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Abs reactive to DNA and DNA/histone complexes are distinguished by the presence of positively charged amino acids, such as arginine, in the heavy chain complementarity-determining region 3 (CDR3). The presence of these amino acids partly results from atypical V_{H}-D-J_{H} rearrangements such as D-D fusions and D inversions. Previous results in our laboratory demonstrated that newborn autoimmune MRL/MpJ+/+ mice undergo these unusual recombinations more frequently when compared with normal C3H/HeJ controls. In addition, the heavy chain junctions in newborn MRL mice demonstrated a preferred usage of V_{H}-proximal D genes and distal J_{H} genes suggestive of secondary gene rearrangements. In this study we explore the possibility that adult MRL B220^{+}IgM^{+} pre-B cells, which have not yet undergone Ag selection, exhibit similar rearrangement patterns. Indeed, MRL pre-B cells possessed more atypical rearrangements (D-D fusions) than those of C3H/HeJ mice. However, the biased use of upstream D genes and downstream J_{H} genes observed in the newborn MRL mice was not present in the pre-B cell library. These results suggest that the heavy chain rearrangement process persists later during B cell life in lupus-prone mice and lead us to propose a model of heavy chain receptor revision in the periphery of autoimmune mice. The Journal of Immunology, 2000, 165: 4487–4493.

Immunoglobulin gene rearrangement is a complex and ordered process that originates with recombination of D to J_{H}, then V_{H} to D-J_{H} genes at the heavy chain locus. Once a functional heavy chain is created, a similar process drives rearrangement at the light chain locus, eventually resulting in production of Abs capable of responding to a diverse number of foreign Ags.

In the autoimmune disease systemic lupus erythematosus, B cells produce Abs reactive to self Ag such as DNA or chromatin (1–5). The B cells producing these Abs are deleted, anergized, or edited in normal individuals (6–9). Although somatic mutation and V gene usage are partly accountable for the specificity of these autoreactive Abs, unconventional Ig gene rearrangements at the heavy chain locus such as D-D fusions may also be responsible (10–12). D-D fusions result from the joining of two heavy chain D genes and create a drastic change in the amino acid sequence of the heavy chain complementarity-determining region 3 (CDR3). The presence of positively charged amino acids such as arginine increase the affinity for Ab binding to DNA or DNA complexed to nuclear proteins such as histones (4, 10, 13). Although D-D fusions are uncommon in the normal Ab repertoire, these unusual rearrangements have frequently been observed in Abs with antigenic specificities (12, 14).

Previous results in our laboratory demonstrated that these unusual rearrangements (D-D fusions and D inversions) occur more frequently in the Ab repertoire of newborn autoimmune-prone MRL/MpJ+/+ (MRL) mice when compared with C3H/HeJ (C3H) normal controls (15). In addition, the autoimmune strain used more frequently upstream D genes and the most D-distal J_{H} genes. In comparison, the nonautoimmune C3H mice tended to use the most 3′ D gene, DQ52, and the most D-proximal J_{H} gene, J_{H} 1. This suggests that MRL mice may have undergone secondary gene rearrangements that delete evidence of a primary rearrangement. Thus, the MRL strain may be prone to generate secondary gene rearrangements at the heavy chain locus that are more likely to include atypical junctions (15).

In this study, we wanted to determine whether similar rearrangement patterns and atypical junctions are also present in the MRL adult pre-B cell repertoire. Thus, we analyzed the heavy chain gene rearrangements in B220^{+}IgM^{+} cells in both MRL and C3H mice. Again, the MRL strain demonstrated an increased frequency of unconventional Ig heavy chain rearrangements when compared with C3H mice. However, the pattern of D and J_{H} use was different in adult pre-B cells compared with newborns. Therefore, we propose a model of secondary gene rearrangements at the heavy chain locus in MRL mice, which explains the differences in gene usage between the newborn and adult libraries and the frequent occurrence of atypical rearrangements in MRL mice.

Materials and Methods

Mice

Male and female animals from the lupus-prone MRL/MpJ^{+}+ (MRL) and the nonautoimmune C3H/HeJ (C3H) strains were obtained from The Jackson Laboratory (Bar Harbor, ME; Ref. 16). C3H was chosen as a control for MRL because the MRL strain is partly derived from C3H and both strains share the same Ig_{H}j allotype. The animals were maintained in our facility and sacrificed at 3 mo of age.

Flow cytometry

Bone marrow cells were obtained as previously described (17, 18). Briefly, a single-cell suspension was obtained by flushing femurs from five mice with ice-cold staining medium (deficient RPMI 1640 medium without 1-glutamine or phenol red (Cellgro, Herndon, VA) containing 10 mM HEPES, 3% FBS, and 0.1% NaN_{3}). The cells were then mixed with a 1 ml syringe and treated with 0.165 M NH_{4}Cl to eliminate erythrocytes. After washing with staining medium, the bone marrow cells were incubated with FITC-RA3.6B2 anti-B220 mAb (Southern Biotechnology Associates, Birmingham, AL) and PE-goat anti-mouse IgM (Jackson Immunoresearch, 0022-1767/00/$02.00

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3 Abbreviations used in this paper: CDR3, complementarity-determining region 3; MRL, MRL/MpJ+/+ mice; C3H, C3H/HeJ mice; RF, reading frames.

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West Grove, PA) for 15 min at 4°C, followed by 3 washes in staining media. Flow cytometric analysis and sorting of the B220+ IgM- bone marrow cells was conducted at the Temple University FACS core facility using an Epics Elite equipped with an autocloning attachment. The sorted fractions were reanalyzed to verify that their purity exceeds 95%. We further verified that there was virtually no (<0.34%) light chain expression in the sorted pre-B cell populations (data not shown; Ref. 19).

Primers
DNA was extracted from the B220+ IgM- bone marrow cells by proteinase K digestion and was then subjected to PCR amplification. The PCR primers and protocols were adapted from previously described methods (20–22). Amplifications were conducted using nested PCR as follows. In the first PCR, DNA was amplified with a mixed set of antisense JH primers and protocols were adapted from previously described methods (15) and either a sense J558 primer (GGGCAAGGCCACATTGACTG TAG) or a sense 7183 primer (GGGGCAGTTCACCATCCTCGAG). In the second PCR, 10 µl of the first PCR product was reamplified with primers that were internal to those used in the first PCR. The internal JH primers were identical with those previously used (15), while the internal VH primers were as follows: J558 sense (GTAGACAAATCCTCCAGCA CAGC) and 7183 sense (GAGACAATGCCAAGAACCCCTG). All PCR were conducted in a volume of 50 µl containing 1.5 mM MgCl2, dNTPs (200 µM each), primers (0.4 µM each), and 2 units Taq DNA polymerase. Cycling conditions were as follows: an initial 5-min denaturation at 94°C, 35 cycles (1 min at 94°C, 2 min at 50°C, 1 min at 72°C), and a final 5-min extension at 72°C. After the second (internal) PCR, amplification products were separated on a 1% agarose gel and bands of the appropriate size were isolated with the QIAEX II agarose gel extraction kit (Qiagen, Chatsworth, CA). The purified products were directly ligated into the pGEM-T vector (Promega, Madison, WI) and transformed into JM109 (Promega, San Diego, CA).

Statistical analyses
All analyses were conducted with the Prism software (Release 2.01; GraphPad, San Diego, CA).

Results

Pre-B V_H-D-J_H rearrangement library

B220+ IgM- pre-B cells were sorted from MRL and C3H bone marrow. Ig gene rearrangements were amplified using a nested PCR with primers specific for the 7183 and J558 V_H germline genes and for each of the four J_H genes. A total of 180 MRL and 153 C3H V_H-D-J_H clones were sequenced and analyzed for this study. There was no significant difference in the frequency of productive vs nonproductive rearrangements between both strains (73% productive rearrangements in MRL mice and 67% in C3H mice). These sequences are available from GenBank under accession numbers AF265709 to AF266041.

Atypical rearrangements

Conventional heavy chain Ab gene rearrangements result from the combining of single VH, D, and J_H genes in the appropriate orientation, whereas we define atypical rearrangements as D-D fusions and D inversions. D-D fusions are possible due to a cryptic heptamer embedded within most D genes that simulates a 23-bp recombination signal sequence (26). Further, D inversions result from the fact that D genes are flanked by symmetric recombination signal sequences with a 12-bp spacer that allows recombination in either orientation.

As previously observed in a newborn V_H-D-J_H rearrangement library (15), MRL mice displayed more atypical rearrangements than their Igh allotype-matched C3H controls (48/180 for MRL vs 25/153 for C3H) (p = 0.016 using the Fisher’s exact test; Table I). This difference was mostly due to D-D fusions in that MRL mice exhibited 44 D-D fusions, whereas C3H mice had only 15 D-D fusions. The increased frequency of atypical junctions in MRL mice was observed for both productive and nonproductive rearrangements (Table I).

D and J_H gene usage

The D genes at the heavy chain locus can be divided into four gene families. The DFL16 family is comprised of two D genes,
DFL16.1 and DFL16.2, which are \( V_H \) proximal. The next \( D \) family, DSP2, is the largest and is composed of 10 known members, including DSP 2.x, which we have observed to be frequently used in MRL and C3H mice (15). The last two families are composed of single genes, DST4 and DQ52. The DQ52 gene is the most \( J_H \)-proximal \( D \) gene, mapping only 700 bp upstream of \( J_H \) 1.

In a previous study of newborn MRL and C3H mice, we observed a clear difference in the pattern of \( D \) gene usage (15). Although C3H mice tended to use DQ52 in the majority of their heavy chain rearrangements, MRL mice used mostly members of the more upstream \( D \) gene family, DSP2 (15). In the present study, there was also a significant difference in the overall distribution of \( D \) genes used between the MRL and C3H pre-B cells (Table II), although the difference was less pronounced than in the newborn repertoire. Most notably, MRL mice used the \( J_H \)-distal DFL16 gene family more often than their C3H counterparts. The pattern of \( D \) gene usage was similar for productive and nonproductive rearrangements in MRL and C3H mice (Table II).

As in the newborns, both strains again frequently recombined a particular DSP family member, DSP 2.x, in most of their normal and atypical heavy chain gene rearrangements. MRL mice used this particular \( D \) segment in 16% of both types of rearrangements, whereas the C3H mice recombined this particular gene in 23% of conventional rearrangements and 28% of their atypical rearrangements (data not shown). The predominance of the DSP 2.x gene is interesting in that it has frequently been observed in Abs with autoreactive specificities (27–31). The repeated usage of DSP 2.x may be a property of the \( Ighj \) heavy chain allotype shared between the strains. DSP2.x may be unique to this allotype or it may possess distinct recombination signal sequences or regulatory elements that favor recombination to this particular gene.

Productively rearranged heavy chain \( D \) genes can be read in three different reading frames (RF; Ref. 32, 33). The majority of rearrangements use RF1 which encodes a glycine-tyrosine-rich neutral amino acid sequence. Often, RF2 usage results in the expression of a truncated \( D_4 \) protein that is selected against, whereas RF3 frequently contains a premature stop codon (34). As in the newborn library, both MRL and C3H mice favored RF1 and there was no significant difference between the two strains (Table III).

There was also an overall significant difference in \( J_H \) gene usage in the MRL and C3H libraries (Table IV). Most notably, both strains overused \( J_H \) 3, a bias that was not observed in the newborn library (15). The \( J_H \) 3 over-representation could be due to the fact that different primers were used in the present study and that certain primer pairs may favor amplification over others. This assay bias, although theoretically possible, is unlikely in that the adult library was created using the same primers for the \( J_H \) genes and only those used to amplify \( V_H \) gene families were changed. Biased \( J_H \) 3 usage has also been observed in other repertoire studies in autoimmune and nonautoimmune mouse strains, although it is yet unclear why the over-representation was detected (35, 36).

**\( N \) and \( P \) nucleotides**

The junctional diversity of \( Ab \) genes is enhanced through the addition of \( N \) and \( P \) nucleotides. \( N \) nucleotides are added by TdT, which is absent in newborn mice and up-regulated in the adult (20, 37). As expected, almost all of the adult pre-B cell sequences contained \( N \) nucleotide additions (Table V), whereas newborn MRL and C3H mice showed a limited number of \( N \) nucleotides (15). Further, there was no significant difference in the amount of \( N \) nucleotide additions between the two strains. MRL mice possessed an average of 5.71 (± 3.54) \( N \) nucleotides per CDR3, and C3H mice had an average of 5.34 (± 3.31) \( N \) nucleotides per CDR3. \( P \) nucleotides are presumably created by uneven cutting of DNA that resolves hairpins created by intermediate coding joints of the Ab genes (38). As with \( N \) nucleotide additions, there was no significant difference between MRL and C3H adult pre-B cells in the frequency of \( P \) nucleotide additions (Table V).

**CDR3 properties**

The deduced amino acid sequences for the productive MRL and C3H \( V_H \)-\( D \)-\( J_H \) rearrangements are listed in Fig. 1. The heavy chain CDR3 is critical for interaction with Ag. In particular, specific residues such as arginine have been linked to mediating CDR3 binding of Abs to DNA or DNA/histone complexes (4, 10, 29). Overall, the CDR3 sequences deduced from productive pre-B cell rearrangements displayed similar properties for both strains (Table V).
FIGURE 1. Deduced amino acid sequences from productive V<sub>H</sub>-D<sub>J<sub>H</sub> rearrangements in MRL and C3H mice. The amino acid sequences include the second invariant V<sub>H</sub> cysteine (position 92) and the invariant J<sub>H</sub> tryptophan (position 103; Ref. 24). MRL sequences start with the letter M, while C3H sequences begin with the letter C.
FIGURE 2. Atypical rearrangements in autoimmunity: a putative mechanism involving secondary Ig heavy chain rearrangements. A, At the pro-B cell stage, D to JH rearrangements take place on both alleles. B, The first (upper) allele undergoes a VH to DJH rearrangement. If the rearrangement on the first heavy chain allele is productive, the B cell will proceed through normal maturation stages. Subsequent D to JH rearrangements continue on the other (lower) allele, resulting in a D-D fusion (C) or a biased usage of upstream D and downstream JH sequences (not depicted). D, The productive rearrangement on the first allele is inactivated (designated by the X), for instance by a nonproductive VH replacement, but the doomed B cell may be rescued by a productive VH to D-DJH rearrangement on the second allele. E, The B cell now possesses a new productive rearrangement on the second allele that contains a D-D fusion or is biased in its D and JH usage. A and B take place centrally whereas, in our model, the events in D and E occur in the periphery.

V, Fig. 1). For instance, there was no significant difference in length between the MRL and C3H CDR3 sequences (12.14 ± 2.562 amino acids for MRL and 11.29 ± 2.768 for C3H) and their average arginine contents were similar (0.37 ± 0.62 arginine per CDR3 for MRL and 0.31 ± 0.51 arginine per CDR3 for C3H). Taken together, these data suggest that at least some of the events leading to the presence of antichromatin CDR3 sequences in MRL mice occur later than the pre-B cell stage.

Discussion

The development of systemic lupus erythematosus is characterized by the presence of antinuclear Abs capable of binding DNA and nucleosomes (1–4). These Abs differ from conventional Abs in that they frequently possess positively charged amino acids such as arginine in the CDR3 of their heavy chain (4, 10). Atypical VH-D-JH rearrangements such as D-D fusions and D inversions favor the presence of these residues allowing binding to nuclear epitopes (10). Previous results in our laboratory revealed that in the lupus-prone MRL mouse strain, an increased number of B cell precursors with atypical VH-D-JH rearrangements may favor the production of antinuclear Abs (15). The analysis of PCR-amplified heavy chain rearrangements from newborn mice showed that this strain possesses more unusual rearrangements of D-D fusions and D inversions than C3H controls (15).

In this study, we wanted to evaluate the VH-D-JH rearrangements amplified from adult MRL and C3H B220 high IgM high pre-B cells which have not yet undergone Ag selection. Our current results confirm our earlier study (15) and suggest that MRL mice indeed exhibit an intrinsic defect that leads to the development of B cell precursors with atypical Ig heavy chain gene rearrangements. It is interesting that most of the difference between the strains is due to D-D fusions in that MRL mice had 44 D-D fusions compared with 15 in C3H. This observation is revealing in that while D inversions are uncommon, they are “conventional” in that they follow established recombination rules. However, D-D fusions may be more predictive of an inherent recombination defect because of the unconventional nature of this particular rearrangement which breaks the 12/23 rule.

When comparing the current results to our previous report (15), we observed that the bias in upstream D and downstream JH gene usage previously seen in newborn MRL rearrangements was not present in the adult libraries. A significant difference is that the neonatal libraries were unselected and contained a large proportion of mature B cells, whereas the adult libraries were restricted to pre-B cells. Therefore, the difference in D and JH usage pattern between the newborn and adult libraries in MRL mice may be related to the stage of B cell maturation. Our results with the newborn library led us to propose that MRL mice may undergo more secondary D-JH rearrangements because they typically recombine more VH-proximal D genes to more distal JH genes when compared with C3H controls. Because of this biased gene usage and the presence of atypical rearrangements in MRL mice, we hypothesized that the mechanisms of atypical heavy chain gene rearrangement and secondary gene rearrangements were related. In a conventional secondary rearrangement, an upstream D gene recombines to a downstream JH gene, thus eliminating the intervening DNA sequence and any evidence of a primary rearrangement. In a D-D fusion, an upstream D gene combines with a preformed D-JH rearrangement forming a D-DJH complex. However, although our MRL pre-B cells contained a significant number of D-D fusions, we did not observe the strong bias in D and JH gene usage seen in the newborn library (15). This suggests that during autoimmunity D-D fusions may arise from mechanisms other than incomplete secondary rearrangements. For example, VH genes could directly recombine with downstream D genes in accordance with the 12/23 rule. This VH-D product may then rearrange to a downstream D-JH complex resulting in a heavy chain with a D-D fusion (26). It has also been suggested that TdT may play a role in both the production of anti-DNA Abs and Ig gene usage. TdT-mediated N nucleotide additions increase the length of the CDR3 thus increasing the potential to generate arginine residues in the Ag binding site (39). C57BL/6-Fas(lpr) mice deficient...
in TdT had significantly fewer arginines in their CDR3 and a lower frequency of anti-DNA Abs (39). Further, Tuillelon and Capra (40) recently demonstrated that the presence or absence of TdT can modulate the choice of V<sub>H</sub>, D, and J<sub>H</sub> use independent of B cell Ag stimulation. Nevertheless, the mechanism by which TdT could affect Ig gene choice during rearrangement remains unknown and our data do not indicate a quantitative difference in TdT activity between MRL and C3H mice.

The analysis of the V<sub>H</sub>-D-J<sub>H</sub> libraries from newborn (15) and adult pre-B cells (this study) leads us to propose a model of D-J<sub>H</sub> revision in the periphery of autoimmune mice (Fig. 2). After D to J<sub>H</sub> rearrangements on both alleles at the pro-B cell stage, the first allele undergoes a V<sub>H</sub> to D<sub>H</sub> rearrangement (Fig. 2, A and B). If the rearrangement on the first heavy chain allele is productive, the B cell will proceed through normal maturation stages. However, subsequent D to J<sub>H</sub> rearrangements may continue on the other allele, resulting in a biased usage of upstream D and downstream J<sub>H</sub> sequences or in D-D fusions (Fig. 2B; Ref. 41). Later in B cell life, the productive rearrangement on the first allele is inactivated (for instance by a nonproductive V<sub>H</sub> replacement), but the B cell may be rescued by a productive V<sub>H</sub> to D<sub>H</sub> (or V<sub>H</sub> to D-D<sub>H</sub>) rearrangement on the second allele (Fig. 2D; Ref. 42). The B cell now possesses a new productive rearrangement on the second allele that will be biased in its D and J<sub>H</sub> usage and may also contain D-D fusions that will predispose the B cell to react with DNA or DNA-protein complexes (Fig. 2E).

This model obviously applies only to situations where the first V<sub>H</sub>-D-J<sub>H</sub> rearrangement is productive. This situation occurs often enough to make our model meaningful and biologically relevant. The model also proposes that B cell development in autoimmune mice will manifest some unique properties. The first is that D to J<sub>H</sub> rearrangements will be ongoing on the second allele, even after a successful V<sub>H</sub> to D<sub>H</sub> rearrangement on the first allele. This is not unlikely, especially when one considers that secondary D to J<sub>H</sub> rearrangement is mechanistically similar to a secondary V<sub>K</sub> to J<sub>k</sub> rearrangement (8, 9). Secondary light chain rearrangements occur routinely during the process of receptor editing, and recent work suggests that both central and peripheral editing can occur more frequently in lupus patients (43–45). Further, D-J<sub>H</sub> replacements were observed in an Abelson murine leukemia virus-transformed B cell line providing evidence that an initial D-J<sub>H</sub> recombination does not prevent secondary recombinations (41).

Another critical element of our model is the rescue of a doomed B cell by a V to D-J<sub>H</sub> rearrangement on the second heavy chain allele. In our model, the B cell should be doomed because its initial V<sub>H</sub>-D-J<sub>H</sub> rearrangement has become inactivated. A likely reason for this event could be a stop codon resulting from the somatic hypermutation process, but this may also result from an additional rearrangement event within the V<sub>H</sub>-D-J<sub>H</sub> region. Most V<sub>H</sub> genes contain embedded heptamers that may serve to mediate V(D)J recombination-type events (46). When the heptamer located in the 3′ region of the V<sub>H</sub> segment is used, the rearrangement may be functional, leading to a so-called V<sub>H</sub> replacement, but rearrangements at other heptamers will be nonfunctional and remain undetected because they result in the death of the B cell (47, 48). Studies with transgenic mice as well as recent human work suggest that such recombination within pre-existing V<sub>H</sub>-D-J<sub>H</sub> rearrangements are more common than previously thought (49–52). RAG1 and RAG2 are likely to be required for these types of V<sub>H</sub> replacements. This is supported by studies showing that these enzymes can be expressed in the periphery although it is unclear whether this is due to reactivation or continuous expression in B cells that have left the bone marrow only recently (53–56). Irrespective of the mechanism, the involvement of RAG1 and RAG2 in the recombination on the first allele would indicate that they are also available to mediate the recombination on the second allele.

Another critical factor is the existence of apoptosis defects during lupus. Although no single consistent defect has been identified, there is evidence that apoptosis is impaired in lupus individuals (57–60). This decrease in apoptosis efficiency may be critical in this situation because it would allow enough survival time for the productive rearrangement to take place on the second allele, whereas a normal B cell would otherwise die before being rescued. An additional important element for autoimmunity is the timing of this secondary rearrangement. As mentioned above, the rearrangements on the second allele will be biased toward the use of certain D and J<sub>H</sub> genes. This is of particular relevance during systemic autoimmunity where self-reactive Abs often express the downstream J<sub>H</sub> 4 gene, an observation consistent with our hypothesis that successive rearrangements may have taken place on the second allele (61–64). Further, these secondary rearrangements may include D-D fusions that favor reactivity with chromatin Ags. Because these “rescue-type” rearrangements will take place in the periphery, they will not be subject to the rigorous mechanisms of central tolerance and they may even occur within a context of B cell activation, resulting in the expansion of potentially self-reactive clones.

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