Anti–DNA B Cells in MRL/lpr Mice Show Altered Differentiation and Editing Pattern

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Abstract

We have studied the regulation of anti–DNA B cells in transgenic mice with a heavy chain transgene (3H9H/56R). This transgene codes for a heavy chain that forms anti–double-stranded DNA (dsDNA) antibody when paired with most members of the endogenous Vκ repertoire, but certain L chains, referred to as Vκ editors, do not sustain dsDNA binding in combination with 3H9H/56R. In the nonautoimmune 3H9H/56R BALB/c, most B cells generated do not bind DNA because the transgene itself is edited or is associated with a Vκ editor. A minor population of B cells (30%) bind dsDNA and express the λ1 light chain (known to sustain 3H9H/56R DNA binding). These 3H9/56R/λ1 B cells coexpress a κ editor, and we propose that the down-regulation of the anti-DNA BCR caused by the dual L chain expression may prevent activation of this κ/λ population. These κ/λ B cells are sequestered in the marginal zone. Here, we studied the influence of autoimmunity on expression and regulation of 3H9H/56R. In 3H9H/56R MRL/lpr mice, the expression of anti-dsDNA is vastly accelerated. Anti–dsDNA B cells use noneditor κs but, in addition, most anti–dsDNA B cells have edited the heavy chain transgene. λ1 B cells (without the coexpression of a κ editor) are found and the κ/λ1 MZ population is absent. Our results suggest that improper editing and failure to sequester autoreactive B cells may contribute to the breakdown of tolerance in MRL/lpr mice.

Key words: lupus • autoimmunity • isotype switch • tolerance • marginal zone

Introduction

Why tolerance fails is poorly understood, but transgenic models have narrowed the range of possibilities. Central tolerance to the facultative self-antigens, MHC in MRL-lpr/lpr mouse (MRL/lpr;* reference 1) and HEL in lpr/lpr mice (2), remains intact. Thus tolerance is broken at a later stage of development in the lpr model of autoimmunity. This leaves many possibilities because autoreactive B cells are regulated in various ways in healthy mice, for example, by inactivation or sequestration (3–5).

Anti-DNA is a good model for studying the sites at which tolerance is broken. Regulation of anti-DNAs in healthy mice takes place at several levels: anti–double-stranded DNA (dsDNA) is regulated in the same manner as anti-MHC, by receptor editing or deletion (6–8); anti–single-stranded DNA is regulated by inactivation similar to soluble hen egg lysozyme (4, 9). In addition, low affinity anti-DNAs are found at high frequencies in B1 cells (10) or the marginal zone (MZ; reference 11) and may escape self-antigen activation in these sites. The 3H9H/Vκ8 transgenes code for an anti–single-stranded DNA, and the B cells of this transgenic mouse are normally regulated by inactivation (12). On the MRL/lpr background, 3H9H/Vκ8 B cells undergo clonal expansion and mutation, indicating that tolerance by inactivation is broken in this model of autoimmunity (13).

The 3H9H/56R anti-DNA transgenic expresses several types of anti-DNAs and is a convenient model for studying the breakdown of self-tolerance in an autoimmune mouse (14). Hybridoma panels derived from LPS-activated splenic B cells of healthy mice show that the majority of B cells do not express anti-DNA because the 3H9H/56R is edited or paired with one of the few L chains that veto binding. A minor fraction of B cells express anti-DNA, but these express more than one antibody. A prominent example of such an "allelically included" B cell expresses a DNA-binding antibody made up of the 3H9H/56R and an L chain that sustains binding such as λ1 and a non–DNA-binding

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*Abbreviations used in this paper: dsDNA, double-stranded DNA; MRL/lpr, MRL-lpr/lpr mouse; MZ, marginal zone; sd-tg, site-directed transgene.

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antibody made up of the combination of 3H9H/56R and an L chain editor. These κ/λ B cells are found in the MZ B cell population (11). Thus, regulation of 3H9H56R B cells takes place by receptor editing and by an unknown mechanism that prevents these B cells from entering a secondary immune response. Consequently, these mice do not develop autoimmune disease although they do express IgM anti-DNA antibody.

The 3H9H/56R MRL/lpr mouse provides the opportunity to study whether receptor editing and the arrest of the κ/λ population are intact. Anti-DNA expression in the transgenic MRL/lpr mouse is clearly influenced by the 3H9H/56R. The transgene vastly accelerates the rate at which IgG anti-DNAs appear. In this paper, we have analyzed the B cells and a hybridoma panel from an 8-wk-old 3H9H/56R MRL/lpr mouse that expresses high titers of IgM and IgG anti-DNA to determine the source of these autoantibodies.

Materials and Methods

Mice. Generation of site-directed heavy chain transgenic 3H9H/56R BALB/c mice is described in Li et al. (14). The line was backcrossed for >10 generations to MRL/lpr mice purchased from The Jackson Laboratory.

Flow Cytometry. Mice used in FACS® analysis were between 6 and 8 wk old. Single cell suspensions were prepared from spleen and stained with the following antibodies: PE-conjugated anti-κ (187.1) (Southern Biotechnology Associates, Inc.); FITC-conjugated anti-CD19 (1D3); biotin-conjugated anti-λ (R26–46); FITC-conjugated anti-CD21/CD35 (7G6); and PE-conjugated anti-CD23 (B3B4) (BD Biosciences). Biotin-conjugated anti-idiotype antibody 1.209 has been described previously (6). Biotin-conjugated antibodies were revealed by APC-conjugated streptavidin (PharMingen). Dead cells were excluded by propidium iodide staining. Stained cells were analyzed using a FACSort™ flow cytometer (Becton Dickinson) and CellQuest (Becton Dickinson) software.

B Cell Hybridoma Generation. Spleen cells from a 12-wk-old 3H9H/56R BALB/c mouse were stimulated in vitro for 3 d with 20 μg/ml LPS (Sigma-Aldrich), and unmanipulated spleen cells from an 8-wk-old 3H9H/56R MRL/lpr mouse were fused in separate experiments with SP2/0 myeloma cells. Hybridomas were plated at limiting dilution in 96-well plates, and single colonies were expanded for analysis. Genomic DNA was prepared as described previously (15).

ELISA. Antibodies secreted by hybridomas were analyzed by solid-phase ELISA as described previously (15). Plates were coated with anti-Ig and developed with alkaline phosphatase–labeled anti–mouse H and L chains (to determine Ig secretors), anti-IgM, anti-IgG, anti-κ, or anti-λ (Southern Biotechnology Associates, Inc.). The enzyme activities were revealed by the substrate *p*-nitrophenyl phosphate (Sigma-Aldrich) and optical densities were read at 405 nm. DNA binding assays were performed as described previously (7).

PCR. PCR for detection of the 3H9H/56R transgene has been described in Li et al. (14). PCR testing for homozygosity of the lpr gene is described in Chan and Shlomchik (16). Light chain rearrangement at the κ locus was analyzed using two types of PCR. Vs PCR was performed as described in Brard et al. (13). The Vs primer amplifies 80–90% of all mouse Vκ genes (17), and was used together with a J2 reverse primer (to detect Vκ/J1 rearrangement) or with a J5 reverse primer (to detect Vκ/J4 and Vκ/J5 rearrangement; reference 18). The Vκ/J2 rearrangement cannot be determined by the Vs PCR because the fusion partner SP2/0 is positive in this reaction. To compensate for that, another type of PCR was performed using L5 primer that detects 50–60% of Vκ genes (19) together with the J2 reverse primer. Cκ deletion was detected using Vs as a forward primer and RS-101 as a reverse primer (20). PCR that detects λ rearrangement was performed as described previously (15).

Heavy Chain Sequencing. To identify point mutations or VH replacements in the heavy chain, we first sequenced the PCR products of LD/CDR3 that detects the 3H9/56R heavy chain transgene (14) from hybridomas that were transgene-positive. Those hybrids that were transgene-negative can be explained by point mutations in the CDR3 of the transgene or by VH replacements. They were analyzed by an LD/JHCH PCR that uses a reverse primer downstream of CDR3 (13), and the PCR products were sequenced to compare with the transgene. For those clones that are negative for both the LD/CDR3 and LD/JHCH PCR, the PCR using VH5.3 as forward primer and JHCH primer as reverse primer was performed to reveal the VH replacement or rearrangement of the endogenous allele. The VH5.3 is a degenerate primer that can amplify ~65% of VH genes (8). The sequences and positions of all these primers have been shown in Brard et al. (13). The PCR products were gel-purified by QIAquick Gel Extraction Kit (QIAGEN), and the sequence was generated at the core facility of the Department of Molecular Biology (Princeton University).

κ Light Chain Sequencing. Total RNA was prepared from hybridomas by TRIzol (GIBCO BRL). Reverse transcription was done using the First-Strand cDNA Synthesis Kit (Amersham Biosciences). The expressed κ light chain was amplified by a forward degenerate Vκ primer (5′-GCCATGGAPRTQLWLMTSACCCAGTCTCCA-3′; reference 21) and a reverse Cκ primer (5′-TGGATGGTGGGAAGATG-3′; reference 22). The PCR products were sequenced using the Cκ primer. For those clones that express the λ light chain, the out-of-frame rearranged κ sequence was obtained by doing a Vs/J5 PCR on genomic DNA from the hybridoma.

Results

Anti-DNA Antibodies in 3H9H/56R Mice. Anti–DNA B cells are differentially regulated in healthy mice. In the conventional anti-DNA transgenics on a BALB/c genetic background, no anti-DNA is expressed (4). Significant levels of IgM anti-DNA are seen in the site-directed transgene (sd-tg) 3H9H/56R BALB/c at the age of 6–8 wk (Fig. 1). There seems to be a gradual loss of anti-DNA IgM, although some mice tested at 12 wk still have significant titer. We do not understand this difference. However, no IgG anti-DNA is expressed. Thus, the IgG anti-dsDNA characteristic of Lupus is tightly regulated in BALB/c mice.

The presence of 3H9H/56R also affects anti-DNA expression in the MRL/lpr mouse. IgM anti-DNA titers are higher in the sd-tg than the non-tg MRL/lpr. The unique and Lupus-associated features of the MRL/lpr emerge in our analysis of IgG anti-DNA. The 3H9H/56R significantly accelerates the expression of IgG anti-DNA. We see significant levels as early as 6 wk and titers peak by 8 wk,

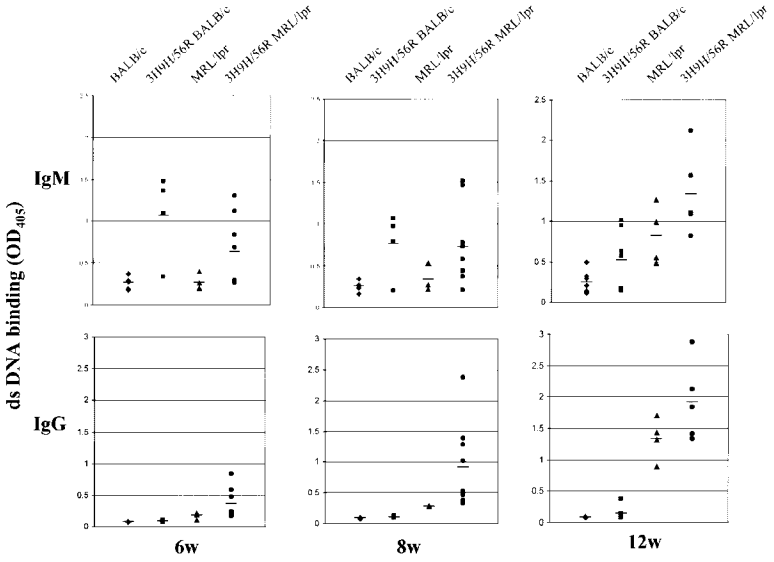

Figure 1. Anti-dsDNA in the sera of BALB/c and MRL/lpr. 3H9H/56R BALB/c, 3H9H/56R MRL/lpr, and their nontransgenic littermates were bled at 6, 8, and 12 wk. (Numbers of animals tested: BALB/c 6w, $n = 5$; 3H9H/56R BALB/c 6w, $n = 4$; MRL/lpr 6w, $n = 4$; 3H9H/56R MRL/lpr 6w, $n = 8$; BALB/c 8w, $n = 5$; 3H9H/56R BALB/c 8w, $n = 4$; MRL/lpr 8w, $n = 3$; 3H9H/56R MRL/lpr 8w, $n = 9$; BALB/c 12w, $n = 7$; 3H9H/56R BALB/c 12w, $n = 7$; MRL/lpr 12w, $n = 4$; 3H9H/56R MRL/lpr 12w, $n = 5$.) Serum samples were tested for anti-dsDNA IgM and IgG by ELISA at 1:100 dilution.

whereas our MRL/lpr express IgG anti-dsDNA by ~10–12 wk of age, and our BALB/c mice never express such autoantibodies (Fig. 1). Thus, the sd-tg influences the rate of IgG anti-DNA production in the MRL/lpr.

The 10–12-wk lag in autoantibody production in autoimmune mice has generally been interpreted to mean that tolerance deteriorates with age. Our accelerated appearance of anti-DNA in the 3H9H/56R MRL/lpr argues for a different interpretation, namely that the appearance of autoreactive B cells determines the rate at which IgG anti-DNA are expressed. Ordinarily, the probability that anti-DNAs arise and escape central tolerance is low, but this probability is vastly increased in the transgenic mouse because almost every B cell is anti-DNA from the beginning. A similar argument has been advanced to explain the relatively low frequency of expression of certain autoantibodies such as Sm in aged MRL/lpr mice (23) and might also apply to differences in the time at which different autoantibodies appear, for example, antichromatin before anti-DNA in MRL/lpr mice (24). The sd-tg might accelerate the onset of anti-DNA production by direct production of anti-DNA, but alternatives such as increasing self-antigen presentation must be considered. We have examined these alternatives by analysis of the genes coding for the anti-DNAs expressed by hybridomas from the 3H9H/56R MRL/lpr.

Genetic Control of Anti-DNA from a 3H9H/56R MRL/lpr Mouse: Hybridomas. We assumed that the early expression of anti-DNA would be directly due to the anti–DNA H chain and that the anti-DNAs would be encoded by the 3H9H/56R. This prediction was tested by sequencing anti-DNAs expressed by hybridomas derived from an 8-wk-old sd-tg. There is considerable evidence that such panels are representative of the B cells that account for the antibody profile of a mouse: among the evidence is similarity of isotype and specificities. Another argument comes from the observation that unmanipulated MRL/lpr B cells yield hybridomas indicating the presence of activated B cells (fusion efficiencies with unmanipulated B cells from healthy mice are low). Presumably these B cells are responsible for the serum antibody.

Fusions using spleen cells from unmanipulated spleen B cells from 3H9H/56R MRL/lpr mice also yielded high frequencies of hybridomas (Table I). A peculiarity of this and other MRL/lpr hybridoma panels is the low percentage of secreting lines: only 32 (42%) secrete immunoglobulin. A similar percentage was found in a second fusion of this transgenic and other anti-DNA transgenics on the MRL/lpr background (unpublished data). This percentage is much lower than in 3H9H/56R BALB/c transgenics using either in vitro LPS-activated B cells (14) or KLH-immunized B cells (8) for the fusions. Some of these hybridomas have deleted the sd-tg and a fraction of these have κ rearrangements as well, suggesting that they are B cell-derived. Others have no κ rearrangement and their cellular origin is in doubt. In this work, we have concentrated on the bona fide B cell hybridomas.

The properties of the mAb panel parallel the serum antibody pattern of 8-wk-old tg MRL/lpr mice (Fig. 1 and

Table I. *Comparison of the Hybridomas from 3H9H/56R BALB/c and 3H9H/56R MRL/lpr*

	3H9H/56R BALB/c[a]	3H9H/56R MRL/lpr[b]
Isotypes	81 IgM; 2 IgG	7 IgM; 25 IgG
dsDNA binder	10 out of 83 (12%)	11 out of 32 (34%)
3H9H56R Tg–positive clones	69 (83%)	8 (25%)
Cκ deletion	9 (11%)	6 (19%)

Results are from a single mouse of each background that expresses the sd-tg in a heterozygous state.
[a]LPS-stimulated fusion.
[b]Spontaneous fusion.

Table I). About 30% secrete anti-dsDNA. Of these, 9/11 are IgG and 2/11 are IgM. The rest of the mAbs do not bind DNA even though the majority are IgGs. A similar distribution of DNA-binding mAbs was seen in a systematic study of non-tg MRL/lpr mAbs. In this case, the non–anti-DNA mAbs had other Lupus specificities such as anti-IgG, anti-Sm, and antihistone as well as a population that was not reactive to a panel of Lupus autoantigens (25). We presumed acquisition of specificities other than anti-DNA in the sd-tg MRL/lpr would be accompanied by transgene modification such as VH replacement (8) and/or L chain editing (6).

Heavy and Light Chain Usage in Anti–DNA B Cells. We studied the heavy and light chain rearrangement in the hybridomas of transgenic mice using VH and VL region PCRs (Table II). Only 8/32 (25%) Ab secretors from 3H9H/56R MRL/lpr have an intact 3H9H/56R sd-tg. Loss of the sd-tg was due to VH and DH replacement (clones 25, 26, 46, and 75). Why the sd-tg is lost in the rest of the secretors is not understood. In the 3H9H, the sd-tg

Table II. *Characteristics of Hybridomas from 3H9H/56R MRL/lpr*

Clone	Isotype	dsDNA binding	H chain tg	L chain usage	Cκ deletion
28	IgM	+++	+	vκ21-10/J2	–
54	IgM	+++	+	vκ19-15/J5	–
5	IgG	++	+	vκba9/J5	–
48	IgG	+++	+ with mutation	vκ23-45/J2	+
57	IgG	+++	+ with mutation	λx	–
31	IgM	–	+	vκ21-D/J1	–
44	IgM	–	+	λx	+
69	IgG	–	+ with mutation	vκ21-1/J5	–
15	IgG	+	endogenous allele expression	vκcr1/J1	–
26	IgG	+	V-replacement	vκcb9/J4	–
35	IgG	+	–	ND	+
39	IgG	++	–	vκbd2/J5(OF); λ1	+
46	IgG	++	V-replacement	vκ19-23/J4	–
75	IgG	+++	V-replacement	vκhg24/J4(OF); λ1	+
17	IgM	–	–	J2	–
30	IgM	–	–	J1	–
4	IgG	–	–	J1	–
18	IgG	–	–	J2 and J4	–
21	IgG	–	–	ND	–
21	IgG	–	–	ND	–
22	IgG	–	–	J2	–
23	IgG	–	–	J2 and J5	–
25	IgG	–	V-replacement	J1	–
29	IgG	–	–	J1 and J5	–
33	IgG	–	–	J1	–
34	IgG	–	–	J1 and J5	–
47	IgG	–	–	ND	–
50	IgG	–	–	ND	–
60	IgG	–	–	ND	–
70	IgG	–	–	λ1	–
72	IgG	–	–	J1 and J4	–
73	IgG	–	–	J5	+
76	IgG	–	–	J2 and J5	–

All 32 hybridomas that secrete immunoglobulin are listed. Their isotypes (IgM or IgG) and dsDNA binding capacities are shown. The heavy chain transgene status in each hybridoma is shown as positive or negative according to the PCR described in Materials and Methods. Point mutations, VH replacements, and endogenous allele expression are also indicated. The light chain rearrangement and expression are shown according to both PCR and sequencing results. The expression of λ is confirmed by ELISA. OF, out-of-frame rearrangement on the κ allele. ND, light chain rearrangements not determined. Those clones that were positive in the PCR detecting Cκ deletion are also indicated.

Table III. *Comparison of Anti–DNA B Cells from 3H9H/56R BALB/c and 3H9H/56R MRL/lpr*

	3H9H/56R BALB/c		3H9H/56R MRL/lpr	
		L chain usage		L chain usage
H chain transgene +	9 IgM:	λ1 and a κ editor	2 IgM:	Vκ21-10; Vκ19-15
			3 IgG	Vκba9; Vκ23-45;
	0 IgG			1 unknown κ
H chain transgene −	1 IgM:	unknown κ	0 IgM	
			6 IgG	Vκcr1; Vκcb9;
	0 IgG			Vκ19-23; λ1

The isotypes, heavy and light chain usage of anti–DNA B cells from 3H9H/56R BALB/c and 3H9H/56R MRL/lpr are shown.

was truncated by DH rearrangement to the internal heptamer (8). These clones now express H chains coded for by the untargeted allele.

The 3H9H/56R MRL/lpr model provides an opportunity to ask whether L chain editing occurs in this model of autoimmunity. In healthy mice, all sd-tg antibodies are always associated with κ or λ editors with the exception of double expressers such as the 3H9H/56R κ/λ population (11). Anti-DNA mAbs from autoimmune mice are associated with many κ chains and λ1 but not the set of κ chains and λx known to edit anti-DNAs. These antibodies include H chains coded for by the 3H9H/56R gene or other closely related members of the J558 family (26). However, edited mAbs are found among the 3H9H/56R MRL/lpr hybridomas. Clones 31 and 44 each expresses an editor (Vκ21-D and λx, respectively), and neither of them binds DNA (Table II). Thus central tolerance is at least partially intact in the MRL/lpr as described previously (1). In addition, we find 3H9H/56R anti-DNAs with noneditor κs or λ1 (Tables II and III). These may have arisen because central tolerance is incomplete in MRL/lpr mice or because of ongoing rearrangement that replaces κ editors with L chains that sustain DNA binding.

A property of Lupus-associated autoantibodies is somatic mutation. The pattern of mutations shows evidence for antigen selection and mutations in anti-DNAs have been shown to create and modify DNA binding (27). Given that these MRL/lpr mice inherit a relatively high affinity anti-DNA, whether they undergo mutation and whether anti-DNA activity is modified is relevant. We could address this question because certain 3H9H/56R antibodies are not edited. The IgM anti-DNAs have no mutations (This applies just to VH; we are not sure about the germline sequences of the L chains expressed in this strain). The IgG anti-DNAs have multiple mutations and the mutation pattern shows evidence for selection. 10/14 replacements occur in CDRs or other sites that contribute to DNA binding, and 5/10 mutants are to arginines or asparagines, which are residues that favor DNA binding (Fig. 2). Clone 69 does not bind DNA even though it is associated with a κ chain that should sustain DNA binding (22). Lack of DNA binding might result from arginine 97 to serine mutation or the potentially negative effect of the mutation of the highly conserved tyrosine 93 to cysteine.

In the anti-dsDNA IgG clones with VH replacements (clones 46, 26, and 75), mutations are also found (Fig. 3). It

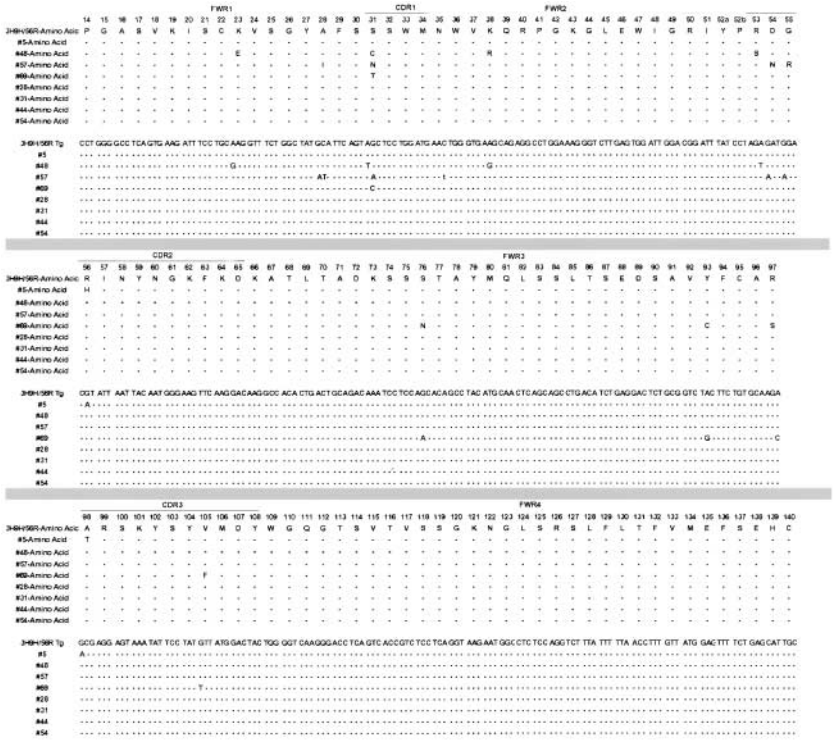

Figure 2. Heavy chain sequences of B cells that retain the H chain transgene. The nucleotide and deduced amino acid sequences of heavy chains from hybridomas that are transgene-positive are aligned with 3H9H/56R transgene sequence. Mutations in the IgG-secreting clones (5, 48, 57, and 69) are indicated. A silent mutation in the FWR2 of clone 57 is shown in lowercase letter.

is interesting to note that the arginine mutation in the FR2 of clone 75 is shared by clone 57 (and other anti-DNAs in the literature; reference 26). This site is superficial and could contribute to DNA binding. In these VH genes, arginines are found in the newly formed D junctions due to either N-addition or point mutation (Fig. 3 B). Another source of an arginine codon is a VH replacement that captures a short sequence from the end of the recipient VH gene (in this case the transgene). In the transgene as well as most VH genes, this sequence includes an arginine codon (Fig. 3 B, clones 46 and 75). These mechanisms by which anti-DNAs are modified or created play a central role in autoantibody formation in the non-tg MRL/lpr. It is surprising that the sd-tg resorts to these mechanisms because the inherited 3H9H/56R (along with most L chains) contributes to high affinity DNA binding. Therefore, it appears the emerging 3H9H/56R B cells must have undergone prior modifications that necessitate de novo development of anti-DNA. These could include VH replacement, somatic mutation (in two instances critical Args in 3H9H/56R have mutated to a neutral amino acid), and L chain editing.

3H9H/56R MRL/lpr Mice Have Fewer κ/λ Double-positive MZ B Cells than 3H9H/56R BALB/c Mice. Early work (14) showed that the λ1, in combination with 3H9H/56R, binds dsDNA. Such autoreactive B cells are seen in 3H9H/56R BALB/c. However, they always coexpress a κ editor such as Vκ21-D or Vκ38C (14). Nonautoimmune 3H9H/56R tg mice also have a population of B cells that coexpress κ/λ light chains (11). The frequency of this population increases with age and reaches a maximum of ~30% of the $B220^+$ B cells population (11). These mice do not express these anti-DNAs, and we presume that they are negatively regulated by inactivation or sequestration. Indeed the majority of the κ/λ population is in the MZ.

In 3H9H/56R MRL/lpr hybridoma panel, we found two clones that express λ1 without the coexpression of a κ

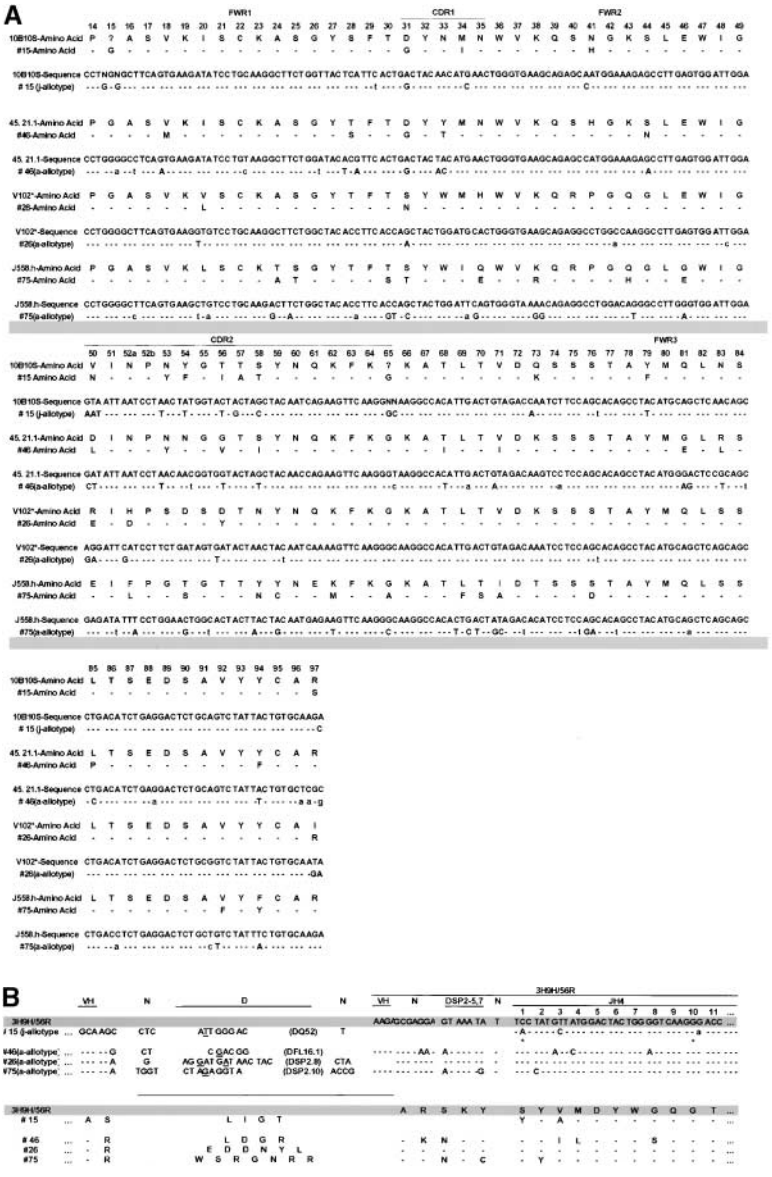

Figure 3. Heavy chain sequence of B cells that have edited the H chain transgene. (A) The nucleotide and deduced amino acid sequences of the V regions of heavy chains from hybridomas that are transgene-negative (clones 15, 46, 26, and 75) are shown. VH gene usage and point mutations compared with each of their germline sequences from the NCBI database are indicated. (B) CDR3 regions of the heavy chains of hybridomas (clones 46, 26, and 75) that had VH replacement are shown. The N-additions at the newly formed D-junctions, the remaining D and J4 region of the 3H9H/56R transgene and point mutations are indicated. Another heavy chain from clone 15 is generated by endogenous rearrangement on the untargeted allele, indicated by the differences at the JH region (marked by asterisk) between the transgene (a-allotype) and endogenous JH4 (j-allotype). The mutations compared with its germline sequence are also shown.

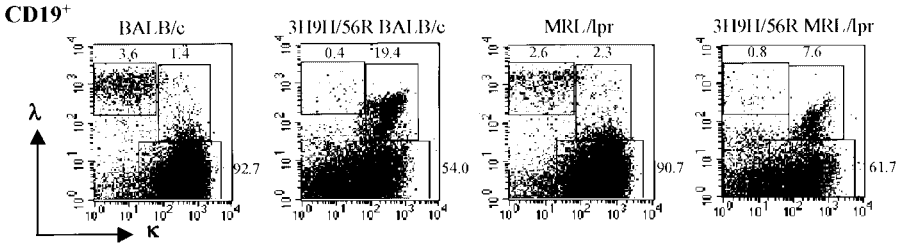

Figure 4. κ and λ expression in spleen B cells. Spleen cells from 3H9H/56R BALB/c, 3H9H/56R MRL/lpr, and their nontransgenic littermates were stained with anti-CD19, κ, and λ. Percentages of κ^+, λ^+, and κ/λ^+ cells in a $CD19^+$ gate are indicated. Representative of experiments using three different mice of each kind.

editor (Table II, clones 39 and 75). Instead, both of them have one κ allele that has out-of-frame rearrangement and Cκ deletion on the other allele. We wondered whether they were derived from the κ/λ population, and whether the kind of tolerance represented by this phenotype was operating in the MRL/lpr.

The peak frequency of κ/λ double-positive B cells is lower in 3H9H/56R MRL/lpr (7.6% of the $CD19^+$ spleen B cell) than in 3H9H/56R BALB/c (19.4%; Fig. 4). The κ/λ double-positive B cells of 3H9H/56R BALB/c are in the marginal zone shown by markers such as $CD21^{high}$ and $CD1^{high}$. Most of the κ/λ double-positive B cells in 3H9H/56R MRL/lpr are $CD21^{low}$ and $CD1^{low}$, indicating that they are not in the marginal zone (Fig. 5). As has been previously reported, there is a general decrease in CD21 and CD23 level in MRL/lpr mice (28). However, there is no generalized defect in MZ B cell development. As shown in Fig. 6 A, non-tg MRL/lpr mice has a larger MZ B cell population ($CD21^{high}$, $CD23^{low}$; 10.8% of lymphoid gate) compared with non-tg BALB/c (1.9%). The exaggerated MZ of MRL/lpr mice has also been reported before (29). Most interestingly, although the 3H9H/56R transgene causes a big increase of the MZ B cells population in BALB/c, it dramatically reduces that population in MRL/lpr (Fig. 6 A). Several mouse strains with transgene have shown larger MZ B cell compartment, which can accommodate self-reactive B cells (for review see reference 30). The reduction of MZ B cell population in3H9H/56R MRL/lpr shows some unique feature of MRL/lpr not only different from BALB/c, but also different from other autoimmune mice such as NZB/W, as pronounced marginal zone compartment was observed NZB/W mice with an anti-DNA μH transgene (31).

Expression of the heavy chain transgene was investigated by using a mAb, 1.209, that recognizes the 3H9 heavy chain. 3H9H/56R MRL/lpr mice have fewer 1.209 cells in the spleen (2.9%) than 3H9H/56R BALB/c (10.3%), suggesting that there is more heavy chain editing in MRL/lpr mice. In addition, although the majority (67%) of the Id^+ B cells of 3H9H/56R BABL/c are in the marginal zone (shown by $CD21^{high}$), most of the Id^+ B cells (76%) of 3H9H/56R MRL/lpr are $CD21^{low}$ (Fig. 6 B).

Discussion

Stochastic Processes Determine the Onset of Autoimmunity. Stochastic processes can explain the variable spectrum of autoantibodies in individuals with Lupus. Certain autoantibodies are expressed in subsets of Lupus patients; for example, the Lupus autoantibody Sm is observed in just 25% of MRL/lpr mice. Exhaustive searches for an epigenetic basis for the variability were unsuccessful, leaving the alternative that acquisition of an Sm receptor occurs with a probability that guarantees expression in ~25% of the population. The frequency of anti-DNA is higher than Sm (32) which, according to the stochastic model, means that the probability of acquiring an anti-DNA receptor is higher than that for Sm. That acquisition of anti-DNA receptors is highly likely is supported by studies on the genetic basis of this specificity. DNA binding is strongly correlated with arginine residues in the CDR3 of VH and arginine codons often formed during V(D)J rearrangement (33).

Another variable feature of autoimmunity is the timing of autoantibody expression. Although most MRL/lpr mice express anti-DNA by 10 to 12 wk of age, the onset of anti-DNA expression is variable from mouse to mouse. Even

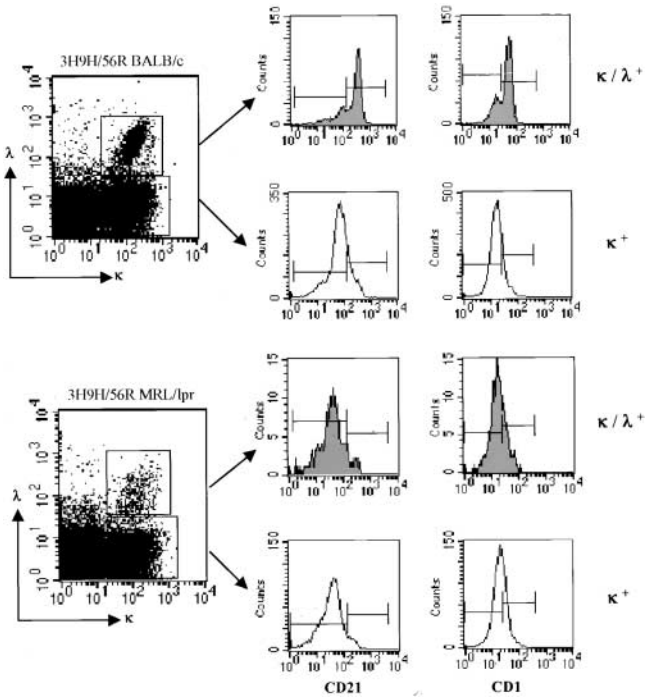

Figure 5. CD21 and CD1 expression levels of κ/λ double-positive cells are different between 3H9H/56R BALB/c and 3H9H/56R MRL/lpr. Spleen cells from 3H9H/56R BALB/c and 3H9H/56R MRL/lpr were stained with anti-CD21, CD1, κ, and λ. Histograms of CD21 and CD1 expression are shown for the κ/λ double-positive (filled area) and κ single-positive (thin line) populations.

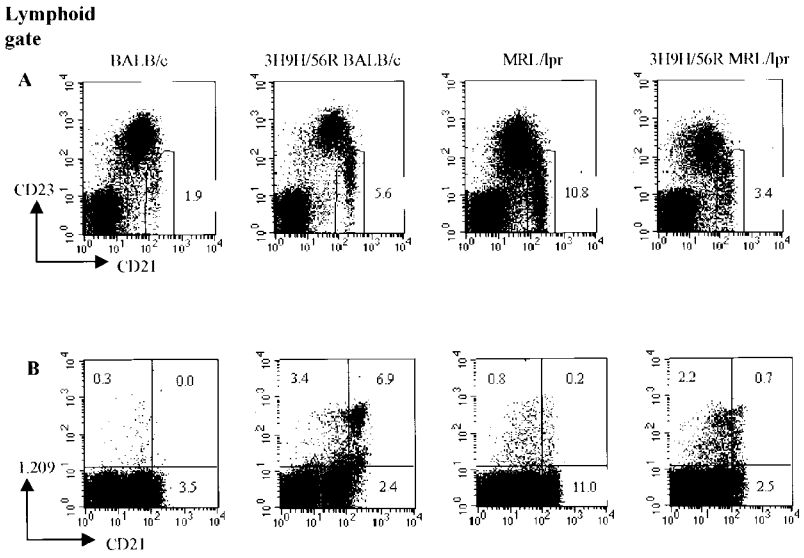

Figure 6. Marginal zone B cells and Id$^+$ B cells in 3H9H/56R BALB/c and 3H9H/56R MRL/lpr. Spleen cells from 3H9H/56R BALB/c, 3H9H/56R MRL/lpr, and their nontransgenic littermates were stained with anti-CD21, CD23, and anti–Id 1.209. Percentages of MZB cells (CD21^{high}CD23^{low}), Id$^+$CD21^{high}, Id$^+$CD21^{low}, and Id$^-$CD21^{high} cells from a lymphocyte gate are indicated.

though the normal process of V(D)J often yields anti-DNA receptor genes, the frequency may not be high enough to prevent a lag in anti-DNA expression in Lupus mice. (It should be pointed out that influence of the anti-DNA sd-tg on the frequency and rate of anti-DNA expression described here is in the context of the autoimmune MRL/lpr mouse.) If during V(D)J rearrangement, acquisition of an anti-DNA receptor is likely but not a certainty, then the variability of expression is expected. In turn it would be expected that the sd-tg would influence the appearance of anti-DNA expression in MRL/lpr mice. This is the case and anti-DNA expression is accelerated by several weeks in sd-tg MRL/lpr mice (Fig. 1). This result suggests that the acquisition of an anti-DNA receptor may be a rate-limiting step in Lupus and inheritance of a preformed anti-DNA receptor (as in the case of 3H9H/56R mice) overcomes this step.

An obvious prediction of this model is that the sd-tg H chain will dominate the anti-DNA response. Based on our sequence analysis of mAbs derived from the MRL/lpr sd-tg this is not the case, only half of the anti-DNA mAbs H chains recovered from a fusion using unmanipulated spleen B cells are encoded by the sd-tg (Table III). Thus, the effect of the sd-tg on anti-DNA expression is, in part, indirect. B cells with the sd-tg–encoded receptor may be subjected to extended periods of rearrangement leading to reediting. The clones that secrete anti-DNA antibodies and retain the sd-tg use noneditors, i.e., the light chains that sustain DNA-binding such as Vκ21–10, Vκ23–45, Vκ19–15, Vκba9, and λ1 (Tables II and III), and they may have arisen after editing as a result of ongoing rearrangement and reediting to recover the initial (anti-DNA) specificity. Anti-DNAs encoded by endogenous VH genes have CDR3 regions characteristic of Lupus anti-DNAs. Such CDR3 sequences are thought to arise by extended periods of rearrangement that creates these sequences and leads to their utilization by inactivation of the original V(D)J allele (in this case, the sd-tg–encoded allele; reference 34). We cannot rule out that these anti-DNAs arose because of a failure to edit as in the case of the sd-tg–encoded anti-DNAs or because of failure of the sd-tg to efficiently exclude rearrangement, as in the case of the anti-DNAs encoded by the untargeted allele. However, the frequency of rearrangement events found in the MRL/lpr hybridomas is high. The frequency of VH replacement and inactivation is much higher than that in LPS-activated tg-B cell hybridomas (14) or B cells from KLH-immunized transgenics (8). Even the 11 hybridomas that secrete anti-DNAs have low frequencies of sd-tg retention: five clones, two IgM (clones 28 and 54) and three IgG (clones 26, 46, and 75), retain the transgene. The other six clones, all IgG, have replaced or inactivated the sd-tg. Along with the extensive loss of the sd-tg, we also find a high frequency of Cκ deletion in these hybridomas, hence we favor the reediting hypothesis as an explanation for the origin of anti-DNAs in the Lupus mice.

Nonspecific and Specific Activation in MRL/lpr. The origin of anti-DNA in Lupus is complex and expression has been linked to different sets of B cells. Autoantibodies are expressed by the hyperactive B cells of the MRL/lpr. These antibodies have the range of self-specificities expressed by mitogen-activated B cells. Typically they are IgM and in LPS panels such "natural" or "germline autoantibodies" are not mutated (35). mAbs with these properties are found in this panel. The anti–DNA H chain is coded for by the sd-tg and is associated with L chains that sustain DNA binding. Here, the tg VH is unmutated (Fig. 2, clones 28 and 54). The anti-dsDNAs characteristic of Lupus are IgG, oligoclonal, and mutated. This type of anti-DNA is also found among the sd-tg MRL/lpr.

We wish to know if there is a connection between these populations. One possibility is that they are of independent origin. The sd-tg may not completely exclude VH rearrangement (9), and a population of B cells with VH replacements or endogenous V(D)J genes may arise early in development and contribute to the adult autoantibody repertoire. This pathway would not explain the accelerated

onset of anti-DNA in the sd-tg, if anything, one might expect delayed expression of autoantibodies by this model. Alternatively, the IgM or hyperactive population may be the precursor of the IgG-secreting B cells. Regulation of this population may be lost or the population—by virtue of its anti-DNA receptor—may gain the potential for entering the memory B cell population. The first case might lead to the shift to tg-encoded IgG anti-DNA as in the case of clone 48 and 57. The second case could yield the diverse repertoire of anti-DNAs as in clones 26, 46, and 75. Here, the tg might contribute to the frequency of B cells that can present antigen to autoreactive T cells. The fate of these B cells is uncertain, they are likely to undergo isotype switch and mutation. In addition, V gene rearrangement might be stimulated leading to the novel V gene repertoires found among these IgG anti-DNAs.

Failure to Sequester Self-reactive B Cells May Lead to Autoimmunity. The 3H9H/56R BALB/c expresses high frequencies of tolerant B cells that arise by "phenotypic editing." This form of editing occurs when secondary (editing) rearrangements do not delete the autoreactive V gene, but take place on its allele or at other L chain isotypes (λ1, λ 2, etc.). These B cells express two L chains, for example, two κ chains (6), one of which is an editor, or as is frequently the case in 3H9H/56R, λ1 and a κ editor (14). We propose that dual expression dilutes the anti-DNA receptor and renders the cell inactive. The κ/λ B cells are concentrated in the MZ, and sequestration to this site may prevent autoreactive B cells from entering germinal centers and developing the properties of pathogenic B cells. We examined these κ/λ B cells in MRL/lpr and found that the sequestration is defective. Failure to sequester self-reactive B cells also has been observed in B1 cells. Anti–erythrocyte B1 cells become activated and differentiated in antibody-producing cells in mesenteric lymph nodes and lamina propria of intestine in Fas-deficient mice (36). Anti-Sm B1 cells in normal mice also fail to develop in MRL/lpr mice (37). Together, failure to sequester self-reactive B cells in regions such as the MZ or peritoneal cavity may contribute to the loss of tolerance.

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