAb released histones that subsequently bound to immobilized DNA to reconstruct histone/DNA complexes [3]. In short, binding assays revealed that most of the mAb obtained by screening against whole chromatin had the prototypical specificity for dsDNA/histone complexes (Table 1). The result was revealing because it demonstrated that

prototypical ANA clones, which are normally subjected to self-tolerance, can arise without SHM, although infrequently and with delayed kinetics.

To look for evidence of clonal selection, we sequenced heavy and light chain V region genes expressed by the hybridomas. Of 30 hybridomas analyzed this way, only two shared identical sequences (Table 1 bold sequences and Fig. 5), indicating very little clonal expansion. Negligible clonal expansion was independent of serum ANA titers, since it was true for both mice. It seems unlikely that this was due to uniform BCR engagement by all of the clones, and thus uniform proliferation among clones because their mAb produced widely varying binding curves in immunoassays against whole chromatin (Fig. 3). However, it is known that the cytoplasmic tail of IgG confers a survival or selection advantage to developing memory B cells [68,69], which cannot occur in mice deficient in AID. In addition, somatic mutations may also promote selective clonal expansion [70]. To determine if the absence of class switch recombination (CSR) and SHM could account for lack of clonal expansion seen in the ANA response of B6.*Nba2 Aicda*^{-/-} mice, we performed a control fusion with splenocytes of a B6 *Aicda*^{-/-} mouse that had been immunized with a hapten-carrier conjugate. Sequences of the V region genes expressed by these hybridomas revealed that there was considerable clonal expansion, as 70% of the hapten-reactive hybridomas belonged to multimember lineages (Fig. 5). Collectively, these results suggested that B6.*Nba2 Aicda*^{-/-} B cells emerging in the BM with an autoreactive BCR directed against the nucleosome were generally compromised in their ability to participate in an autoimmune response.

3.4. Unusual J κ usage by ANA in B6.*Nba2 Aicda*^{-/-} mice

During B cell development in the bone marrow, B cells that recognize high-avidity self-antigens undergo secondary light-chain rearrangements involving distal J κ gene segments or V λ genes. To test for evidence of receptor editing, we assayed serum and hybridoma culture supernatants for anti-chromatin antibodies that used lambda light chains and inspected light chain sequences for J κ use. None of the hybridomas expressed V λ genes, nor did sera of the aged cohort of B6.*Nba2 Aicda*^{-/-} mice contain detectable V λ -containing antibodies against chromatin (data not shown). However, as seen in Table 1, J κ 5 usage appeared to be unusually high in hybridomas from both mice, and none of the hybridomas used J κ 4. When compared to that of 188 published ANA hybridomas, the use of J κ 5 by B6.*Nba2 Aicda*^{-/-} hybridomas was twice as frequent (Fig. 6A). These differences between ANA clones derived from AID-deficient and wild type autoimmune mice provide additional evidence that B cells emerging in the BM with a nucleosome-reactive BCR are not major contributors to the anti-nucleosome response in wild type, i.e. AID, autoimmune mice.

3.5. Limited allelic inclusion among ANA-producing clones

Poor light-chain allelic exclusion has been associated with the production of ANA in some, but not all, mouse models of SLE [10,32,71]. Since our hybridomas exclusively produced kappa⁺ antibody, we performed Southern blots to look for multiple κ -light chain gene rearrangements. The blots revealed that 3 hybridomas carried 2 kappa gene rearrangements (Fig. 6B–C). Upon sequencing multiple cDNA clones from these, we confirmed that in each case, both alleles were productively rearranged. Thus, only 3 of 30 hybridomas expressed

two κ light-chains (Table 1). We conclude that the constraint on receptor editing, which limited dual BCR expression in B6.*Nba2 Tdt*^{-/-} mice, is not a likely explanation for why nearly all dsDNA/histone-reactive IgG clones obtained from these mice in our prior study were generated *de novo* by SHM [28].

3.6. Similar VHCDR3 characteristics of ANA from AID-deficient and TdT-deficient B6.*Nba2* mice

The virtual lack of ANA among germline revertants in our study with TdT-deficient mice could be a consequence of shorter VHCDR3s, or VHCDR3s with lower isoelectric points and/or fewer Arg residues, any of which might result from V-D-J recombination in the absence of TdT [29,30]. To examine this, we compared germline VHCDR3 sequences of our B6.*Nba2 Tdt*^{-/-} ANA with those of B6.*Nba2 Aicda*^{-/-} ANA. As shown in Fig. 7(A–C), there were only minor differences between the two groups in terms of length, number of Arg residues or isoelectric points (pI), none of which reached a $p = 0.05$ level of significance. Moreover, less than 43% of ANA VHCDR3s from B6.*Nba2 Aicda*^{-/-} mice had Arg residues generated by TdT (Fig. 7D).

It was possible that the virtual absence of VHCDR3 differences between the two groups of antibodies was a result of differential VH use by the two strains of mice, as particular VH genes may have specific VHCDR3 requirements for binding to nuclear antigens [72]. To control for this, we compared VHCDR3s of IGHV1 (VHJ558)-expressing ANA from the two strains, because this was the predominant VH family expressed by both. As shown in Fig. 7E, out of 10 clones from the B6.*Nba2 Tdt*^{-/-} mice used V genes from the IGHV1 family. Among CDR3 sequences of these clones, 3 had Arg residues that were encoded by a germline IGHV1 gene, while the remaining 3 clones lacked VHCDR3 Arg residues. For ANA from AID-deficient mice, 20 used V genes from the IGHV1 family. Among CDR3 sequences of these, 13 had either no Arg codons ($n = 5$) or only germline Arg codons ($n = 8$) located within an IGHV1 gene. Together, these data indicate that limitations on junctional diversity in TdT-deficient mice cannot easily account for the major role played by SHM in generating their IgG ANA. Notably, among Arg codons generated by SHM in the *Tdt*^{-/-} set, many ($n = 8$) were located in kappa V genes and a large majority were in VH or V κ CDR1 and CDR2 ($n = 8$) rather than VHCDR3 ($n = 2$), indicating ample opportunities to create ANA by SHM without a heavy reliance on VHCDR3.

3.7. Germline Ig V genes have an intrinsic bias to acquire CDR Arg residues by SHM

It is well established that Arg residues frequently are major contributors to the binding energies of ANA [3,44,57,73]. A prior study from our group involving B6.*Nba2* mice deficient in TdT demonstrated that a large majority of Arg codons generated in ANA via SHM occurs at the germline serine codons: AGC and AGT [28].

This is probably because these are the only codons that can mutate to an Arg codon by 3 different single base changes. In addition, AGC is the most intrinsically favored triplet target of AID [74]. We have also shown that mouse and human germline Ig V genes have a higher frequency of AG(C/T) codons than expected for random codon use, indicating an intrinsic bias to acquire Arg codons via SHM [28]. To determine if this bias is subregion-specific, we

extracted CDRs 1 & 2 and FRs 1, 2 & 3 from all mouse and human germline Ig V genes and analyzed them for AG(C/T) codon frequencies. As shown in Fig. 8, observed frequencies of AG(C/T) codons in combined heavy chain CDR1 & 2 were ~2–4 fold higher than expected. And they were ~4–6 times higher than expected in light chain CDR1 & 2. This was true regardless of whether the “expected use” was taken as random codon use, i.e. 1/61 (0.016), or whether it was taken from the codon usage tables for mice and human genes. In contrast, when the FR sequences were analyzed, observed frequencies of AG(C/T) codon use were much closer to expected frequencies. These results indicate that the bias for potentially “dangerous” AG(C/T) codons was largely restricted to those parts of the binding site most likely to engage antigen. Strikingly, this trend held consistently across VH, V κ and V λ families of mouse and human genes.

4. Discussion

An earlier hybridoma sampling study involving B6.*Nba2* *Tdt*^{-/-} *Jh*^{+/-} *Igk*^{+/-} mice revealed that almost all clones producing IgG antibody against dsDNA/histone complexes were created from nonautoreactive antecedents by SHM. Here, we pursued a complementary analysis using B6.*Nba2* *Aicda*^{-/-} mice in which VHCDR3 diversity and receptor editing were unrestricted, while SHM could not occur. We found that the autoantibody response was largely directed against cytoplasmic antigens, that few mice produced high titers of antibodies reactive with dsDNA/histone complexes, and that this response was delayed despite no competition from clones that would otherwise have been created by SHM. Clonal expansion of dsDNA/histone-reactive clones was negligible and their J κ use was uncharacteristically skewed in favor of J κ 5. Thus, the kinetics, quantity and quality of the ANA response were not characteristic of what is normally observed in autoimmune B6.*Nba2* mice with functional AID. Moreover, additional observations impart clarity to our prior study in autoimmune *Tdt*^{-/-} *Jh*^{+/-} *Igk*^{+/-} mice. Few ANA-producing clones from the autoimmune *Aicda*^{-/-} mice had undergone secondary light chain gene rearrangements to express more than one BCR, and VHCDR3 Arg content and charge were similar to those of ANA from *Tdt*^{-/-} *Jh*^{+/-} *Igk*^{+/-} mice. Therefore, we conclude that restrictions on VHCDR3 diversity and receptor editing in the latter mice were not significant factors contributing to the high frequency of ANA created by SHM. The importance of SHM in generating ANA is further reinforced by our finding that frequencies of particular codons most prone to mutate to encode Arg are inordinately and consistently high in CDRs but not in FRs of mouse and human germline VH, V κ and V λ genes. This striking observation indicates that antibody V genes are poised to mutate towards nuclear specificities. Collectively, these results provide a substantial body of evidence to support the main conclusion of our study that most IgG-producing clones reactive with the predominant histone/DNA nuclear target are generated *de novo* via SHM. This is consistent with studies showing that encounters with abundantly expressed nuclear antigen compromise the survival or functional capabilities of autoreactive B cells. On the other hand, autoreactive somatic mutants that arise during an immune response should have an advantage in only having to traverse peripheral checkpoint(s) in self-tolerance that follow antigen stimulation and SHM. We think it unlikely that our findings are an idiosyncrasy of the *Nba2* model because it is derived from the classical

(NZB $\times$ NZW)F1 model of spontaneous SLE. Moreover, results of a carefully conducted reversion study involving human SLE ANA are in agreement with our conclusion [25].

As in our prior study, we focused on the response to histone/DNA complexes because experiments involving Ig Tg mice have shown that B cells with this specificity are normally censored by self-tolerance mechanisms [2–10]. This is consistent with results of complementary studies revealing the presence of exposed nuclear material on surface membranes of apoptotic cells [75,76]. Yet when these clones participate in autoimmunity, they possess characteristic features of a T cell-dependent immune response [11–13]. They also make up the largest and most consistent component of the ANA response, as reinforced again by the observation that most of the hybridomas isolated here produced such Ab despite being screened for reactivity against whole chromatin. There is considerable confusion in the literature regarding ANA specificities because such mAb frequently carry nuclear antigen derived from cell cultures into the immunoassay [3]. This view was reaffirmed by contrasting results presented here involving DNase-treated and untreated mAb.

Although the ANA response in B6.*Nba2 Aicda*^{-/-} mice was limited, other autoAbs were prevalent. It is unclear how much of this antibody was derived from clones that are normally eliminated by self-tolerance mechanisms. In wild type mice, for example, B cell tolerance to soluble monomeric self-antigen may be infrequent [77]. Similarly, in an Ig Tg model, anti-La B cells mature normally, even in a non-autoimmune background, and self-tolerance is primarily confined to the T cell repertoire [78]. Anti-Sm BCR-Tg B cells also develop and enter the periphery in nonautoimmune mice, however they are arrested in a pre-plasmablast stage of differentiation [79]. Thus, the relevance of SHM in producing autoimmune B cells with a particular specificity likely depends upon the availability and avidity of the self-antigen.

We cannot exclude the possibility that the predominant response against cytoplasmic antigens in B6.*Nba2 Aicda*^{-/-} mice is due to the autoimmune influence of the AID deficiency [80–83]. Other groups have reported autoantibodies in mice and humans lacking functional AID [84,85], although in these studies the auto-antibodies were nuclear-reactive. In control B6 *Aicda*^{-/-} mice, we did observe Ab responses to cytoplasmic antigens (albeit weak), as assessed by staining of fixed HEp-2 cells. The discordance regarding ANA in sera of B6 *Aicda*^{-/-} mice among different laboratories may be a reflection of differences in genetic background or in animal husbandry [86].

Because AID-deficient mice cannot undergo CSR, we cannot directly assess how frequently dsDNA/histone-reactive clones in B6.*Nba2 Aicda*^{-/-} mice would be able to fully differentiate into IgG + plasma cells had they expressed AID. Nevertheless, based on the low frequency of such ANA, the delayed kinetics with which they arose, associated with limited clonal expansion, and uncharacteristic J κ usage, we can infer that germline-encoded nucleosomespecific B cells rarely escape and differentiate into IgG plasma cells in autoimmune AID-sufficient mice. This deficiency is almost certainly due to censorship following encounters with self-Ag. In this regard, an inordinately high frequency (~40%) of hybridomas using J κ 5 supports the interpretation that many of these clones from our

autoimmune AID-deficient mice have indeed engaged self-antigen during central development and edited their receptors by rearranging new Ig light chain genes. In a prior survey and in the one reported here, *J κ 5* usage by ANA from autoimmune AID-sufficient mice was only ~15% and ~18% respectively, similar to its usage by nonautoreactive mAb (~15%) [87]. A failure to make the IgM to IgG transition may have beneficial physiological consequences, as several groups have reported that IgM ANA confer protection against inflammation and nephritis [88–92].

The finding that VHCDR3 diversity was not substantially different between dsDNA/histone-reactive clones from autoimmune TdT-deficient and AID-deficient mice in terms of length, charge and Arg content is in agreement with a prior study showing minimal effects of a TdT deficiency on the ANA response of auto-immune (NZB $\times$ NZW)F1 mice [93]. In contrast, a TdT deficiency in autoimmune MRL *lpr/lpr* mice was reported to result in shorter VHCDR3s and a reduced Arg content among ANA [29,30,94]. This disagreement among studies could be due to different mechanisms driving autoimmunity in different strains of mice. Notably, the B6.*Nba2* model we used is most closely related to the (NZB $\times$ NZW) F1 model [45]. An alternative explanation is that not all groups were examining the same ANA. Long, charged VHCDR3s are often associated with polyreactive Ab [95]. This is a recurrent issue in the published literature and is the reason why we eliminate bound nuclear material from ANA prior to assaying their antigenic specificities, so that our work can be selectively focused on a subset of ANA (dsDNA/histones-reactive) to which B cell self-tolerance is known to be attained under physiological circumstances [3,96–99]. V-D-J recombination during early B cell development generates a high frequency of auto/polyreactive clones that is estimated to be 55–75% [95]. However, the frequency with which such clones, and particularly ANA clones, is generated by SHM is unknown. Any estimation is confounded by a number of variables, not the least of which is the extent of somatic mutation in a given clone responding to a given immunogen. Somatic mutations accumulate preferentially in CDRs due to a bias in triplet sequences targeted by AID [74,100]. AG(C/T) Ser codons are prone to mutate to Arg codons due to this bias and because only these codons can do so by any one of 3 different single base changes [44]. In our analyses of V gene sequences expressed by ANA hybridomas from autoimmune B6.*Nba2 Tdt^{-/-} Jh^{+/-} Igk^{+/-}* mice, we found that two thirds of all somatic mutations producing Arg codons occurred at germline AG(C/T) codons [28] and that human and mouse germline VH, V κ and V λ genes contained high frequencies of AG(C/T) serine codons. Here, we extended this analysis to subregions of germline V region genes and found that high frequency use of AG(C/T) Ser codons was selectively confined to the CDRs. It is striking that this trend holds consistently for mouse and human VH, V κ and V λ genes. This observation suggests that SHM frequently generates V regions with a propensity to bind nucleic acids, which lends further support to our conclusion that SHM is the major generator of IgG nucleosomespecific autoantibodies in SLE. Although it has been suggested that high mutability in CDRs may be important for optimal immunity [101], the benefit of having a high frequency of potentially “dangerous” serine codons in CDR germline V region genes is nevertheless unclear. One possibility is that Arg residues enhance the avidity of BCR and Ab directed against enveloped viruses that display widely-spaced antigenic determinants together with nuclear debris acquired upon budding from the host cell [102,103]. In any case, this situation implies that highly efficient

self-tolerance mechanisms normally censor autoreactive B cells that are generated via SHM. Otherwise SLE would be the rule rather than the exception.

Supplementary Material

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Fig. 1.

Lack of prototypical ANA in 6 months-old AID deficient autoimmune mice. (A) IgM Abs to total calf chromatin in sera of 6 months-old AID deficient mice. (B) Igκ Abs against the dsDNA/histones complex. Serum Ig concentration was previously quantified for each sample and results are expressed as (autoantigen counts) – (BSA counts) at an IgM concentration of 1 μg/ml. (C) Igκ Abs against dsDNA/histones from B6.Nba2 (n = 11) and wild type B6 (n = 9) mice at 6 months of age. Results are expressed as (autoantigen OD) – (BSA OD) at the indicated serum dilutions. (D–E) Autoantibodies against several non-nuclear autoantigens in sera from 7 months-old B6.Nba2 *Aicda*^{-/-} mice (n = 8) as detected by an autoantigen array. Serum samples were adjusted to contain the same concentration of IgM. Results are expressed as the ratio of the IgM signal-to-noise-ratio (SNR) for B6.Nba2 *Aicda*^{-/-} sera to that of wild type B6 sera [(SNR sample)/(mean wild type B6 SNR + 2 standard deviations)] for each indicated autoantigen. (D) Common autoantigens associated with a HEP-2 cytoplasmic stain. (E) Other common SLE non-nuclear autoantigens.

Fig. 2.

Prototypical anti-dsDNA/histone Ab of B6.Nba2 *Aicda*^{-/-} mice at 12 months of age. (A) IgMκ anti-dsDNA/histone Ab in sera of B6.Nba2 *Aicda*^{-/-} mice (n = 8, 12 months of age). (B) IgMκ anti-dsDNA/histone Ab in sera of B6.Nba2 mice (n = 5, 8 months of age), B6 *Aicda*^{-/-} mice (n = 5, 12 months of age) and wild type B6 mice (n = 10, 12 months of age). B6 *Aicda*^{-/-} and wild type B6 data shown as mean + SEM. Dotted lines indicate the mean of wild type B6 + 2SD at the highest serum concentration tested. Assays for A & B were performed at the same time. (C) Relative serum concentration of IgMκ. (D) HEP-2 stain. Results are shown as the mean fluorescence intensity (MFI) in the FITC channel.

Fig. 3. Variable affinities of anti-nuclear mAb from two B6.*Nba2 Aicda*^{-/-} mice. Binding data were adjusted to account for IgMκ concentration.

Fig. 4.

Monoclonal Ab specificity is masked by DNase I-sensitive component(s) in culture supernatant. Each symbol indicates a different mAb. (A) Mock treated mAb supernatant binding to total histones alone or dsDNA/histones. (B) Same as (A) but mAbs were incubated overnight in the presence of DNase I. (C) Summary results of (A) and (B). Area under the curves (AUC) in (A) were divided by the AUC in (B).

Fig. 5. Limited clonal expansion by ANA-producing B cells of B6.*Nba2 Aicda*^{-/-} mice. Doughnut graph (left) represents ANA clones described in Table 1. Right doughnut graph represents anti-Ars clones obtained from an immunized B6 *Aicda*^{-/-} mouse with VHCDR3s of multimember lineages shown in panel on right. Lineage assignments were based on heavy and light V gene sequences.

Fig. 6.

Jκ distribution and dual light-chain expression among ANA from B6.Nba2 Aicda^{-/-} mice. (A) Jκ use among ANA-producing clones in Table 1 (n = 30) versus published IgG ANA (n = 188). *P* values were calculated by Fisher's Exact Test, and *p* values < 0.05 are shown. (B) Schematic illustration of the unrearranged Jκ locus (not to scale). (C) Example of a Jκ Southern Blot with the Jκ probe described in (B), showing a clone with two kappa rearrangements (lane #4). Light-chain gene rearrangements are listed in Table 1.

Fig. 7.

Similar VHCDR3 characteristics of ANA from TdT-deficient and AID-deficient autoimmune mice. (A) Number of amino acid residues in VHCDR3 of ANA. (B) Number of arginine residues in VHCDR3. (C) VHCDR3 isoelectric point. (D) Percentage of VHCDR3 arginine residues created by TdT for clones derived from AID-deficient mice. (E) Origin of VHCDR3 arginine residues among ANA using the same VH family. VHCDR3 were defined using the IMGT definition.

Fig. 8. High frequency of AG(C/T) serine codons in CDR but not FR among germline mouse and human V genes. Ratios of observed versus expected frequencies for AG(C/T) codons among CDRs and FRs of germline mouse (A) and human (B) V genes. Expected frequencies were defined by equal random probability of using a non-stop codon (1/61) or by codon frequency usage based on 52,926 mouse codons or 40,662,582 human codons.

Table 1

Heavy and light chain sequences of anti-chromatin hybridomas.

Clone #	Heavy chain	Light chain ^a	Specificity ^b
<i>Mouse #1</i>			
A3 1-6	IGHV1-67/IGHD2-9/IGHJ3	IGKV4-74/IGKJ5	dsDNA/histones
A3 3-2	IGHV1-26/IGHD4-1/IGHJ4	IGKV4-74/IGKJ2	dsDNA/histones
A3 1-5	IGHV1-55/IGHD1-1/IGHJ2	IGKV16-104/IGKJ1	dsDNA/histones
A3 6-6	IGHV10-1/IGHD2-4/IGHJ1	IGKV8-28/IGKJ5	dsDNA
A3 1-1	IGHV1-61/IGHD3-2/IGHJ2	IGKV19-93/IGKJ2	dsDNA
A3 5-3	IGHV2-5/IGHD4-1/IGHJ3	IGKV4-53/IGKJ5	dsDNA/histones
A3 3-4	IGHV8-12/IGHD1-1/IGHJ1	IGKV6-20/IGKJ2 and IGKV13-84/IGKJ2	dsDNA/histones
2A 8-6	IGHV8-8/IGHD1-1/IGHJ3	IGKV3-7/IGKJ2	dsDNA/histones
2A 1-1	IGHV5-4/IGHD3-2.2/IGHJ1	IGKV1-110/IGKJ1	dsDNA/histones
2A 7-1	IGHV1-64/IGHD1-1/IGHJ3	IGKV1-110/IGKJ1	dsDNA
2A 3-7	IGHV1-76/IGHD1-1/IGHJ1	IGKV14-111/IGKJ5	dsDNA/histones
2A 3-2	IGHV1-55/IGHD4-1/IGHJ2	IGKV2-137/IGKJ2 and IGKV1-135/IGKJ1	dsDNA/histones
2A 3-6	IGHV1-26/IGHD3-2/IGHJ3	IGKV4-74/IGKJ2	dsDNA/histones
2A 5-4	IGHV1-62/IGHD2-1/IGHJ2	IGKV4-74/IGKJ5	dsDNA/histones
2A 6-2	IGHV1-72/IGHD2-1/IGHJ2	IGKV4-74/IGKJ5	dsDNA/histones
2A 4-3	IGHV8-8/IGHD1-1/IGHJ2	IGK14-126/IGKJ2	dsDNA/histones
<i>Mouse #2</i>			
B1 1-1	IGHV1-80/IGHD1-1/IGHJ1	IGKV10-96/IGKJ2	dsDNA/histones
B1 2-1	IGHV1-26/IGHD1-1/IGHJ4	IGKV4-74/IGKJ5	dsDNA/histones
B1 4-2	IGHV1-64/IGHD2-4/IGHJ2	IGKV1-110/IGKJ5	RNA-like
B1 3-1	IGHV1-49/IGHD5-8/IGHJ1	IGKV1-110/IGKJ1	dsDNA/histones
B1 4-1	IGHV8-12/IGHD4-1/IGHJ2	IGKV4-74/IGKJ1	dsDNA/histones
B1 5-1	IGHV1-80/IGHD1-1/IGHJ1	IGKV10-96/IGKJ2	dsDNA/histones
B1 3-4	IGHV1-26/IGHD2-4/IGHJ3	IGKV4-74/IGKJ2	dsDNA/histones
B1 5-2	IGHV5-2/IGHD2-5/IGHJ3	IGKV1-110/IGKJ1	dsDNA/histones
B1 6-1	IGHV1-42/IGHD1-1/IGHJ1	IGKV4-74/IGKJ5	dsDNA/histones
B1 5-3	IGHV1-82/IGHD3-2/IGHJ2	IGKV3-7/IGKJ1 and IGKV3-12/IGKJ1	dsDNA/histones
2B 5-7	IGHV1-26/IGHD2-3/IGHJ2	IGKV4-74/IGKJ5	dsDNA/histones
2B 4-1	IGHV1-81/IGHD2-4/IGHJ3	IGKV9-123/IGKJ2	dsDNA/histones
2B 5-4	IGHV8-12/IGHD1-1/IGHJ4	IGKV4-70/IGKJ5	dsDNA/histones
2B 3-1	IGHV1-62/IGHD2-1/IGHJ2	IGKV12-44/IGKJ2	dsDNA/histones

^a Presence of one or two light-chains were determined by Southern-blot. Multiple light-chain sequences were analyzed in the case of the clones with more than one kappa-chain. No Vλ expression by ELISA was detected among the hybridomas.

^b Specificity was determined after DNase I treatment of the hybridomas culture supernatants. RNA-like specificity was determined by positivity to ssDNA with a cytoplasmic staining pattern on a Hep-2 assay. The dsDNA specificity was determined by positivity in the ssDNA assay with a homogenous nuclear staining pattern in the Hep-2 assay. All dsDNA/histones specific mAbs did not react to histones or dsDNA alone, and had a nuclear Hep-2 pattern.