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Editors and Editing of Anti-DNA Receptors

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Summary

Receptor editing is a means by which immature bone marrow B cells can become self-tolerant. Rearrangements of heavy (H) and/or light (L) chain genes are induced by encounter with autoantigens to change the specificity from self to nonself. We have developed site-directed transgenic mice (sd-tg) whose transgenes code for the H chain of antibodies that bind DNA. B cells that express the transgenic H chain associate mainly with four of the 93 functional Vκ genes of the mouse. Numerous aspartate residues that might inhibit DNA binding by the V_H domain distinguish these L chain Vκ sequences, but engaging these Vκ editors often requires multiple rearrangements. Among the edited B cells is a subset of multispecific cells that express multiple receptors. One consequence of multispecificity is partial autoreactivity; these multispecific B cells may contribute to autoimmunity.

Introduction

B cells achieve self-tolerance by deleting autoreactive receptors. This is accomplished in part by receptor editing; a process that replaces expressed V genes with upstream V genes (Gay et al., 1993; Radic et al., 1993b; Tiegs et al., 1993). If the replacement generates a nonautoreactive V_H/V_L combination, then the formerly autoreactive B cell can escape to the periphery. Efficient self-tolerance depends on a variety of factors. Among them are the nature of the antigen-antibody interaction and the ability of the editor (V_L and/or V_H) to veto autospecificity. Because combinations of V_H/V_L residues often determine antibody specificity, replacement of either V_H or V_L can change specificity. Antibodies vary in the relative contributions of V_H and V_L to specificity. Anti-DNA antibodies lie at one extreme in that most contacts with DNA are made through arginine (R) residues located in the CDRs of V_H (Radic et al., 1993a; Jang et al., 1998; Shlomchik et al., 1989). Consequently many V_L regions are unable to inhibit binding (Ibrahim et al., 1995) and editing often requires multiple rounds of rearrangement until an effective L chain editor is found (Luning Prak et al., 1994).

The 3H9 H chain model has allowed us to study the role of V_H and V_L editing in tolerance induction (Chen et al., 1997, 1995). We now wish to study how affinity for DNA influences editing. To this end, we have developed two site-directed H chain transgenics that are derived from mutants of the V_H3H9 with either one or two additional arginine substitutions (designated 3H9H/56R and 3H9H/56R/76R). These sites were chosen because they frequently are mutated to arginine in anti-DNAs of SLE and Lupus-prone mice (Radic and Weigert, 1994). Either arginine substitution increases the affinity for DNA relative to 3H9 and the combination, 56R and 76R, or it has an additive effect (Radic et al., 1993a). The requirements for editing in these models should be more demanding than in the 3H9 H chain transgenic because L chain editors must now inhibit additional DNA contact residues. We find that only a few L chains can prevent 3H9H/56R DNA binding and that even established κ chain editors cannot completely inhibit DNA binding with 3H9H/56R/76R. As a result, there are more rearrangements per mature B cell at the κ locus leading to B cells that express both κ and λ chain. Some anti-DNA B cells escape self-tolerance and are found in the periphery of these mouse models.

Results

We have constructed two site-directed anti-DNA transgenic mice (Figure 1A). By in vitro mutagenesis, 3H9H/56R and 3H9H/56R/76R were derived from the H chain of an anti-DNA antibody, 3H9, by *in vitro* mutagenesis. The 3H9H/56R mutant has a single base substitution in V_H3H9 that replaces an aspartate with an arginine at position 56 in CDR2. The 3H9H/56R/76R mutant has 56R and an additional single base change that replaces a serine with an arginine at position 76 in FR3 (Figure 1B). The construct used previously to generate the 3H9H site-directed transgenic (sd-tg) was modified for targeting the 3H9H/56R and 3H9H/56R/76R genes to the V_H locus, replacing DQ52 and JH regions by homologous recombination (Chen et al., 1995). Thus the three constructs are identical except for the single and double base changes that lead to the arginine substitutions. We have modeled the V_H domains of 3H9, 3H9/56R and 3H9/56R/76R (Figure 1C), and calculated the electrostatic potential of each model. It is clear that increased DNA binding affinity goes hand in hand with increased positive potentials in the region of CDR3 and along the V_H and V_L interface. The combination of arginines at 96, 53, and 56 produces a continuous region of positive potential, suggesting that the negative phosphate groups of DNA may approach the combining site along the H chain CDR, parallel to the V_H-V_L interface. R76 creates a separate region of positive potential. To compare editing in the three transgenics we have generated hybridoma libraries from LPS activated splenic B cells.

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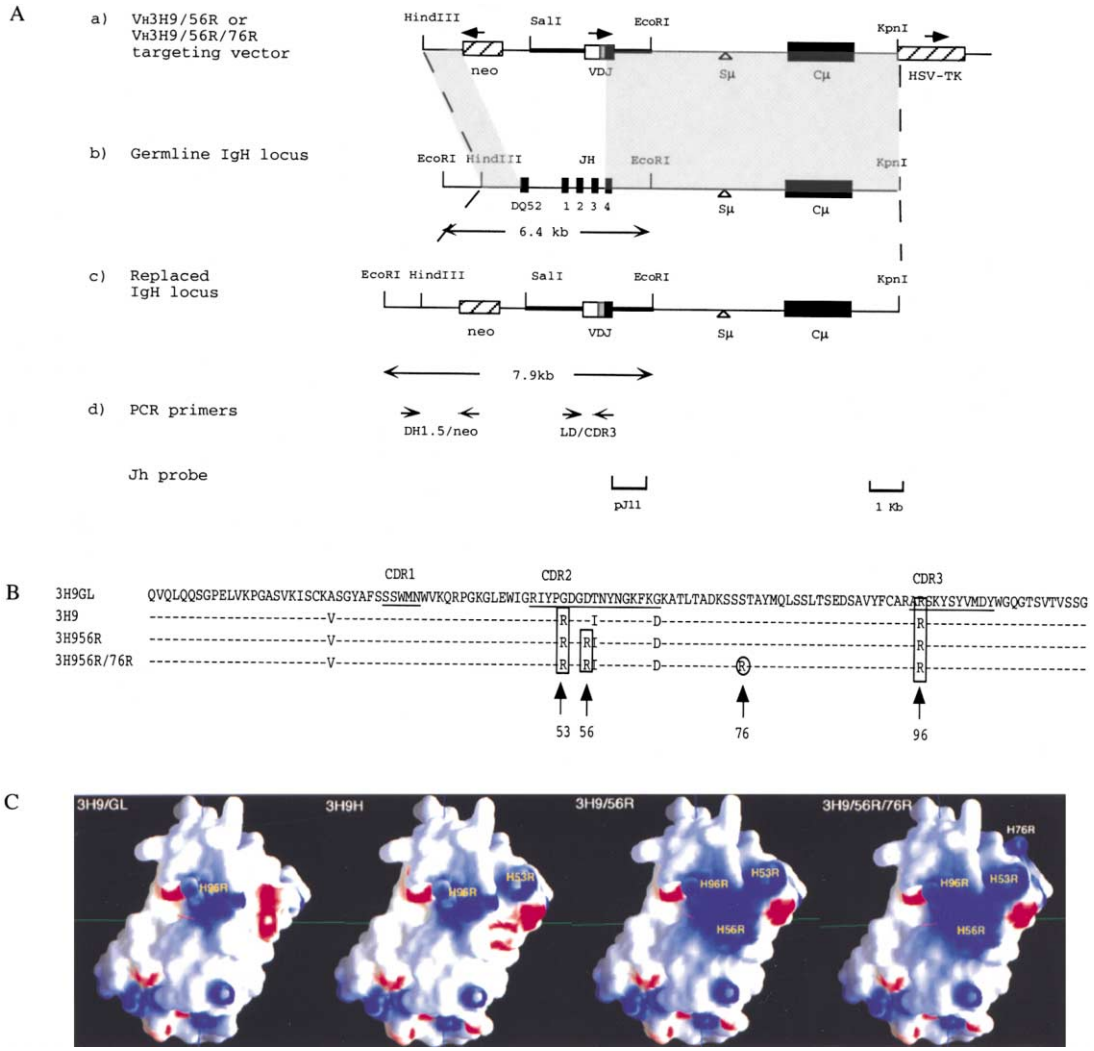


Figure 1. 3H9H/56R and 3H9H/56R/76R Site-Directed Transgenics

(A) Diagram of the strategy used to generate site-directed transgenic mice of 3H9 mutants by homologous recombination. Arrows indicate the transcriptional orientations of the neomycin phosphotransferase (NEO), immunoglobulin heavy chain (VDJ) and thymidine kinase (HSV-TK) genes. Homologous sequences to facilitate recombination between the targeting vector (A-a) and the germline IgH locus (A-b) are indicated as shaded areas. Southern blotting with a heavy chain J region probe, pJ11 (Chen et al., 1995), yields a 7.9 kb EcoR1 band for the targeted IgH locus (A-c) and a 6.4 kb band for the germline locus (A-b). The positions of the J_H probe (pJ11) and the PCR primers are shown in (A-d).
(B) Amino acid alignments of 3H9 V_H mutants. The 3H9H/56R mutant has a single base substitution in V_H3H9 that replaces an aspartate with an arginine at position 56 in CDR2. The 3H9H/56R/76R mutant has an additional single base change that replaces a serine with an arginine at position 76 in FR3. The mutants were created by site-directed mutagenesis (Radic et al., 1993a).
(C) Electrostatic potentials of variable regions of the 3H9 H chain and its mutants. 3H9GL (3H9 germline) is another model, in which all the mutations unique to the 3H9H have been reverted to their germline codons except the arginine at the position 96 (96R) in CDR3. 96R in 3H9 was created by VDJ recombination and the low-affinity of 3H9GL for DNA has been attributed to the 96R residue (Radic et al., 1993a). The heavy chain template, a chimeric Oxy-Cope catalytic antibody Az-28 (PDB code: 1D5B), was chosen from the Protein Data Bank (http://www.rcsb.org/pdb) based on its sequence similarity to 3H9. Swiss Model reconstructed predicted models of 3H9 and its mutants (http://www.expasy.ch/swissmod/SWISS-MODEL.html). Electrostatic potentials at the surfaces of the reconstructed models were calculated with GRASP (Nicholls et al., 1991) and represented on a color scale from blue for positive potential, to white for neutral, to red for negative potential.

H and L Chain Usage in 3H9, 3H9/56R, and 3H9/56R/76R Hybridoma Abs

The majority of mAbs from the three transgenics fail to bind DNA (see below) and they fall into two categories: those that have retained and those that have lost the sd-tg H chain. The sd-tg is often deleted or inactivated by rearrangements at the transgene site. As shown previously for 3H9, these rearrangements represent replacements by upstream V_H genes or inactivation by D_H gene insertion. D_H invasion is a common form of transgene inactivation and is accompanied by expression of H chain from the untargeted allele in hybridomas that secrete antibody. There is a correlation between arginine content and transgene loss as seen in studies of transgene expression (Table 1) but it is unclear whether the loss is due to editing or positive selection of endogenous rearrangements at an early stage of B cell development.

Table 1. H- and L-Chain Editing of LPS-Stimulated B Cells

	3H9H (%)	3H9H/56R (%)		3H9H/56R/76R (%)	
Total clone tested	155	83		139	
Vh-sd-Tg$^+$	147 (94.8)	69 (83)		52 (37)	
Mouse V genes	3H9H$^+$ (%)	3H9H/56R$^+$ (%)	dsDNA$^+$	3H9H/56R/7 6R$^+$ (%)	43 dsDNA$^+$
Vκ1	2 (1.4)	0 (0)	0	1 (1.9)	0
Vκ2	0 (0)	2 (2.9)	0	0 (0)	0
Vκ4/5	3 (2.0)	0	0	2 (3.8)	0
Vκ9	0 (0)	0 (0)	0	1 (1.9)	1
Vκ12/13	94 (64.0)	0 (0)	0	0 (0)	0
Vκ20	3 (2.1)	3 (4.3)	1	1 (1.9)	1
Vκ21D	17 (11.6)	54 (78.3)	2	0 (0)	0
Vκ23	0 (0)	1 (1.4)	1	1 (1.9)	1
Vκ38c	2 (1.4)	7 (10.1)	4	40 (76.9)	36
Vκs$^-$	7 (4.8)	2 (2.9)	1	1 (1.9)	1
Other Vκs$^+$	19 (12.9)	0	0	5 (9.6)	3

Splenocytes from 3H9/56R or 3H9/56R/76R H chain transgenic mice were subjected to in vitro LPS-stimulation for 3 days before fusion with Sp2/0 (see Experimental Procedures). Data presented here are from one hybridoma panel of each model. Transgene-bearing hybrids were identified by PCR using primers located in the leader and CDR3 regions of the 3H9H gene (Figure 1A). Only sd-tg positive clones were analyzed for Vκ usage. Vκ12, Vκ20, Vκ21D, Vκ38c rearrangements were detected by specific PCR primers indicated in the Experimental Procedure section. Other rearranged Vκs were identified by sequence of Vs or L5/Jκ PCR products. The Vκs group includes those samples in which no κ rearrangement could be detected by PCR using Vκ20, Vκ21D, Vκ38c, Vκ12/13, Vκs, or L5 primers. The other group includes the uncharacterized rearranged Vκ genes that were positive in Vκs or L5/Jκ PCR. Four hybridomas from 3H9H/56R and one clone from 3H9H/56R/76R fusion were excluded, two for polyclonal genotypes and three for lack of amplification in all Vκ L-chain assays (despite amplification of the H-chain transgene).

In this study we have focused on the hybridomas that retained the transgene. To confirm that these B cells are tolerized by L chain editing, we characterized the range of Vκ genes that they expressed. We assume that all κ chains can pair with 3H9H based on studies on a 3H9γ2b (membrane exonless) mouse. A large repertoire of κ chains paired with the 3H9γ2b and most of these antibodies bound DNA, which were not deleted because the 3H9H is constitutively secreted (Ibrahim et al., 1995; Thiebe et al., 1999). In contrast, the 3H9H tg, which is associated with the endogenous H chain locus, is associated with a restricted repertoire of L chains (Table 1) and these antibodies do not bind dsDNA (Gay et al., 1993; Radic et al., 1993b; Chen et al., 1997). The absence of dsDNA binding in this case is due to the use of editor L chains, which veto H chain DNA binding. As the L chains that veto DNA binding by 3H9H constitute a minority of the entire Vκ repertoire (Ibrahim et al., 1995; Thiebe et al., 1999), their overrepresentation is far greater than expected by chance alone (Radic et al., 1993b). The pattern of distal Jκ segment usage by some of the expressed Vκ genes suggests that this skewed Vκ repertoire is generated at least in part by L chain editing, not just by negative selection (Radic et al., 1993b; Luning Prak et al., 1994).

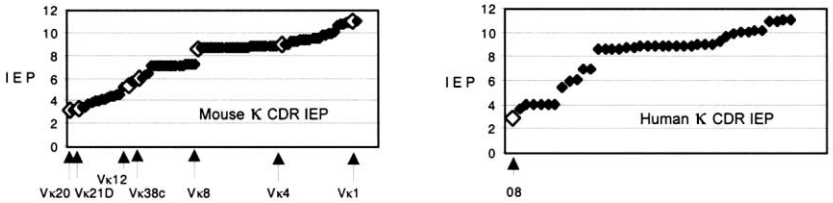
The Vκ usage of 3H9H/56R and 3H9H/56R/76R B cells is different from and more restricted than 3H9H B cells. In this and in previous studies, the majority of 3H9 antibodies are associated with two members of the Vk12/13 group. The 3H9H/56R and 3H9H/56R/76R are not associated with Vκ12/13, which can veto 3H9H; instead they are mainly associated with just three Vκs: Vκ20, Vκ21D, and Vκ38c (Table 1). This implies that the structural requirements for inhibiting DNA binding of the 3H9/56R and 3H9/56R/76R VH regions are more stringent than those for 3H9. The extra arginine residue(s) in 3H9H/56R and 3H9H/56R/76R may require Vκs that offset these additional DNA binding sites.

Editor Light Chains Can Have Low Isoelectric Points

Vκs associated with 3H9H and its mutants in normal mice have low IEPs, whereas Vκs associated with 3H9H in autoimmune mice that sustain or implement DNA binding have high IEPs (Figure 2A). In certain cases such as Vκ21D, the editing function can be attributed solely to the aspartate residues in CDR1. Vκ21D is a member of a closely related group of Vκ regions, Vκ21, yet Vκ21D is the only member in the Vκ21 group that edits. Vκ21D differs from its most related group member, Vκ21E, by two aspartates in CDR1, pinpointing these sites as critical for successful editing (Figure 2B). The calculated electrostatic potentials of the Vκ editors compared to Vκ1, a Vκ that sustains DNA binding, is shown in Figure 2D. Editors Vκ21D and Vκ20 have a broad region of negative potential centered about VL CDR1 at the VH-VL interface. Vκ38c has a much smaller region of negative potential even though it is well represented among 3H9H/56R$^+$ and especially 3H9H/56R76R$^+$ hybridomas (Table 2). Among the human Vκ genes O8 has the lowest IEP (Figure 2A).

However, acidic IEPs of CDRs do not necessarily qualify Vκs for editing anti-DNA. Several Vκs with extremely low IEPs, such as Vκfl12 and VκCi12 (Thiebe et al., 1999) with IEPs of 3.4 and 3.5, respectively, have not been seen in either 3H9H/56R or 3H9H/56R/76R B cells. Conversely, some efficient editors have nearly neutral IEPs, implying that the positions of acidic residues or combinations of acidic residues within CDR must be relevant and that framework acidic residues (which were excluded from these IEP calculations) might also be important. For example: even though Vκfl12 and VκCi12 have low IEPs, they lack aspartate residues in CDR1. Conversely, Vκ38c (IEP 6.1) is an efficient editor of 3H9H, 3H9H/56R, and 3H9H/56R/76R. Vκ38c shares two CDR aspartates with the highly acidic editors, and the location of the Vκ38c CDR1 aspartate residue is similar to the key aspartate in Vκ21D (Figure 2C). Vκ38c also has

A



B

		CDR1	CDR2	CDR3	IEP
21-5	DIVLTQSPASLAVSLGQRATISC	RASKSVSTSGYSYMH	WYQQKPGQPPKLLIY	LASNLES GVPARFSGSGSGTDFTLNIHPVEEEDAATYYC	QHSRELP	6.32
21-10	D----------	----------	----------	-----E- ----------D--------EEED------	----E--	6.32
21-12	D----------	----------	----------	-----E- ----------D--------EEED------	----E--	6.32
21-1	D----------	---E--EYY-T-L-Q	----------	A---VE- ----------D-S------EEDDI-M-F-	-Q--KV-	4.98
21-2	D----------	---E--DNY-I-F-N	-F--------	A---QG- ----------D-S-----MEEDDT-M-F-	-Q-KEV-P	4.60
21-3	D----------F-	---Q--DYN-I----	-F--------	A----E- -I----------D--------EEED------	-Q-IED-	4.44
21-4	D----------	K--Q--DYD-D---N	----------	A----E- -I----------D--------EEED------	-Q-NED-	4.03
21-7	D----------	---Q-----S-----	----------K	Y----E- ----------D--------EEEDT-----	---WEI-	5.80
21-9	D----------	Q--E---FA-T-L--	----------	R----E- ----------ESD---T-D--EEDD--M---	MQ-MEDP-	4.07

C

Vk1(bb1)	RSSQSLVHS-NGNTYLH	KVSNRFS	SQSTHVP-	11.23
Vk4(ag4)	SASSSVSSS-----NLH	RTSNLAS	QQWSGYPP	9.05
Vk8(8-19)	KSSQSLLNSGNQKNYLT	WASTRES	QNDYSYP-	8.59
Vk38c(gj38c)	KASQDINK------YIA	YTSTLQP	LQYDNLL-	6.08
Vk12(12-46)	RASENIYS------NLA	AATNLAD	QHFWGTP-	5.22
Vk9(cb9)	QATQDIVK------NLN	YATELAE	LQFYEFP-	3.75
Vk21D(21-4)	KASQSVDYD--GDSYMN	AASNLES	QQSNEDP-	3.40
Vk20(bt20)	ITSTDIDD------DMN	EGNTLRP	LQSDNLP-	3.36
Vk20(bw20)	ITSTDIDD------DMN	EGNTLRP	LQSDNMP-	3.06
O8	QASQDIS-N-----YLN	DASNLET	QQYDNLP-	3.06

D

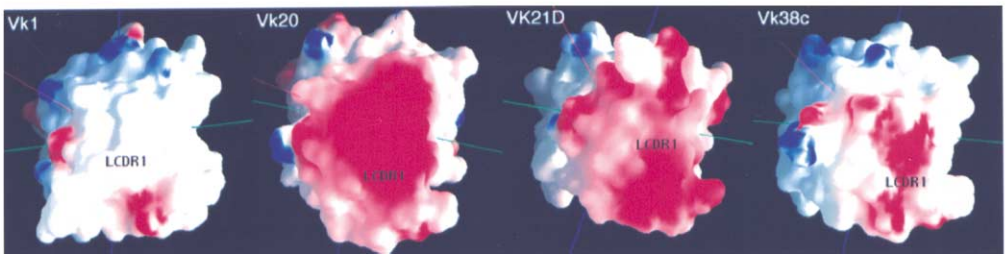


Figure 2. Isoelectric Points (IEPs) and Amino Acid Sequences of L chain Editors

(A) Isoelectric points (IEPs) of CDRs of Vκ chains. Amino acid sequences of the functional mouse and human Vκ genes were obtained from GenBank (http://www.ncbi.nlm.nih.gov/igblast). Only CDRs were used for the IEP calculation. IEPs of the proteins were calculated using MacVector software (International Biotechnologies, Inc., New Haven, CT).

(B) Amino acid sequences of the murine Vκ group 21. CDRs are underlined. The Vκ21-5 sequence was used as the standard for comparison. Identical amino acids (in other family members compared to 21–5) are represented by dashes except aspartate (D) and glutamate (E), which are highlighted with bold letters. 21–5, 21–10 and 21–12 have identical amino acid sequences but their nucleotide sequences differ. Vκ21-4 (Thiebe et al., 1999) is equivalent to Vκ21D.

(C) Amino acid sequences in the CDRs of selected κ chains; O8 is a human κ chain and the rest are mouse κ chains. The amino acid residues having potential influence on DNA binding, D and E, are highlighted in bold. The mouse κ names used in the GenBank immunoglobulin database are indicated in parentheses. In association with 3H9 H chain, Vκ1 and Vκ4 bind DNA; Vκ8 binds DNA with low-affinity. Vκ12, Vκ9 and Vκ38c do not bind DNA. Vκ38c, Vκ20 and Vκ21D in combination with 3H9H/56R do not bind DNA. Vκ38c in combination with 3H9H/56R/76R bind DNA poorly.

(D) Electrostatic potentials of variable regions of Vκ1, Vκ20, Vκ21D, and Vκ38c. Predicted models and electrostatic potentials were obtained as described in the Figure 1C legend.

an aspartate in FR3 that could interact with VH CDRs (see discussion, Figure 4).

DNA Binding of 3H9/56R and 3H9/56R /76R Antibodies

The mAbs of hybridoma panels were tested for dsDNA binding using our standard solution phase assay (Radic et al., 1993a). In previous studies we have shown that 3H9H-associated mAbs rarely bind dsDNA. Here we find that a significant fraction of 3H9/56R and most of 3H9/56R/76R mAbs bind dsDNA. These mAbs were evaluated by comparing their affinities for dsDNA to 3H9/Vκ8 (very low relative affinity) and 3H9/Vκ1 (a high relative affinity anti-dsDNA) (Ibrahim et al., 1995).

The 3H9/56R mAbs are associated with three different editor κ chains, Vκ20, Vκ21D, and Vκ38c. Each editor can completely inhibit dsDNA binding, with the exception of several examples associated with the Vκ38c editor. As discussed below, the basis of dsDNA binding is the coexpression of λ1 L chain. Most of the 3H9/56R/76R

Table 2. κ Rearrangements of 3H9H/56R$^+$ and 3H9H/56R/76R$^+$ Hybridomas

3H9H/56R		3H9H/56R/76R	
Genotype (Rearrangements)	Number of Hybrids	Genotype (Rearrangements)	Number of Hybrids
Vκ38c Rearrangements		Vκ38c Rearrangements	
38c-J4, Vκ-J5	1	38c-J1, Vκ-J1inv	1
38c-J4, Vκ-J5, Cκdel	1	38c-J1inv, Vκ-J1	1
38c-J4, Cκdel	1	38c-J1inv, Vκ-J1inv	1
38c-J4, 21D-J2, Vκ-J2inv	1	38c-J1inv, Vκ-J1inv, Vκ-J5	1
38c-J5	2	38c-J2	1
38c-J5, Vκ21-J2	1	38c-J2, Vκ-J4	1
subtotal	7	38c-J2inv, Vκ-J2inv	2
		38c-J2inv, Cκdel	1
Vκ20 Rearrangements		38c-J4	1
20-J2, Vκ-J4	1	38c-J4, Vκ-J2inv	1
20-J5, Cκdel	2	38c-J4, Vκ-J4, Vκ-J1inv	1
subtotal	3	38c-J5,	10
		38c-J5, Vκ-J1	1
Vκ21 Rearrangements		38c-J5, Vκ-J1inv	1
21D-J1	2	38c-J5, Vκ-J2	1
21D-J2	26	38c-J5, Vκ-J2inv	2
21D-J2, Vκ-J1	2	38c-J5, Vκ-J1inv, Vκ-J2inv	1
21D-J2, Vκ-J1inv, Cκdel	1	38c-J5, Vκ-J4, Vκ-J2inv	1
21D-J2, Vκ-J2	1	38c-J5, Vκ-J5	6
21D-J2, Vκ-J4	1	38c-J5, Vκ-J5, Vκ-J2inv	1
21D-J2, Vκ-J5	7	38c-J5, Cκdel, Vκ-J1inv	1
21D-J2, Cκdel	2	38c-J5, Cκdel, Vκ-J2inv	1
21D-J4	2	38c-J5, Vκ-J5, Vκ-J1inv, Vκ-J2inv	1
21D-J4, Vκ-J1	2	subtotal	40
21D-J4, Vκ-J1, 38c-J2inv	1		
21D-J4, Vκ-J2	2	Miscellaneous	
21D-J4, Vκ-J1inv, Vκ-J5	1	Vκ-J2inv	3
21D-J4, Cκdel	1	Vκ-J4	3
21D-J4, Vκ-J1inv, Cκdel	1	Vκ-J4, Cκdel	1
21D-J5, Vκ-J5	1	Vκ-J5	3
subtotal	54	Vκ-J5, Cκdel	1
		Cκdel	1
Miscellaneous		subtotal	12
Vκ-J1	1		
Vκ-J1, Cκdel	1		
Vκ-J2, Cκdel	1		
Cκdel	2		
subtotal	5		
Total	69		52

Depicted are the κ light chain rearrangements identified in 69 3H9/56R H-chain positive hybridomas from a 3H9H/56R knockin mouse and 52 3H9/56R/76R H-chain positive hybridomas from a 3H9H/56R/76R knock-in mouse. General features of these hybridomas are shown in Table 1. κ rearragnements were determined by Vκs or L5 PCR assays and editor rearrangements were confirmed by Vκ-specific amplifications. The number of rearrangements identified represents an underestimate of the true number of rearrangements (see text for details). Vκ (refers to any Variable κ gene segment amplified by one of the generic Vκ primers, L5 or Vs), J1 (Jκ1), J2 (Jκ2), J4 (Jκ4), J5 (Jκ5), Vκ-J1 (a Vκ to Jκ1 rearrangement), inv (inversion), Cκdel (RS deletion by rearrangement of a Vκ to the RSS downstream of Cκ) Retter and Nemazee, 1998). 3H9H/56R transgene DNA positive hybridomas are divided into four genotype classes based on the identity of the editor Vκ segment: Vκ38c, Vκ20, Vκ21D, or none of these three (miscellaneous). 3H9H/56R/76R$^+$ clones are divided into two groups: Vκ38c and miscellaneous.

mAbs bind dsDNA even though they are associated with Vκ38c. The relative affinity for dsDNA is low but significantly higher than the 3H9/56R-Vκ38c mAbs (Figure 3). The only difference between the two mAbs is the serine to arginine substitution in the FR3 of 3H9H/56R/76R. This extra arginine must account for the dsDNA binding of the 3H9/56R/76R-Vκ38c antibody and suggests that this site is not accessible to editing by Vκ38c.

Many B Cells Express Two Different L Chain Isotypes

Some mAbs from hybridomas with the 3H9/56R H chain and L chain editors do bind dsDNA (Figure 3). We considered that these Vκ rearrangements might be nonfunctional or mutated, but DNA sequence analysis indicates that neither is the case (data not shown). Instead, the observed DNA binding results from a λ chain-associated antibody, λ1, in combination with 3H9H/56R. As shown in an earlier study, this combination binds dsDNA (Radic et al., 1993a). The rearrangement of λ1 in some 3H9H/56R hybridomas was shown by PCR using Vλ and Jλ1 primers and their expression of λ was confirmed by detection of the λ L chain in hybridoma supernatants. (Other 3H9H/56R hybridomas had rearranged λ1 but these examples neither bind DNA nor express λ chain. We assume that these cases have nonproductive λ1 rearrangements).

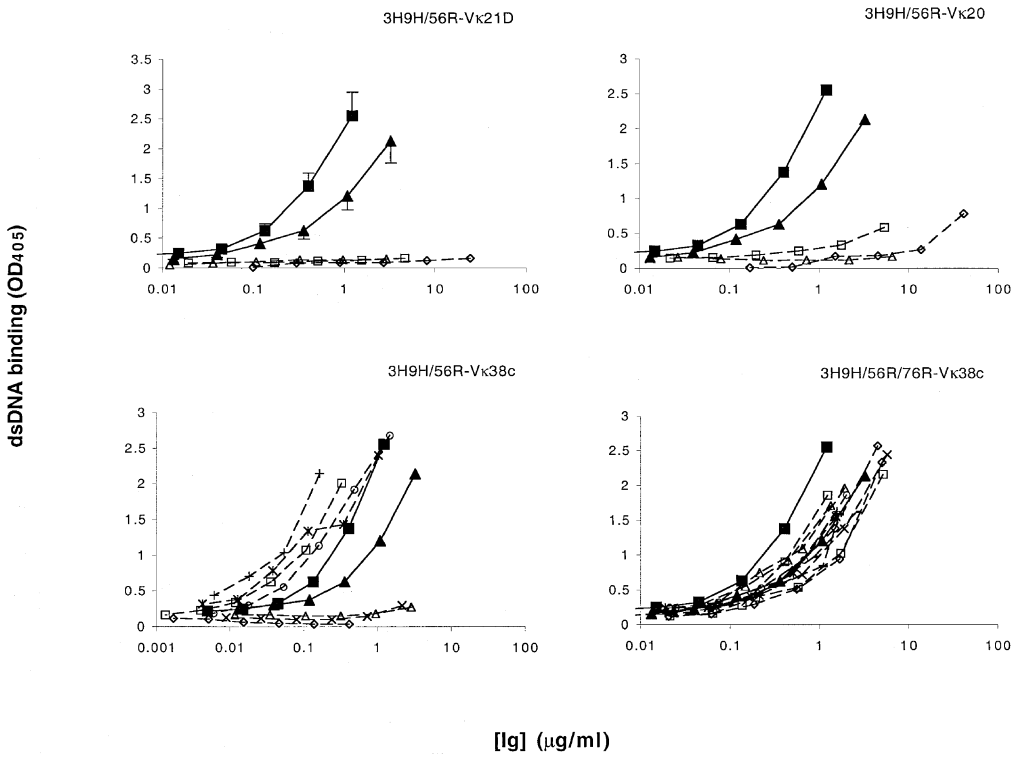


Figure 3. dsDNA Binding of Supernatants Secreted by *3H9H/56R* or *3H9H/56R/76R* Hybrids

All samples are shown as open symbols with dashed lines. 3H9/Vκ1 (■) (our unpublished data) and 3H9/Vκ8 (▲) (Chen et al., 1997) antibodies were used for high- and low-affinity controls, respectively. Only three representatives out of 54 samples in the Vκ21D group are shown (in the upper left panel) because all but two were ds-DNA non-binders. All three samples in theVκ20 group are shown in the upper right panel. All seven samples in the 3H9H/56R-Vκ38c group are presented in the lower left panel. All four dsDNA binders in the 3H9H/56R-Vκ38c group were λ positive (detected by λ1 PCR and/or ELISA; see experimental procedures for details). In 3H9H/56R/76R-Vκ38c panel (lower right), examples shown here are those 3H9H/56R/76R$^+$-Vκ38c$^+$ cells in which no other rearrangement was detected in the κ locus.

The eight hybridomas from 3H9H/56R mice with productive λ rearrangements also express κ according to our ELISA assay. Most, six out of the eight, have rearranged a κ editor supporting the idea that the 3H9H/56R-λ1 combination binds DNA. Two of the λ$^+$ clones deserve additional mention. Neither clone 56-33 nor clone 56-3 binds DNA. These clones may express other λ chains such as λX that inhibit DNA binding (Chen et al., 1994).

Edited B Cells Have Rearranged Multiple Times

Many hybridomas show evidence for extensive L chain editing. First, there is a bias toward Jκ5 rearrangement. Vκ gene segments rearrange to Jκ5 in 25% to 30% of normal B cells, but Jκ5 rearrangements are found in 44% of 3H9H/56R$^+$ clones, excluding the Vκ21D group (see below), and in 63% of 3H9H/56R/76R$^+$ clones (Table 2). In 3H9H/56R/76R-Vκ38c B cells we can estimate the Jκ usage of the silent or aberrantly rearranged κ alleles. Nine out of fifteen noninverted, non-38c Vκ rearrangements from this group of hybridomas were to Jκ5. This distal Jκ bias might be even greater were it not for high frequency of Cκ deletion of the noneditor allele (Table 2). Since positive selection cannot explain the Jκ5 bias in silent or nonproductively rearranged κ alleles, we regard the skewing toward Jκ5 among noneditor L chain rearrangements in these hybridomas as evidence of multiple rearrangement attempts. A second genetic feature that is suggestive of extensive receptor editing, Cκ deletion, is found in a substantial number of hybridomas. Out of 69 3H9H/56R$^+$ hybridomas, 10 type PCR positive for Cκdel (Table 2). Out of 52 3H9H/56R/76R$^+$ hybridomas tested, 6 have Cκdel rearrangements (Table 2). A third indicator of extensive rearrangement is the relative frequency of deletional and inversional rearrangements. Since functional Vκ genes are oriented in deletional and inversional orientations to the same extent (Shapiro and Weigert, 1987; Thiebe et al., 1999), single rearranged B cells should have equal frequencies of inversions and deletions. With the exception of the Vκ21D group, clones from both 3H9H/56R and 3H9H/56R/76R exhibit a large number of rearrangements by this criterion. Using 3H9H/56R/76R-Vκ38c hybridomas as an example, 24 out of 30 rearrangements to proximal Jκ segments appear to have undergone inversion. This observation is based on a given rearrangement typing positive with a Jκ2 primer but typing negative with a Jκ5 primer, indicating that it is no longer in proximity to Jκ5. Based on the observed number of inversions we estimate about 3 rearrangements per clone (ELP and MW in preparation).

Vκ21D-Edited B Cells in 3H9H/56R$^+$ Hybridomas Have Different L Chain Genotypes

Among 3H9H/56R$^+$ hybridomas with Vκ21D rearrangements, most Vκ21D rearrangements are to Jκ2 and the

silent κ allele is usually unrearranged (Table 2). The high frequency of this genotype is not simply due to the expansion of a single, edited B cell clone because most Vκ21D hybridomas are distinguishable based on the status of their other H and L chain loci. For example, among the 26 hybridomas in which Vκ21D-Jκ2 was the only identified κ rearrangement, seven different genotype/phenotype combinations were observed, e.g., different H chain configurations. Fourteen 3H9H/56R$^+$ clones with Vκ21D-Jκ2 rearrangements had evidence of additional and different κ rearrangement(s). Taken together, this diversity in L chain and H chain genotypes indicates that most of the hybridomas with Vκ21D-Jκ2 rearrangements are clonally unrelated. Moreover, hybridomas derived from LPS-treated spleen cells (as in this case) tend to be of independent origin. A high frequency of Vκ21D-Jκ2/κ^0 could result from preferential rearrangement of this Jκ-proximal Vκ gene. J proximal V genes are preferentially rearranged in the mouse H chain locus (Yancopoulos et al., 1984), however this has not been convincingly documented for κ (Kaushik et al., 1989; Lawler et al., 1989). Alternatively, Jκ proximal V genes may only have a brief opportunity to rearrange, due to deletion by upstream Vκ rearrangement; hence a genotype suggestive of essentially primary rearrangement of Vκ21D would be the most likely to emerge. However, neither model explains the striking Jκ2 preference. Vκ21D can and does rearrange to other Jκ segments; in our 3H9H/56R$^+$ panel there were eight hybridomas with Vκ21D rearrangements involving non-Jκ2 segments (one to Jκ1, six to Jκ4, and one to Jκ5). One possible explanation currently under investigation may be a sequential order of Jκ rearrangement in which Jκ1 and Jκ2 are rearranged before Jκ4 and Jκ5.

Discussion

Anti-DNA is the most common of all self-specificities in autoimmune disease (Tan, 1988). One reason for this is that many proteins can bind DNA merely through surface-active basic amino acids. This is especially true for the arginine side chain, which can form so called bidentate interactions with G-C base pairs (Seeman et al., 1976) and electrostatic interactions with phosphate groups. Mutations to arginine occur frequently in FR3 and this region, in addition to the CDRs, is exposed on the surface of the antibody and can contribute to antigen binding (Figure 4). Mutations to arginine are a recurrent theme in anti-DNAs (Radic et al., 1993a), (Shlomchik et al., 1989). In vitro mutagenesis shows that single amino acid substitutions to arginine can create specificity for DNA and multiple substitutions to arginine have an additive effect on affinity for DNA (Radic et al., 1993a). The scaffold upon which these substitutions arise seems unimportant since anti-DNA antibodies are encoded by a variety of V genes in mouse or human.

It is easy for antibodies to acquire arginine residues. One route is by somatic mutation. In fact the codon bias in the CDRs of many V genes (Chang and Casali, 1994) favors mutation to arginine. For example, CDR serine codons are usually AGY and two different single base changes in this codon lead to an arginine codon. Since serine is among the most common germline-encoded CDR amino acids (Kabat et al., 1991), there is a high probability of becoming anti-DNA during clonal expansion. This is in fact how the specificity for dsDNA arose in the 3H9 antibody (Shlomchik et al., 1987b). Another source of arginine codons is the diversity segment, D_H. Certain reading frames and orientations of D_H segments are enriched in arginine codons, leading to the formation of anti-DNA B cells early in development (Eilat et al., 1988; Shlomchik et al., 1987a).

The arginines of anti-DNAs are mainly in V_H. This obviously follows for arginine codons created by V(D)J rearrangement and N addition, but the majority of somatic mutations to arginine codons arise in V_H as well. If arginines play a central role in DNA binding then one would expect the H chain to be the dominant specificity-determining domain of anti-DNAs. H chain dominance has been shown directly; isolated H chains bind DNA (Polymenis and Stollar, 1995a), affinity labeling shows preferential association of oligonucleotides with H chain (Jang et al., 1996), and in vitro, mutagenesis of H chain arginine codons eliminates or modifies DNA binding (Radic et al., 1993a). H chain dominance contributes to the high frequency of anti-DNA B cells. If one subunit is sufficient for DNA binding, then a variety of V_H/V_L combinations will share specificity, in contrast to specificities defined by particular V_H/V_L combinations. For example, 3H9H binds DNA in association with many different Vκs and Vλ1 (Ibrahim et al., 1995).

The high probability that anti-DNAs arise during the formation of the preimmune repertoire and during clonal expansion puts individuals at risk of developing anti-DNA autoimmunity. However, anti-DNA B cells are efficiently regulated even in anti-DNA transgenic mice that begin with an abnormally high precursor frequency of anti-DNA B cells. This is mainly due to L chain editing. Editing is possible because certain L chains can modify the DNA binding of the 3H9H, 3H9H/56R, and 3H9H/56R/76R. These L chain editors have V regions with very acidic CDRs (particularly because of aspartate residues) (Figure 2) and have the capacity of preventing or significantly reducing DNA binding (Figure 3). L chain editors are likely to work for many anti-DNA V_Hs. Although many different genes encode murine and human anti-DNA V_Hs, they have arginines at positions analogous to those of 3H9H/56R/76R. The affinity of an anti-DNA with arginines just in CDR3 is significantly decreased by the aspartates in the CDR1 of the editor, Vκ20 (Jang et al., 1998). Therefore, it is likely that just a few strategically positioned acidic residues within a subset of Vκs are adequate for editing most anti-DNAs.

The aspartate residues that distinguish master L chain editors are at or near the surface of the antibody and create a region of negative potential along the V_H-V_L interface (Figure 2D). Mutagenesis of the aspartate residues in CDR1 of Vκ20, to neutral amino acids results in a significant increase in DNA affinity of the 2C10 antibody (Jang et al., 1998). The ability of these aspartates to inhibit DNA binding might simply be a matter of negative charge repulsion. Alternatively, the V_L aspartate residues may prevent binding by directly neutralizing V_H arginines. In order for opposite charges to cancel, they must be able to reach each other. Models of 3H9H/56R/76R combined with editors (Figure 4) illustrate how the proximity of aspartates and arginines can reduce the negative and positive surface potentials of the individual VLs and 3H9H/56R/76R. But substantial positive regions remain

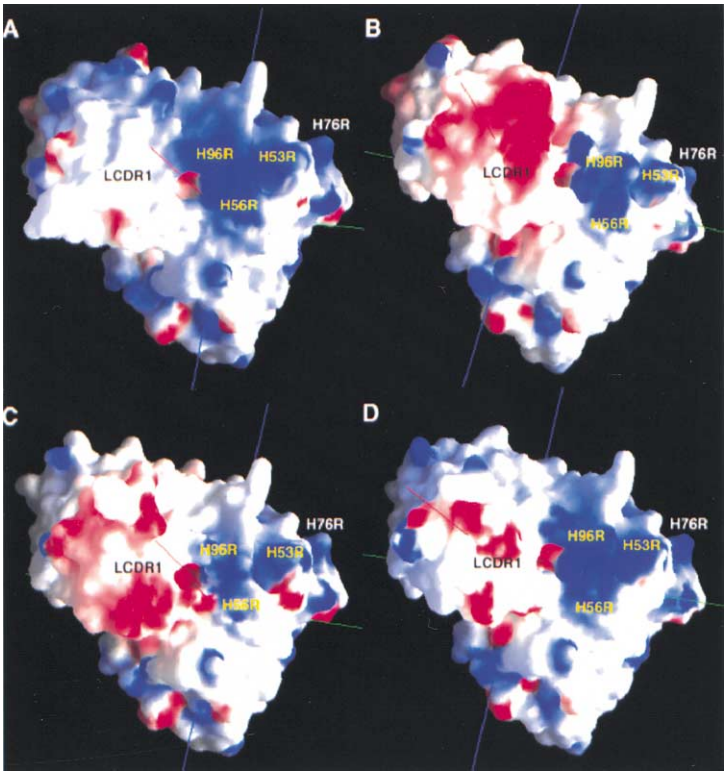


Figure 4. Electrostatic Potentials at the Molecular Surface of the Models of Anti-DNA mAb

3H9H/56R/76R H chain paired with different L chains: Vκ1 (A), Vκ20 (B), Vκ21D (C) and Vκ38c (D). The same template (PDB code: 1D5B) was chosen from the Protein Data Bank as a structure template to re-model the target molecules. The sequences of target molecules and template were sent to the Swiss Model web site (http://www.expasy.ch/swissmod/SWISS-MODEL.html) to reconstruct the structures of the target molecules. Electrostatic potentials at the surface of remodeled molecules were calculated with GRASP (Nicholls et al., 1991) and represented on a color scale from blue for positive potential, to white for neutral, to red for negative potential.

in 3H9H/56R/76R, suggesting that some arginines in 3H9H/56R/76R are too far from residues in the L chain to achieve adequate charge neutralization. However, the range of conformational shifts in the H/L dimer is not well known. Shifts in the relative positions of the H and L chains have been observed, as have shifts of individual CDR domains and internal motions of amino acid sidechains. It is worth pointing out that the sidechains of arginine as well as of its potential partners, glutamate and aspartate, are among the longer, more flexible sidechains in proteins. In addition, Tramontano et al., have identified one specific requirement for a large-scale shift in the combining site. As described by these authors, the presence or absence of a bulky sidechain at position 71 in FR3 is correlated with a 6.3 Å conformational shift in the H chain CDR2 (Tramontano et al., 1990). A smaller side chain, such as the alanine at position 71 in the 3H9 H chain, could allow CDR2 to move closer relative to the remainder of the combining site. Although the side chain flexibility and the H chain CDR2 conformational shift may not be sufficient to render all of the exposed arginines in 3H9H/56R/76R accessible to the negatively charged residues in the editor L chains, it is conceivable that these, along with other, as yet unknown alternative Fv structures, could block access of DNA to the 3H9 combining site. If this is the case, interactions between arginines in the transgenic H chains with negative charges in editor L chains may be expected to reach across the combining site in a zipper-like fashion and block the binding of DNA. As the distances between H chain arginines and negative charges in the combining site of the L chain increase, the task of blocking DNA binding becomes progressively more difficult, if not impossible for the most distant arginine, 76R in FR3. This view is supported by the inability of the Vκ editors to fully suppress DNA binding of the 3H9/56R/76R H chains.

The scarcity of L chains that prevent or reduce binding in the H chain sd-tg mice, profoundly affects the B cell repertoire. Only 4 out of the 93 functional Vκ genes edit the DNA binding of the 3H9/56R and 3H9/56R/76R VH regions. Consequently, B cells that retain the H chain sd-tg often undergo multiple editing attempts to veto or decrease affinity for DNA. Multiple editing attempts lead to B cell repertoires that are biased to Jκ5 and may also increase the frequency of λ B cells. Although the effect on the λ B cell frequency is not obvious in this setting because of the limited mouse Vλ repertoire, the influence on λ frequencies among human B cells could be profound. The large, diverse human Vλ repertoire may include efficient editors of anti-DNA VH regions and other self-specificities and can thus play an important role in establishing self-tolerance (Lee et al., 2000).

Receptor editing involves rearrangement in B cells that express a functional, albeit anti-self, receptor. Consequently editing might compromise allelic and isotypic exclusion. As κ and λ loci rearrange independently of each other (Liu et al., 2000; Ramsden and Wu, 1991; Yamagami et al., 1999), it is thought that exclusion is accomplished by the combination of recombination termination signals and differential rates of rearrangement between κ and λ (Arakawa et al., 1996). Concurrent rearrangement of a productive κ and λ is minimized by a rate of λ rearrangement that is much slower than the κ rate (Mehr et al., 1999; Ramsden and Wu, 1991). Recombination is thought to stop when a functional receptor is expressed (Alt et al., 1984). Hence a productive κ rearrangement prevents further κ and λ rearrangement. If recombination is reinitiated because of the autoreac-

tivity of a κ-associated receptor, then the λ loci can resume recombination and the longer it takes to edit, the greater becomes the probability of a productive λ rearrangement. Thus the κ/λ dual-expressing B cells in 3H9H/56R mice probably arise by the following sequence of events. During the course of editing a 3H9/56R-κ anti-DNA antibody, a B cell rearranges a λ gene productively. Because the 3H9/56R-λ combination binds DNA, the cell continues to edit until a κ editor is successfully rearranged, for example, Vκ38c. But, because λ rearrangements cannot be deleted in the mouse, this B cell still expresses λ and remains partially autoreactive. We have shown that even though the 3H9H/56R-κ/λ B cell is autoreactive, mechanisms that supplement editing appear to regulate these anti-DNA B cells (Li et al., in press).

Partially autoreactive B cells may pose a special risk of causing autoimmunity. These B cells are poised to become fully autoreactive if the editor is lost. Loss of an editor or the editing function can come about by a nonsense mutation (Brard et al., 1999) or a missense mutation. An example of the latter is the MRL/lpr antibody, 2C10 (Jang et al., 1998). The 2C10 antibody is associated with the Vκ20 editor, yet binds DNA. This Vκ20 has a mutation to asparagine in CDR1 next to aspartates that edit DNA binding. The additional asparagine at this site might directly interact with aspartate or enhance affinity for DNA. Reinitiation of recombination can also destroy an editor. Since Vκ editors are often rearranged to Jκ5, further rearrangement on the same allele would result in loss of the editor by RS inactivation. A consequence of exhaustive κ light chain editing (including RS deletion) would be an enrichment of λ-associated autoantibodies and this is indeed the case among human lupus autoantibodies (Paul et al., 1992; Ravirajan et al., 1998; Roben et al., 1996; Seskak et al., 1987).

Thus, ironically, mechanisms of receptor editing can reinstate autospecificities that editing initially served to eliminate. Because editing ordinarily happens in immature B cells, the edited population should be tolerant to self-antigens found in the milieu of the bone marrow. These self-antigens are thought to include the contents of blebs on apoptotic cells, predominantly nucleic acid-protein complexes (Casiola-Rosen et al., 1994), and we have shown that the 3H9 antibody binds to bone marrow B cells undergoing apoptosis (Xu et al., 1998). Thus loss of tolerance by inappropriate rearrangement and mutation will result in the reappearance of antibodies directed to bone marrow self-antigens and may explain the prevalence of these autospecificities in both natural and induced autoimmunity (Tan, 1988).

Experimental Procedures

Generating Site-Directed Transgenic Mice

The aspartic acid at position 56, alone or in combination with the serine at position 76 of the V_H3H9, has been replaced with arginine(s) by site-directed mutagenesis to generate the mutated V_H3H9 segments, 3H9H/56R and 3H9H/56R/76R, respectively (Radic et al., 1993a). A 3.7 kb SalI-EcoR1 DNA fragment containing the 3H9 mutant, prerearranged VDJ was joined to the Cμ region as described previously (Erikson et al., 1991). Because of changes introduced during cloning of the mutants, this 3.7 kb fragment is ~400bp shorter at the 5′ end than the cloned 3H9 fragment, but this should not influence transgene expression because the truncation is far upstream of the promoter. The neomycin phosphotransferase gene (Neo) and the thymidine kinase gene (TK) were cloned into our construct in the orientation and sites shown in Figure 1 as described previously (Chen et al., 1995). The transfection and identification of the targeted genes were performed as described previously with minor modifications (Luning Prak and Weigert, 1995; Chen et al., 1995). Briefly, the targeting vectors carrying 3H9H/56R or 3H9H/56R/76R mutants were linearized by Not I digestion. 20 μg of the linearized DNA were transfected into E14-1 ES cells (2×10^7 cells) using electroporation conditions of 0.25 kV, 500 μFD in 800 μl of the ES cell culture medium at room temperature. ES cell culture was carried out as described previously (Luning Prak and Weigert, 1995; Chen et al., 1995). G418 resistant colonies were screened for homologous recombination events by a DH1.5 /neo PCR (Chen et al., 1995). Candidate ES cell clones were confirmed by Southern hybridization using a JH probe (pJ11). A 7.9 kb fragment was observed for each 3H9 mutant in addition to the 6.4 kb wild-type germline band (data not shown). Targeted ES cells were injected into C57Bl/6 blastocysts. Chimeric mice were crossed to C57Bl/6 mice to screen for germline transmission. Sd-tg mice were backcrossed to the BALB/c background. Most of the mice used in this study were 10 to 12 weeks old and at the second backcross generation for the 3H9H/56R/76R model and the ninth backcross generation for the 3H9H/56R and 3H9H models on the BALB/c background. Mouse genotypes were determined by PCR analysis of genomic DNA from tail biopsies.

PCR

All PCRs were carried out in 1 × buffer II (Perkin Elmer) with a final concentration of 200 μM of each dNTP (Boehringer Mannheim), 50 pmol of each primer, 1.5 mM $MgCl_2$, and 1 unit of AmpliTaq Gold (Perkin Elmer). All PCR amplifications were performed using a Peltier-type thermal cycler (Hybaid). Primers and conditions used for H and some L chain PCR assays have been described previously (Luning Prak et al., 1994; Luning Prak and Weigert, 1995; Brard et al., 1999; Chen et al., 1995). For PCR detection of the sd-tg H chains, a primer homologous to the leader sequence (LD, MW114) and an sd-tg-specific primer in the CDR3 region (MW162) of the V_H3H9 gene were used. The mutations in 3H9 were confirmed by sequencing. For PCR detection of the rearranged κ genes, the PCR conditions and all of the primers (the forward primers: Vκ12/13, Vκs, and L5; the reverse primers: Jκ2 and Jκ5) were described previously (Luning Prak et al., 1994). To detect Jκ usage of the editor rearrangements, Vκ-specific forward primers (Vκ21-4: 5′-GCCAGCCAAAGTGTTGATTATG-3′; Vκ38c: 5′-AAGGCAAGCCAAGACATTAACAAGTATATAGCT-3′; Vκ20: 5′-ACCAGCACTGATATTGATGAT-3′) were used in combination with the standard reverse primers (Jκ2; Jκ5). The Vκs and L5/Jκ2 or Jκ5 PCRs were performed to detect the Jκ usage by unknown Vκs. In our experience the Vκs primer detects approximately 90% of Vκ genes (Schlissel and Baltimore, 1989). The Vκs primer also detects the Vκ/Jκ2 rearrangement contributed by the Sp2/0 fusion partner in all hybridomas. However, the L5 primer detects multiple Vκ genes without picking up Vκ/Jκ2 rearrangement from Sp2/0. Neither the Vκs nor the L5 primer detects theVκ38c gene. Cκ-deletion was detected using Vκs as a forward primer and RS-101 as a reverse primer. The RS-101 primer is homologous to the recombination signal sequence located downstream of Cκ. The other type of Cκ deletion detected by IRS1/RS101 PCR was not performed in this study (Retter and Nemazee, 1998). The PCR primers and conditions for detecting rearrangement of the λ genes, λ1 and λX, were carried out as described previously (Luning Prak et al., 1994).

Sequencing

The identities of some editor genes were confirmed by sequencing the Vκs or L5/Jκ2 or Jκ5 PCR products. PCR fragments were purified from a 1.5% agarose gel (Ultra Pure Agarose; GIBCO BRL) using QIAquick gel extraction (Qiagen Inc.). Nucleotide sequencing was performed using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase, FS, according to the manufacturer's instructions (Applied Biotechnology, Inc.). Reactions were run on the Applied Biosystem 377 PRISM automated DNA sequencer (PE Applied Biosystems).

ELISA

ELISA determined Ig isotypes and κ chain as described previously (Luning Prak et al., 1994). For detecting λ, a biotinylated rat anti-mouse antibody against λ1, λ2, and λ3; R26-46 (Pharmingen, San Diego, California) and streptavidin-alkaline phosphatase (Vector Laboratories, Inc., Burlingame, California) were used in a solid phase assay. OD_{405} was measured using an automated plate reader (Bio-Rad Laboratories). Purified isotypic mouse immunoglobulins (Sigma, St. Louis, Missouri) were used as standards in the range of 0.0001-1 μg/ml. The Ig concentration in the supernatants was calculated based on a standard curve using Delta 3 software (BioMetallics, Inc., Princeton, New Jersey).

DNA Binding Assay

Double-stranded DNA (ds-DNA) binding was determined in a liquid phase ELISA using biotinylated ds-DNA as described previously (Radic et al., 1993a). For detecting DNA-bound λ antibody, a rat anti-mouse λ (an IgG2α/κ isotype, Pharmingen, San Diego, California) was used. AP-conjugated goat anti-rat IgG was used to detect the rat anti-mouse antibody. Supernatant from a 3H9/Vκ1 hybrid obtained from the fusion of a 3H9/Vκ1/Rag-$2^{-/-}$ mouse (our unpublished data) was used for a high-affinity ds-DNA binding positive control and 3H9/Vκ8 supernatant (Chen et al., 1997) was used as a low-affinity control. OD_{405} of ds-DNA binding were normalized between plates using the same positive control on each plate.

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