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Heavy Chain Revision in MRL Mice: A Potential Mechanism for the Development of Autoreactive B Cell Precursors[1]

Kimberly D. Klonowski and Marc Monestier[2]

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. 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.**

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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 Ags 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).[3] 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 antinuclear 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 *Igh 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 media (deficient RPMI 1640 medium without L-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_4Cl 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,

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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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Table I. *Atypical rearrangements in productive, nonproductive, and total MRL and C3H V_H-D-J_H rearrangements*[a]

Strains	MRL			C3H		
	P	NP	Total	P	NP	Total
Total number of rearrangements analyzed	131	49	180	103	50	153
Total number of atypical V_H-D-J_H rearrangements	32 (24.4)	16 (32.7)	48 (26.7)	18 (17.5)	7 (14.0)	25 (16.3)
Number of D-D fusions	29 (22.1)	15 (30.6)	44 (24.4)	12 (11.7)	3 (6.0)	15 (9.8)
Number of D inversions	3 (2.3)	1 (2.0)	4 (2.2)	6 (5.8)	4 (8.0)	10 (6.5)

[a] Total atypical rearrangements are defined as D-D fusions and D inversions. Values in parentheses represent the percentage of atypical rearrangements indicated. P, Productive; NP, nonproductive.

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$^-$ subpopulation 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 J_H primers (15) and either a sense J558 primer (GGGCAAGGCCACATTGACTG TAG) or a sense 7183 primer (GGGCCGATTCACCATCTCCAGAG). 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 J_H primers were identical with those previously used (15), while the internal V_H primers were as follows: J558 sense (GTAGACAAATCCTCCAGCA CAGC) and 7183 sense (GAGACAATGCCAAGAACACCCTG). All PCR were conducted in a volume of 50 μl containing 1.5 mM $MgCl_2$, 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 cells. After blue/white selection, positive colonies were grown in 5 ml Luria-Bertani/Amp for plasmid purification. The inserts were sequenced using the fmol DNA sequencing system (Promega) with a ^{32}P-labeled primer complementarity to the T7 promoter (GTAATACGACTCACTAT AGGGC). Virtually all plasmids contain a V_H-D-J_H rearrangement and their sequences were analyzed using the GCG program by comparison with known D and J_H germline sequences (23–25). A minimum of four contiguous identical nucleotides was required for assignment to a germline D sequence.

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 gene families 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 V_H, 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 an 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,

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Table II. *D family usage in productive, nonproductive, and total MRL and C3H V_H-D-J_H rearrangements*[a]

Strains	MRL			C3H		
	Productive	Nonproductive	Total	Productive	Nonproductive	Total
DSP	63 (48.1)	20 (40.8)	83 (46.1)	64 (62.1)	33 (66.0)	97 (63.4)
DFL16	19 (14.5)	8 (16.3)	27 (15.0)	5 (4.9)	6 (12.0)	11 (7.2)
Q52	5 (3.8)	1 (2.0)	6 (3.3)	7 (6.8)	4 (8.0)	11 (7.2)
ST4	7 (5.3)	4 (8.2)	11 (6.1)	6 (5.8)	2 (4.0)	8 (5.2)
Others	37 (28.2)	16 (32.7)	53 (29.4)	21 (20.4)	5 (10.0)	26 (17.0)

A minimum of four identical contiguous nucleotides was required for assignment to a given D germline gene. Others refers to D segments that are too short to be identified or D-D fusions. Values in parentheses indicate the percentage of D family use.

Table III. *RF usage in MRL and C3H productive V_H-D-J_H rearrangements*[a]

Strains	MRL	C3H
RF1	70 (85.4)	62 (86.1)
RF2	3 (3.7)	1 (1.4)
RF3	9 (11.0)	9 (12.5)

[a] Only V_H-D-J_H rearrangements using single D genes from the SP2 or FL16 families were included since there is no established bias in Q52 or ST4 RF usage. Values in parentheses indicate the percentage of RF use.

Table V. *Frequency of MRL and C3H V_H-D-J_H rearrangements with homologous junctions and N or P nucleotide additions*[a]

Strains	MRL	C3H
Total number of rearrangements analyzed	180	153
Number of rearrangements with homologous junctions	40 (22.2)	28 (18.3)
Number of rearrangements with N nucleotides	175 (97.2)	146 (95.4)
Number of rearrangements with P nucleotides	69 (38.3)	67 (43.8)

[a] The values in parentheses indicate the percentage of homologous junctions and N or P nucleotides for each strain.

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 most often 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 *Igh j* 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 Dμ 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 ($\pm$ 3.54) N nucleotides per CDR3, and C3H mice had an average of 5.34 ($\pm$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

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Table IV. *J_H germline gene usage in productive, nonproductive, and total MRL and C3H V_H-D-J_H rearrangements*[a]

	MRL			C3H		
Strains	Productive	Nonproductive	Total	Productive	Nonproductive	Total
J_H1	17 (13.0)	2 (4.1)	19 (10.5)	7 (6.8)	3 (6.0)	10 (6.5)
J_H2	25 (19.1)	15 (30.6)	40 (22.2)	10 (9.7)	10 (20.0)	20 (13.1)
J_H3	83 (63.4)	32 (65.3)	115 (63.9)	75 (72.8)	34 (68.0)	109 (71.2)
J_H4	6 (4.6)	0 (0)	6 (3.3)	11 (10.7)	3 (6.0)	14 (9.2)

[a] Values in parentheses are the percentage of J_H use.

MJ-1 CATGYDYDDEAWFAYW
MJ-4 CTSYSTELGFAYW
MJ-6 CAFYYYDGRPPWFAYW
MJ-7 CARSGGNYGSGGAYW
MJ-9 CARPLYDGSYVLAYW
MJ-10 C?KSYDEGFDYW
MJ-11 CASGGAYW
MJ-12 CARYSSGYDWFAYW
MJ-13 CARDYYDGSPWFAYW
MJ-14 CARRDGYYLAYW
MJ-20 CARRWIPYSNSYYYAMDYW
MJ-23 CARLIYYGDYHYFDYW
MJ-27 CARSPWFAYW
MJ-28 CARSLLLRWYPWFAYW
MJ-29 CARKDYSNYFDYW
MJ-32 CARNRNGNPYAMDYW
MJ-33 CARRRGYDGYPAWFAYW
MJ-34 CASSYYSNYVFPWFAYW
MJ-39 CARWGYDGRRFAYW
MJ-42 C?RDGCYWYFDVW
MJ-43 CARAGSSFAYW
MJ-44 CARGWLYYGNSDWYFDVW
MJ-45 CAREDSSGYPFAL?G
MJ-46 CARLFAY?G
MJ-49 CARQ?TARATQAWFAY?G
MJ-53 CARRPYYYDG?YLWFDVWG
MJ-54 CAALPPYYYDGSYWFAYWGQ
MJ-56 CASHYGDLAWFAYWGQ
MJ-57 CARGYDGSPAWFAYWG
MJ-59 CARRQWGYWGQ
MJ-60 CARGGYYSNYLFAYWGQ
MJ-61 CARRSLMVTTGYFDYWG
MJ-62 CARGGIYYYDGSYVDYWGQ
MJ-63 CIYYYDGSSTYPWYFDVWGA
MJ-64 CTIEGYYYDGSFYWYFDVWG
MJ-65 CTIKRRLLRFAYWGQ
MJ-68 CARDSYYSYDAWFAYWGQ
MJ-71 CKRSTLYFDYWGQ
MJ-72 CARVFAYWGQ
MJ-73 CTINYYDGSPWFAYWGQ
MJ-76 CARGSDYYDGSYSAYWG
MJ-77 CARDSYYSYDAWLAYWG
MJ-78 CARRGYDGSWFAYWG
MJ-79 CAREGYSNWFAYWGQ
MJ-82 CARGGGNYVWFAYWGQ
MJ-83 CARSLLRWSSFAYWGQG
MJ-84 CAREGDGYYFDYWG
MJ-85 CGRPGYPWYFDVWG
MJ-86 CARDYYDGSSFAYWGQ
MJ-88 CTYGDYVDYWGQ
MJ-90 CAREWETGAYWG
MJ-95 CTTDGYPAWFAYWGQ
MJ-96 CASRPRAWFAYWGQ
MJ-98 CARPLNGAYWG
MJ-99 CARPYSYYSYPSYWGQ
MJ-100 CA?EGYGDDYAMDYWG
MJ-101 C?RVGAYYTYIDYWG
MJ-102 CATPPYYSNYLFAYWG
MJ-103 CARDHYSFDYWG
MJ-105 CARWHYYDGSYLDYWGQ
MJ-108 CARFYYGNSYWYFDVWGA
MJ-110 CALDGYYVRTWFAYWGQ
MJ-113 CKRSHRFYYDGSPAGMDYWG
MJ-115 CATYYSNYLFAYWGQ
MJ-116 CIYYYDGSSTYPWYFDVWG
MJ-123 CARGDSNSYFDYWG
MJ-126 CARDDGYYKFAYWGQ

M7-1 CARHSWFAYWG
M7-3 CARDSNWDDFDYWG
M7-4 CARDRELAWFAYWG
M7-6 CARHRYGDYVFFDYWG
M7-9 CARHTYYSNYLFAYWGQ
M7-10 CARHGMRTFFAYWGQ
M7-12 CARHRGYGYFDYWGQ
M7-19 CARHHYSNWYFDVWGA
M7-20 CAEGWLPPWFAYWGQ
M7-23 CARDYDGIFDYWGQ
M7-27 CARPYYDGSFAYWGQ
M7-28 CASAFYYDYPLFAYWGQ
M7-34 CARQNYGDHEGYFDVWGA
M7-36 CARHEGLRREGLDYWG
M7-37 CARHPYDGYYVGFAYWGQ
M7-40 CARHPWFAYWGQ
M7-41 CARHDYGKGAYWGQ
M7-43 CARPGWLLLPPSWFAYWG
M7-46 CARQGYYSNYPFAYWGQ
M7-47 CASRAYSNYDWFAYWGQ
M7-50 CAREGHDEDYWG
M7-53 CARHDGYYQAGFAYWG
M7-54 CASPYGDYAYWGQ
M7-55 CARRGTTMVDWYFDVWG
M7-60 CARHPHYYDGSYRYFDVWGA
M7-63 CANYYSNYEEVWFAYWG
M7-66 CA?MIYYYDGPFDYWG
M7-67 CARGYYSYYSYDVDYAMDYWG
M7-69 CARQPYDGYSWFAYWGQ
M7-72 CARGETMVALYWYFDVWG
M7-75 CARQESNYGAWFAYWGQ
M7-77 CARPRYSNFAYWGQ
M7-80 CARQGGWLLDYWGQ
M7-81 CARRGWFKRGPAWFAYWG
M7-82 CAREGIRGFDYWG
M7-88 CARHDRVLFAYWG
M7-91 CARDGYDVSWFAYWGK
M7-92 CARHGYYGWGQ
M7-95 CARPHLYYYDGSWGFAYWGQ
M7-96 CARQGNYVYFDVWGA
M7-101 CARDEEATPWFAYWG
M7-102 CASYSYYSSYWCFDVWG
M7-105 CARHDGYYEAGFAYWGQ
M7-107 CARRYDGPYWYFDVWGA
M7-108 CARRDYADAMDYWGQ
M7-109 CARLLGHGFAYWGQ
M7-110 CARPLLIYYGDSAWFAYWG
M7-118 CANSFAYWGQ
M7-120 CARHNSNYVGDWFAYWGQ
M7-121 CASYYYDGSYYFDYWG
M7-124 CARWLLRGFAYWGQ
M7-126 CARGGALFAYWG
M7-127 CASAFYYDYPLFAYWGQ
M7-128 CARPGWLLLPPSWFAYWG
M7-134 CARGSDYYDGSYSAYWG
M7-135 CATGSRPRYFDVWG
M7-136 CARDYYDGSSFAYWGQ
M7-139 CARKGKRAYWG
M7-142 CARHGVVTPWFAYWGQ
M7-143 CARRANWDAWFAYWGQ
M7-144 CASHGYYWFAYWGQ
M7-150 CARHRGYGYFDYWG
M7-151 CARRYYSYYSYDGFAYWGQ
M7-156 CAREGGPDSSGYDWFAYWGQ

C7-2 CARPDYSNYLSWFAYWG
C7-4 CARHNYPFAYW?Q
C7-9 CARQRGDYDDWYFDVWG
C7-11 CARLDDGSYPYWYFDVWG
C7-14 CARLKVRGFAYWG
C7-16 CARGVYYGDYVGYFDVWG
C7-17 CARPVGNPFAYWG
C7-20 C?RHDFDVWG
C7-24 CARLYYDYHWG
C7-26 CARQGVARDAGYYYAMDFWG
C7-28 CARRFYYWG
C7-34 CASRNWDVEAMDYWG
C7-35 CARPAYYSNGGFAYW?
C7-39 CARQDYDGSYGFAYWG
C7-41 CARHYGDYVGGAWFAYWG
C7-44 CAW?DYSNYLSWFAYWG
C7-48 CARGRGYSNYVFDYWG
C7-50 CARHGYDGYYFDYWG
C7-52 CA?PLANWD?WFAYWG
C7-56 CARQKGDGYYSFAYWG
C7-57 CARHYSFAYWG
C7-58 CARTIGWLPSWFAYWG
C7-68 CARHENSNYLAWFAYWG
C7-78 CARNWDGFAYWG
C7-80 CARRAGYWG
C7-82 CARQGGDYDDWYFDVWG
C7-83 CASPDYWG
C7-86 CARDQTGDWYFDVWG
C7-87 CARTWYMSWFAYWG
C7-88 CARPSNYSLFAYWG
C7-89 CARDDGYYLFAYWG
C7-91 CARYYSYPYYAMDYWG
C7-94 CARDRGYPLPYWG
C7-97 CAREGLDYDYDWYFDVWG
C7-99 CAPGYPAWFAYWG
C7-100 CARLYYDGRERAYFDYWG
C7-105 CARQKGDGYYSFAYWG
C7-109 C?RYYS?PYYAMDYWG
C7-113 CASSYYSNHAWFAYWG
C7-117 CARHNDGSYGFAYWG
C7-119 CARDLSLAYWG
C7-123 CARRLSWFAYWG
C7-124 CARRGAYYSNTWFAYWG
C7-125 CARHYSNYGVAYWG
C7-126 CARGGDIWFAYWG
C7-127 CARDRGTGTGFAYWG
C7-128 CARLEGYFLAYWG
C7-138 CARDYYSYDVRFAYWG
C7-153 CARSSNYGYYYAMDYWG
C7-155 CARQAYYSNPFAYWG
C7-156 CARLANWDVEAYWG
C7-160 CAREALSYFDYWG
C7-161 CARHCLYGDYFDYWG
C7-162 CARHENSNHLAWFAYWG
C7-165 CAREINYSNSWFAYWG
C7-171 CARRYYDGSYGFAYWG
C7-176 CARQ?YSYYSYDDWFAYWG

CJ-2 CARGGLSVFAYWG
CJ-3 CARSRELYYFDYWG
CJ-4 CARLWSNYVAWFAYWG
CJ-8 CARNYYDGSYWFAYWG
CJ-9 CTDGFAYWG
CJ-10 CAGGNYEAYWG
CJ-12 CTIGGDSSGYGWG
CJ-14 CALDSSGPWFAYWG
CJ-18 CARGAYWG
CJ-22 CKLDYDYPWFAYWG
CJ-25 CARIPPELGRKAYWG
CJ-26 CARCSNRPWFAYWG
CJ-35 CANRNHISWFAY?G
CJ-37 CARGGSNYGGFAYWG
CJ-40 CARGDYGNVPYWG
CJ-42 CAS?AYYSYYSYEFAYWG
CJ-49 CAREEGGNYRYWYFDVWG
CJ-58 CASYDYDPFAYWG
CJ-64 CARGRGWLPWFAYWG
CJ-71 C?RSHSYYSYDGFDYWG
CJ-74 CAGWQGGFAYWG
CJ-75 CAIRKIYTMEGFAYWG
CJ-78 CTIRSYSYYSYDGGFAYWG
CJ-79 CARKSNYAYWG
CJ-82 CSLSGDYAMDYWG
CJ-83 CARWDSNLAYWG
CJ-86 CAPYGDF?AYWG
CJ-87 CARRDYSNYEGSAMDYWG
CJ-94 CARLFAYWG
CJ-96 CARPAYWGQ
CJ-97 CASRCLLAYWG
CJ-98 CARELPYYYAMDYWG
CJ-99 CAREAYWG
CJ-106 CARDGYNLYAYWG
CJ-108 CARDSNS?FDYWG
CJ-109 CARRGLYYYYAMDYWG
CJ-115 CVQAYYSNYGFAYWG
CJ-118 CADLAGAWFAYWGQ
CJ-127 CARRSYYSYDLAYWG
CJ-131 CARSLHFAYWG
CJ-134 CANSYYSYDVRFAYWG
CJ-139 CAYYDYDWFAYWG
CJ-143 CARRHYRY?WFAYWG
CJ-144 CARRD?YIFAYWG
CJ-145 CARLRSYYSYDGGAWFAYWG
CJ-148 CARNLFFAYWG

FIGURE 1. Deduced amino acid sequences from productive V_H-D-J_H rearrangements in MRL and C3H mice. The amino acid sequences include the second invariant V_H cysteine (position 92) and the invariant J_H tryptophan (position 103; Ref. 24). MRL sequences start with the letter M, while C3H sequences begin with the letter C.

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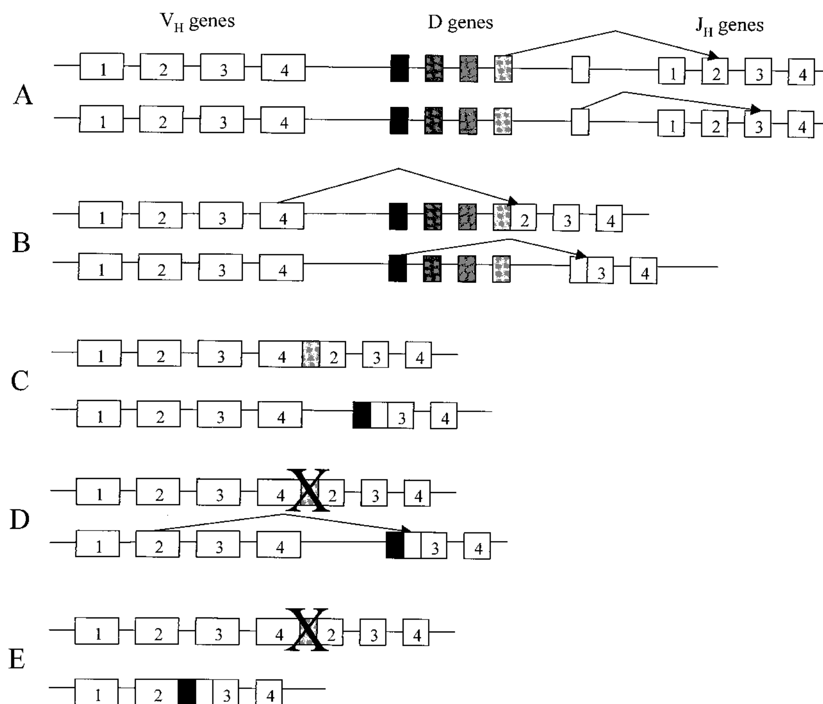

FIGURE 2. Atypical rearrangements in autoimmunity: a putative mechanism involving secondary Ig heavy chain rearrangements. *A*, At the pro-B cell stage, D to J_H rearrangements take place on both alleles. *B*, The first (upper) allele undergoes a V_H to DJ_H rearrangement. If the rearrangement on the first heavy chain allele is productive, the B cell will proceed through normal maturation stages. Subsequent D to J_H rearrangements continue on the other (lower) allele, resulting in a D-D fusion (*C*) or in a biased usage of upstream D and downstream J_H sequences (not depicted). *D*, The productive rearrangement on the first allele is inactivated (designated by the X), for instance by a nonproductive V_H replacement, but the doomed B cell may be rescued by a productive V_H to D-DJ_H 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 J_H 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 V_H-D-J_H 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 V_H-D-J_H 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 V_H-D-J_H rearrangements amplified from adult MRL and C3H $B220^+IgM^-$ 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 J_H 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 J_H 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-J_H rearrangements because they typically recombine more V_H-proximal D genes to more distal J_H 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 J_H 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-J_H rearrangement forming a D-DJ_H 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 J_H gene usage seen in the newborn library (15). This suggests that during autoimmunity D-D fusions may also arise from mechanisms other than incomplete secondary rearrangements. For example, V_H genes could directly recombine with downstream D genes in accordance with the 12/23 rule. This V_H-D product may then rearrange to a downstream D-J_H 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

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in TdT had significantly fewer arginines in their CDR3 and a lower frequency of anti-DNA Abs (39). Further, Tuaillon and Capra (40) recently demonstrated that the presence or absence of TdT can modulate the choice of V_H, D, and J_H 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_H-D-J_H libraries from newborn (15) and adult pre-B cells (this study) leads us to propose a model of D-J_H revision in the periphery of autoimmune mice (Fig. 2). After D to J_H rearrangements on both alleles at the pro-B cell stage, the first allele undergoes a V_H to DJ_H 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_H rearrangements may continue on the other allele, resulting in a biased usage of upstream D and downstream J_H sequences or in D-D fusions (Fig. 2*B*; Ref. 41). Later in B cell life, the productive rearrangement on the first allele is inactivated (for instance by a nonproductive V_H replacement), but the B cell may be rescued by a productive V_H to DJ_H (or V_H to D-DJ_H) rearrangement on the second allele (Fig. 2*D*; Ref. 42). The B cell now possesses a new productive rearrangement on the second allele that will be biased in its D and J_H usage and may also contain D-D fusions that will predispose the B cell to react with DNA or DNA-protein complexes (Fig. 2*E*).

This model obviously applies only to situations where the first V_H-D-J_H 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_H rearrangements will be ongoing on the second allele, even after a successful V_H to D-J_H rearrangement on the first allele. This is not unlikely, especially when one considers that secondary D to J_H rearrangement is mechanistically similar to a secondary Vk to Jk 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_H replacements were observed in an Abelson murine leukemia virus-transformed B cell line providing evidence that an initial D-J_H 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_H rearrangement on the second heavy chain allele. In our model, the B cell should be doomed because its initial V_H-D-J_H 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_H-D-J_H region. Most V_H 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_H segment is used, the rearrangement may be functional, leading to a so-called V_H 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_H-D-J_H rearrangements are more common than previously thought (49–52). RAG1 and RAG2 are likely to be required for these types of V_H 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 re-activation 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_H genes. This is of particular relevance during systemic autoimmunity where self-reactive Abs often express the downstream J_H 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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