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Regulation of Anti-Phosphatidylserine Antibodies

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Summary

The degree of heavy chain (H) editing, the types of Vκ editors, and the pattern of Jκ usage are correlated with a range of the affinity of anti-DNA. This range was determined by the number and location of arginine (R) residues in the VH. We, here, changed a key arginine residue in the VH of anti-DNA transgene to glycine, which sharply reduces the affinity for dsDNA. However, complete reversion of this anti-DNA to germline enhances the affinity for phosphatidylserine (PS). The B cells of this low-affinity anti-DNA and anti-PS transgenic mouse are tightly regulated by receptor editing. Thus, anti-PS B cells are another example of a constitutive self-antigen regulated in the bone marrow.

Introduction

Central tolerance operates efficiently on lymphocytes that bind to membrane-bound self-antigen. The prototypic example, anti-MHC, is subject to central tolerance only when the self-antigen is in the membrane (Nemazee and Burki, 1989; Russell et al., 1991). On the other hand, B cells reactive to soluble self-antigen appear to be subject to peripheral tolerance mechanisms such as inactivation (Goodnow et al., 1988). Anti-DNA B cells are also subject to central tolerance. The 3H9/Vκ4 transgenic, constructed from an anti-ss/dsDNA spontaneously expressed by an MRL/*lpr* lupus mouse, has no peripheral anti-DNA B cells because the anti-DNA BCR is edited (Chen et al., 1995a; Erikson et al., 1991). In mice unable to edit, such as *RAG*$^{-/-}$ mice, the 3H9/Vκ4 B cells are completely deleted at the pre-B cell to immature B cell transition (Xu et al., 1998). This is surprising because DNA would be expected to be present in soluble form. However, a cell surface association of DNA and indeed many of the targets of lupus autoantibodies on dying cell have been demonstrated (Casiola-Rosen et al., 1994). We were stimulated by this finding to test whether 3H9/Vκ4 also binds to apoptotic cells. This is the case: the 3H9/Vκ4 antibody binds uniquely to cells undergoing experimentally induced apoptosis (Xu et al., 1998). 3H9/Vκ4 also binds to bone marrow B cells, and we assume that here too the binding is to dying cells. Hence, the targets of lupus autoantibodies and of the anti-DNA, 3H9/Vκ4, may also fall in the category of surface antigens, and self-reactive bone marrow B cells may be exposed to (and regulated by) these autoantigens early in B cell development.

Alternative self-antigens can also considered. Many anti-DNAs bind related molecules such as anionic phospholipids, 3H9/Vκ4 being a case in point (Cocca et al., 2001). The polyanionic nature of DNA and certain phospholipids, such as phosphatidylserine (PS), provides a possible basis for this crossreaction. Therefore, the tolerogen of 3H9/Vκ4 B cells could be either DNA, anionic phospholipids, or other polyanions. Phosphatidylserine is an attractive candidate because this phospholipid is displayed on the outer surface of apoptotic cells (Fadok et al., 1992).

We could test whether PS is a tolerogen because of our finding that reversion of the somatic mutations in 3H9H (to resurrect the germline 3H9H [3H9H/GL]) significantly decreased affinity for DNA but enhanced affinity for PS (Figure 1 and Table 1) (Cocca et al., 2001). Consequently a site-directed transgenic (sd-tg) mouse using the VDJ coding for the 3H9H/GL should be informative. Here we have compared the 3H9H/GL sd-tg to the 3H9H sd-tg. We find that regulation of the two is similar. Both undergo receptor editing mainly at the κ locus, and there is considerable overlap of the Vκ editors. Hence, PS is an active tolerogen. This finding relates the process of self-tolerance to apoptosis and may help to explain why defects in apoptosis are associated with autoimmunity.

Results and Discussion

The 3H9H/GL sd-tg Model

We have constructed a new site-directed H chain transgenic model, 3H9H/GL (Figure 2). The 3H9/GL H chain, paired with a variety of L chains, binds PS and has low affinity for ss/dsDNA (Cocca et al., 2001). 3H9H/GL was derived from the H chain of an anti-DNA antibody, 3H9, by in vitro mutagenesis. The three somatic replacement mutations in the 3H9 VH gene have been reverted to their germline codons (Figure 1A). This VH sequence is now identical to the coding sequence of the germline VH gene, VMU3.2 (Winter et al., 1985). The construct used to generate the 3H9H series of site-directed transgenics was modified for targeting the 3H9H/GL genes to the VH locus through replacement of the Q52 and JH regions by homologous recombination (Chen et al., 1995b). Thus, the 3H9H/GL is identical to the other H chain sd-tg models except for 3, 4, and 5 nucleotide changes in 3H9H, 3H9H/56R, and 3H9H/56R/76R, respectively.

The 3H9H/GL sd-tg Excludes Endogenous H Chain Rearrangement

A key property of a H chain sd-tg is its ability to exclude endogenous H chain expression. Allelic exclusion is a property of normal H chain expression and/or can indicate receptor editing. Expression of the 3H9H/GL sd-tg

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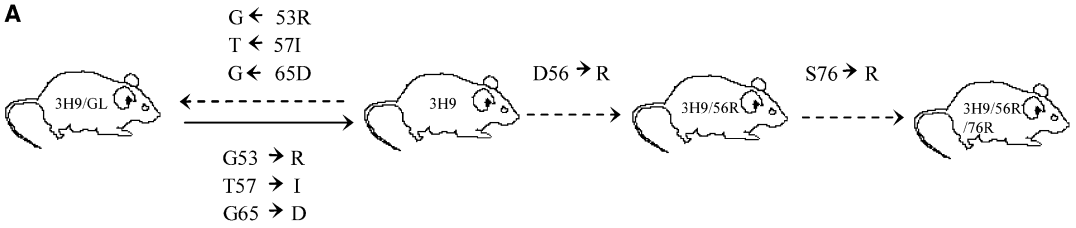
A



B

Antigen specificity and relative binding of 3H9 variants.

ssDNA	dsDNA	Antibodies	DOPS	DOPS+β2GPI
0.02	0.02	D56R, S76R	0.18	0.10
0.01	0.12	D56R	0.26	0.16
1.00	**1.00**	**3H9**	**1.00**	**1.00**
0.67	0.78	D65G	0.40	0.45
0.25	0.29	I57T	0.38	0.48
3.15	---	R53G	---	---
---	---	R53G, I57T, D65G	0.37	0.44

Figure 1. Somatic Replacement Mutations and Their Specificities

(A) The 3H9 clone produces an Ab with higher affinity for both ssDNA and dsDNA than its siblings. We attribute this to the glycine to arginine mutation in CDR2. The 3H9H gene rearrangement is the substrate for in vitro mutagenesis. We have derived higher affinity mutants by sequential mutations to arginine of the aspartate at position 56 and the serine at position 76 of the 3H9 H chain. Here, we explore the effect of reverting the 3H9H to its germline sequence 3H9H/GL. The solid lines represent the mutations that occurred naturally. The dashed lines represent the mutations that were created in vitro.

(B) Antigen specificity and relative binding of 3H9 variants. The data are taken from references (Radic et al., 1993a; Cocca et al., 2001) and normalized to 3H9. The data were derived using bivalent molecules. The DNA binding assays used IgG2b H chains paired with the λ1 L chain, whereas phospholipid binding was assayed by using homodimers of scFv containing the indicated VH and the 3H9 VL. Dashes indicate that readings remained below half maximal OD.

has been studied by measuring the amount of the sd-tg associated IgM allotype (IgM^a) on splenic B cells. The IgM associated with the 3H9/GL VH is derived from a 129/O1a ES cell line (Ig^a allotype). Crosses of the sd-tg to the Ig^b congenic CB17 ([sd-tg x CB17] F_1) mice allow us to distinguish sd-tg expression from endogenous IgM expression. Splenic B cell levels of the sd-tg encoded IgM are comparable to IgM^a levels in nontransgenic F_1 littermates (IgM^a/IgM^b) (Figure 3); thus, the expression level of the sd-tg is normal. In the (sd-tg/CB17) F_1, most B cells express only IgM^a (Figure 3). We presume that the absence of IgM^b means that the sd-tg prevents H chain rearrangement. It is possible that VH replacement occurred just on the targeted allele, but the hybridoma studies show that >92% of the hybridoma panel expresses the intact sd-tg (Table 1).

Almost complete exclusion of VDJ rearrangement and lack of VH replacement is also found in B cells of the 3H9H sd-tg (Table 1). The 3H9H/56R and 3H9H/56R/76R exclude less well: 17% and 63%, respectively, either express H chain from the untargeted allele or have undergone VH replacement (Li et al., 2001). These findings indicate that the 3H9H/GL B cell is either not regulated or regulated mainly by L chain editing.

Hybridoma Panels

To compare the antibody and V gene repertoires of the 3H9H and 3H9H/GL sd-tgs, we made hybridoma panels from in vitro LPS-activated splenic B cells of both sd-tg mice. Previous studies have shown that 3H9 mAbs either do not bind DNA or bind weakly. The majority of mAb do not bind DNA because the 3H9 H chain is associated with an L chain that does not sustain DNA binding (Chen et al., 1997). This is also the case for most of the 3H9H panel surveyed here (Table 1). Only six out of 147 3H9H positive hybrids exhibit significant DNA binding (Table 1 legend). The editor L chains constitute a limited range of the available L chain repertoire and include $V\kappa$s whose CDRs are rich in aspartates, e.g., $V\kappa20$ and $V\kappa21$-4 (Table 1). We think that the interaction of $V\kappa$ aspartates with the VH arginines accounts for the editing capacity of these L chains.

The majority of 3H9H/GL mAbs do not bind DNA presumably because the reversion of the 3H9H mutations (in particular, R53 to G) sharply reduces DNA binding (four mAbs do have significant DNA activity, see Table 1 legend). Hence, we expected that the κ repertoire would be broad (Radic et al., 1993a). However, the 3H9/GL mAbs are associated with a limited $V\kappa$ repertoire that overlaps with the 3H9H panel. 3H9/GL mAbs are mainly associated with $V\kappa12$ or $V\kappa20$ (Table 1). $V\kappa12$ and $V\kappa20$ are members of a set of four L chains that have CDRs with exceptionally low isoelectric points (IEPs) (Li et al., 2001).

We wondered whether the observed L chain restriction influenced the capacity of 3H9/GL (and 3H9) to bind to PS. Arginine also contributes to PS binding (Cocca et al., 2001); hence, the same $V\kappa$ editors that veto arginine-

Table 1. H and L Chain Usage in 3H9HGL and 3H9H sd-tg Hybridomas

H chain	3H9HGL (%)	3H9H (%)
Total clone tested	91 (100)	155 (100)
sd-tg$^+$	84 (92.3)	147 (94.8)
L chain	**3H9HGL$^+$**	**3H9H$^+$**
Total clone tested	84 (100)	147 (100)
Vk12 (−46)	21 (25.0)	94 (64.0)
(−41)	1 (1.2)	0 (0)
Vk20 (bt20)	34 (40.5)	3 (2.0)
(bw20)	1 (1.2)	0 (0)
Vk21 (−4)	1 (1.2)	17 (11.6)
(−5)[a]	1 (1.2)	0 (0)
(−10)[a]	6 (7.2)	0 (0)
Vk38c	0 (0)	2 (1.4)
Vk1	1 (1.2)	2 (1.4)
Vk4	1 (1.2)	3 (2.0)
Other Vks	13 (15.5)	19 (12.9)
Vks$^-$	4 (4.8)	7 (4.8)
λ1	0	1
λx	0	0

Splenic B cells were isolated from 3H9H/GL and 3H9H sd-tg mice and stimulated with LPS (20 μg/ml) for 3 days before fusion to sp2/0. The results shown here represent one mouse for each model. A similar result was obtained from a second fusion of 3H9H/GL B cells (data not shown). Vκ12-46, Vκbt20, Vκ21-4, Vκ38c, λ1, and λX were identified by PCR using specific 5′ primers described in the Experimental Procedures. The other Vκs were identified by sequencing the products of Vκs or L5/Jκ PCRs. All sequencing data were analyzed using the NIH Igblast database (http://www.ncbi.nlm.nih.gov/igblast). There were four clones in 3H9H/GL fusion with ds-DNA binding activity. The supernatants from a clone with a Vκbb1/Jκ1 rearrangement and three unknown Vκ clones bound to dsDNA. There were six ds-DNA binders in 3H9H fusion. Both 3H9H anti-dsDNA clones had a Vκ4/Jκ5 rearrangement. Four 3H9H clones with unidentified Vκ rearrangements also bound to dsDNA.
[a] Vκ21-5 and Vκ21-10 share the same amino acid sequence.

mediated DNA binding might operate on PS receptors. In PS binding assays of hybridoma supernatants, the Vκ12 and Vκ20 L chains efficiently inhibit PS binding by 3H9/GL (Figure 4A). A few mAbs bind PS, and they are associated with Vκs known to sustain DNA binding when combined with 3H9 (such as Vκ1 or Vκ4). This set may have escaped regulation because of low affinity for PS. Some PS binding mAbs are also found in the 3H9H panel (Figures 4B and 4C). Of particular interest are the mAbs with editor L chains. In the 3H9H panel, as in previous studies, the majority of mAbs express an editor L chain and these mAbs do not bind DNA (data not shown). However, this edited set falls into two categories of PS binding: the Vκ12 and Vκ38c bind PS, while Vκ20 and Vκ21 do not. We attribute the differential ability of editors of DNA to inhibit PS to sequence differences in these Vκ editors.

The Structural Basis of PS/DNA Binding and Editing

Residues involved in DNA binding have been identified through in vitro mutagenesis. Arginine plays a key role in DNA binding by the 3H9 VH as shown by the R53 to G or R96 to G mutations (Radic et al., 1993a). The reversion of the R53 somatic mutation of 3H9H to the germline VMU2.3 sequence also significantly reduces dsDNA binding. The R96 codon resulted from either N addition or mutation. The R96 to G substitution reduces DNA binding to background levels. Models of 3H9H show both arginines to be on the surface of the antibody; thus, both arginines could bind DNA. R96 is in the center of the combining site and may influence the shape of the combining site. A similarly placed R in the DNA-1 structure of anti-DNA Fab fragment is hypothesized to prop open the site (Tanner et al., 2001). Thus, R53 may determine the so-called affinity maturation of the 3H9, while R96 may control the shape of the combining site (and the ability of CDR residues to come in contact with DNA).

PS binding is more complex; reversion of all the somatic mutations of 3H9 lowers affinity for DNA but increases affinity for PS. To identify the residue(s) responsible for this effect, the mutations were individually replaced. Replacement of the I57 of 3H9H by T and D65 of 3H9H by G reduces PS binding, but PS binding is restored by G53 of 3H9H to R (Cocca et al., 2001). This pattern suggests that the PS binding site is different in 3H9 and 3H9/GL mAbs. The 3H9 site centers around R53 but the 3H9/GL site lies elsewhere. Alternatively, the presence of an arginine as such may have a greater impact on DNA binding than PS binding. This could result from the pleiotropic interactions between DNA and arginine (Seeman et al., 1976). In contrast, R53 causes only an incremental improvement of PS binding. Thus, the net effect of the reversions is that 3H9/GL binds better than 3H9 to PS, but worse than 3H9 to DNA.

Comparisons of Vκ editor sequences support the separate-site hypothesis. The CDRs of editors have low IEPs because of high frequencies of aspartates, and Vκ20 editing has been shown by in vitro mutagenesis to depend on CDR aspartates (Jang et al., 1998). However, the distribution of aspartates varies between editors, and this could account for the differences in editing efficiencies (Li et al., 2001). Vκ12 edits both DNA and PS activity in 3H9/GL, yet it only suppresses DNA binding without affecting PS binding of 3H9. The key aspartate in Vκ12 is likely to be D56, and this site is close to R96 in VH (Figure 5A). Consequently, D56 could neutralize or block access of DNA to R96. However, the Vκ12 aspartate is far from the potential PS binding site at R53 that is unique to the 3H9 VH. In contrast, the Vκ20 editor has additional aspartate CDR residues at sites in CDR1 that are close to R53 (Figure 5B, left). This may explain why Vκ20 inhibits both DNA and PS binding in combination with either H chain.

The models of the various Vκ editors provide a structural basis for the observation that DNA binding of 3H9 involves cooperation between R53 and R96. Exchange of the 3H9 CDR3 with other CDR3s or mutation of R96 to glycine abolished DNA binding . In an analogous fashion, the proximity of aspartate D56 in Vκ to R96 in VH of 3H9/Vκ12 may suffice for inhibiting DNA binding as the R53 in VH by itself does not support DNA binding.

Addition of R56 to the 3H9H can raise the affinity for DNA to the point that L chain editors require aspartates in CDR1 that can block both R53 and R56 in VH in order to prevent DNA binding (Figure 5B, right). Thus, Vκ21-4, a L chain that has aspartates at CDR1 sites 30, 32, and 34 is the major editor rather than Vκ12. However, electrostatic potentials alone may not fully explain the editing potential of the different L chains. For example, even though the CDR1 of Vκ20 and Vκ21-4 have similar

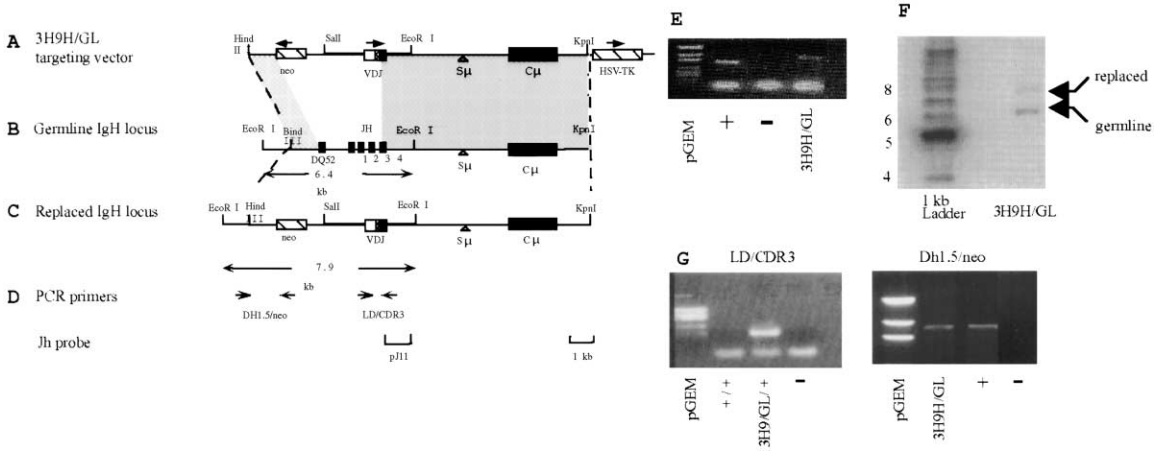


Figure 2. Site-Directed Replacement of the JH Locus with the 3H9/GL VH Gene

Arrows indicate the transcriptional orientations of the neomycin, *VDJ*, and *HSV-TK* genes. Homologous flanking sequences facilitate recombination between the targeting vector (A) and the germline IgH locus (B). The regions of homology are stippled. A 7.9 kb EcoRI restriction fragment is obtained with the replaced IgH locus (C and F) while a 6.4 kb fragment was derived from the germline locus (B and F). The position of the JH probe (pJ11) and the PCR primers is shown (D). PCR analysis of targeted ES cells is shown in (E). Vector DNA with the binding sites for both DH1.5 and neo primers (expected size of the PCR product is 0.9 kb) is used as a positive control. DNA from 3H9H/GL-targeted ES cells was assayed for homologous recombination by DH1.5/neo PCR. The expected size of the DH1.5/neo PCR product in targeted ES cells is 1.5 kb. The pGEM DNA marker (Promega) is loaded in the first lane. Southern blot analysis was performed using EcoR1 digested genomic DNA from transfected ES clones that tested positive by DH1.5/neo PCR. The DNA was hybridized with pJ11. The observed bands at (F) indicate proper targeting, 6.4 kb (germline) and 7.9 kb (targeted) (B and C). PCR analysis of ES cell-derived offspring is shown in (G). Tail DNA of a heterozygous mouse (3H9H/GL/+) and a wild-type littermate (+/+) were tested for the presence of the 3H9H/GL sd-tg by LD/CDR3 PCR (expected size is 0.45 kb). Water was used as a negative control in PCR.

negative charges (Figure 5B, middle and left), Vκ20 may not be as efficient as Vκ21-4 at blocking PS binding. The Vκ21-4 editor may be more efficient at blocking PS binding to sites in the 56R H chain (Figure 5C) because its negative charges at D30 and D32 are carried on a longer CDR1. It is curious that 3H9 is strongly biased to Vκ12 when other editors are at least as efficient at abrogating PS binding. Perhaps Vκ12 is preferentially rearranged. Alternatively, the excess of aspartates may prevent selection by other antigens.

Jκ2 Bias of the Vκ Editor Rearrangements

The L chains of edited 3H9, 3H9/GL, and 3H9/56R (Li et al., 2001) antibodies are mainly rearranged to Jκ2 (Table 2). This is not due to clonal expansion of just a few Jκ2 rearrangements. Several different fusions show this bias, and within a fusion we can demonstrate that many examples are of independent origin because of unique configurations of untargeted or unexpressed alleles. Nor can the bias to Jκ2 be explained by its unique sequence because other Vκ12/Jκ or Vκ20/Jκ combinations also edit anti-DNA H chains. Instead, the Jκ2 bias may reflect the order of rearrangement. For example, if rearrangement usually begins at Jκ2, then one might expect this Jκ bias. However, Jκ representation in random Vκ repertoires shows high frequencies of both Jκ1 and Jκ2 (Wood and Coleclough, 1984); thus, the extreme case is unlikely unless the Vκ12, 20, and 21-4 were to uniquely prefer Jκ2. Yet, this cannot be the case because these Vκ genes can (and do) rearrange to other Jκs in other settings (see below).

Processive rearrangement, Jκ1 then Jκ2, etc., would predict higher frequencies of Jκ1 than observed (Table 2) unless Jκ1 combinations were counterselected. For example, rearrangement of many Vκs to Jκ1 (but not other Jκs) yields an arginine codon (TGG to CGG [Wu et al., 1978]). L chains with an arginine 96 are only seen in anti-DNA antibodies (Wu et al., 1978), and such L chains are edited (our unpublished data). In addition, Jκ1 might have a negative effect on H/L pairing or on

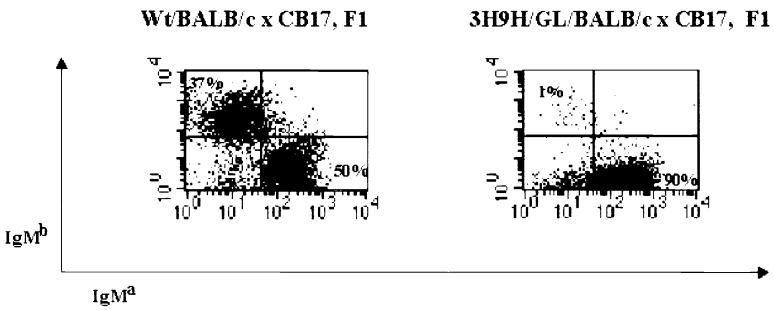


Figure 3. H Chain Allelic Exclusion in 3H9H/GL sd-tg Mice

The 3H9H/GL sd-tg mice on BALB/c background were crossed to CB17 to verify expression of the sd-tg. The targeted allele is of the μ^a allotype, being derived from 129/Ola ES cells, while the CB17 locus is of the μ^b allotype. FACS profiles of splenocytes from 3H9H/GL/BALB/c x CB17 (F_1) and a transgene-negative littermate control show staining of IgM^a and IgM^b populations. Cells were gated on the $B220^+$ population.

Table 2. Jκ Usage in L Chain Editor Rearrangements

	3H9H/GL		3H9H	3H9H/56R	3H9/Vκ4		
	Vκ12 (%)	Vκ20 (%)	V12κ (%)	Vκ21-4 (%)	Vκ12 on targeted allele (%)	Vκ12 on untargeted allele (%)	Total (%)
Total master editor+ clones	22 (100)	35 (100)	94 (100)	54 (100)	20	29	49 (100)
Jκ1	2 (9.1)	0 (0)	15 (16.0)	2 (3.7)	0 (0)	7 (14.3)	7 (14.3)
Jκ2	20 (90.9)	32 (91.4)	63 (67.0)	41 (75.9)	0 (0)	10 (20.4)	10 (20.4)
Jκ4	0 (0)	2 (5.7)	0 (0)	10 (18.5)	0 (0)	0 (0)	0 (0)
Jκ5	0 (0)	1 (2.9)	16 (17.0)	1 (1.9)	20 (40.8)	12 (24.5)[a]	32 (65.3)

For the 3H9H/GL fusion, all Vκ12- and Vκ20-positive clones were used for Jκ usage analysis. For the 3H9H fusion, only Vκ12-positive clones are shown. Jκ usage of Vκ21-4-positive hybrids from 3H9H/56R fusion are reported here in detail while the features of other major editors in the same fusion have been described previously (Li et al., 2001). For comparison, Jκ usage of Vκ12-positive clones is shown from a previous 3H9/Vκ4 study in which the 3H9 H chain sd-tg mouse was crossed to a Vκ4/Jκ4 sd-tg mouse (Chen et al., 1997). In the Vκ4/Jκ4 sd-tg, only the Jκ5 gene segment on the targeted allele was available for secondary rearrangement on the targeted allele. Vκ12 rearrangement on the targeted allele is determined by the following criteria: $V\kappa12^+$, $V\kappa4^-$, and no other κ rearrangements detected by PCR. Vκ12 rearrangement on the untargeted allele is $V\kappa4^-$, and $V\kappa12^+$ rearrangements to Jκ1, Jκ2, Jκ4. Jκ usage in editor rearrangements was detected by PCR using specific editor primers and Jκ2 or Jκ5 as the 3′ primer (as described in the Experimental Procedures).
[a] These rearrangements could be on either the targeted or the untargeted allele.

the range of specificities of Jκ1-associated antibodies. These two conditions, processive rearrangement and deleterious effects of Jκ1 on specificity or pairing, could lead to a Jκ distribution that is highly enriched in Jκ2. In order to explain the low Jκ4+Jκ5 frequency, this model must also assume a sharp decline in rearrangement rate as rearrangement progresses through the Jκ locus. Discontinuities in the process of rearrangement are not unexpected; for example, the occupancy of Jκ4 in the 3H9/Vκ4Jκ4 sd-tg promotes secondary rearrangement to Jκ5, and mice with an abbreviated κ locus are biased toward Vκ12/Jκ1 and Vκ12/Jκ5 (Table 2). Thus, differences in target size or time available for editing may dictate Jκ usage.

Conclusions

Self-tolerance to surface molecules takes place in the bone marrow (Hartley et al., 1991; Lang et al., 1996; Nemazee and Burki, 1989; Russell et al., 1991). This is only natural because lymphocytes that bind to other cells would be trapped. However, a B cell stuck to another cell can become unglued by editing the specificity of its receptor. Binding to PS may be a particular liability. B cells with receptors for PS, a unique component of the outer surface of apoptotic cells, may become attached to dying cells that are destined for removal by a scavenger. Therefore, regulation of anti-PS may be analogous to the regulation of autoantibodies to MHC (Lang et al., 1996). In contrast, DNA is an autoantigen that is not expected to be released from dying cells unless there is a defect in their clearance. Although DNA-protein complexes such as chromatin, and other lupus autoantigens, redistribute to blebs located at the surface of apoptotic cells, they remain secluded from the outside of the cell until the late stages of apoptosis when the plasma membrane becomes permeable. A possible reason that anti-DNA may nevertheless be subject to central tolerance arises because of crossreaction

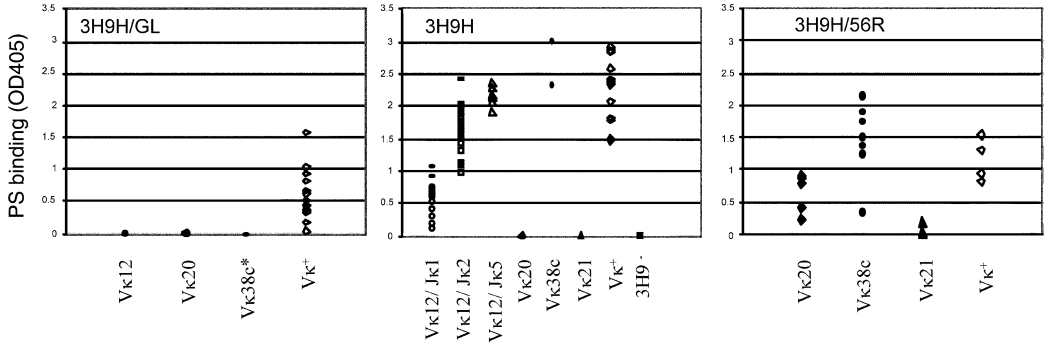


Figure 4. Phosphatidylserine (PS) Binding of Hybridoma Supernatants

PS binding of supernatants of 3H9H/GL, 3H9H, and 3H9H/56R hybrids was performed as described in the Experimental Procedures. All of these hybrids have retained the sd-tg H chain except the $3H9^-$ group in the 3H9H fusion. The results are displayed as groups according to the usage of editor L chains in each fusion. $V\kappa^+$ group includes the hybrids with the rearrangements that were detected by either Vκs or L5 primers. They are further divided into subgroups according the Jκ usage of $V\kappa12^+$ hybrids in the 3H9H fusion. PS binding was displayed as OD_{405} and normalized to an Ig concentration of 1 μg/ml. The fluctuation in the assay was determined by measuring the same samples five times. The range of fluctuation was 0.6 OD units. Therefore, the fluctuation seen in measurements of the same antibody is partially due to the experimental error. Each data point represents an individual mAb. Only the supernatants from the second 3H9H/GL fusion were used for PS binding. *One Vκ38c clone was found in the second 3H9H/GL fusion while none in the first fusion (Table 1). Supernatants of 3H9H/56R hybrids were from a fusion using 3H9H/56R-κ^{del}/κ^+ sd-tg mice (our unpublished data).

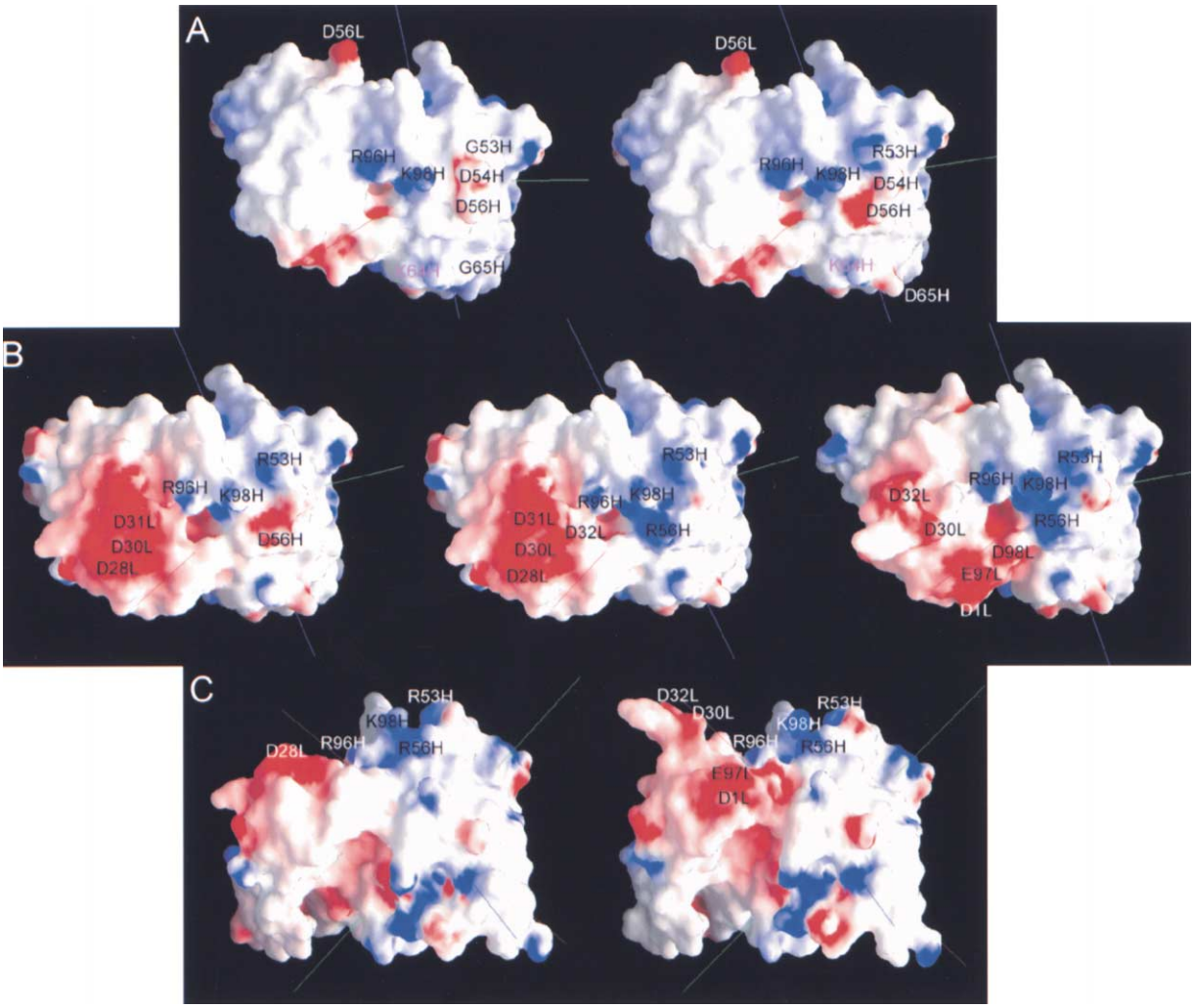


Figure 5. Influence of Aspartates of the Vκ Editors on the Electrostatic Potentials of Variable Regions of 3H9H/GL and 3H9H

A template (PDB code 1I8M) was chosen from the Protein Data Bank (http://www.rcsb.org/pdb) based on its sequence similarity to Vκ12. Predicted models of 3H9/GL-Vκ12 ([A], left), 3H9-Vκ12 ([A], right), 3H9-Vκ20 ([B], left), 3H9/56R-Vκ20 ([B], middle), 3H9/56R-Vκ21-4 ([B], right), 3H9/56R-Vκ20 ([C], left), and 3H9/56R-Vκ21-4 ([C], right) were reconstructed by Swiss Model (http://www.expasy.org/swissmod/sm_webmodel.html). (A) and (B) represent the top looking down into the combining sites. (C) provides the side views of the combining sites. The electrostatic potentials of each model were calculated with GRASP and are represented on a color scale from blue for positive potential, to white for neutral, to red for negative potential.

with phospholipids. This crossreaction has been observed in SLE patients (Lafer et al., 1981). In the case of 3H9 and 3H9/GL, this crossreaction is directed to PS which, as shown here, is a mediator of central tolerance.

These findings portend an intimate relationship between apoptosis and self-tolerance. This relationship is evident from the correlation between apoptosis deficiencies such as Fas (*lpr/lpr*) and autoimmunity. This relationship is thought to mean that autoimmunity results from failure to delete autoreactive lymphocytes. Our results support a different reason. As proposed by Rosen et al. (Casiola-Rosen et al., 1994), apoptosis defects may limit the amount of self-antigen. Thus, anti-PS B cells may escape from the bone marrow of Fas-deficient mice because of insufficient numbers of PS-presenting apoptotic cells. Our hybridoma panels from healthy mice also include significant numbers of PS binding mAbs. Some of these are not associated with editors, and others such as 3H9/Vκ12 retain PS activity in spite of editing. We suspect that these B cells escape because of low affinity for PS. Hence, PS tolerance may be delicately balanced, and any change in PS levels may shift this balance toward autoimmunity and permit anti-PS B cells to escape central tolerance. Such B cells may themselves be a source of autoAb as such or may be a substrate for affinity maturation and formation of anti-DNA by somatic mutation.

Experimental Procedures

Generating Site-Directed Transgenic Mice

Using site-directed mutagenesis, the arginine at position 53, the isoleucine at position 57, and the aspartic acid at position 64 of the 3H9H were reverted to their germline counterparts, glycine, tyrosine, and glycine, respectively, in 3H9H/GL (Figure 1) (Radic et al., 1993a).

A 3.7 kb SalI-EcoR1 DNA fragment containing the 3H9H/GL prerearranged VDJ was cloned into an expression vector with the Cμ region, the neomycin phosphotransferase (*Neo*), and thymidine kinase (*TK*) genes as shown in Figure 2A and described previously (Chen et al., 1995b; Li et al., 2001). The expression vector contains flanking sequences (indicated as shaded areas) that are homologous to the germline DH and JH/Cμ region (Figures 2A and 2B). The transfection and identification of the targeted genes were performed as described previously (Chen et al., 1995b; Li et al., 2001). ES cell culture was carried out as described previously (Chen et al., 1995b; Luning Prak and Weigert, 1995). G418 resistant colonies were screened for homologous recombination events by a DH1.5/neo PCR (Figures 2D and 2E) (Chen et al., 1995b). Candidate ES cell clones were confirmed by Southern hybridization using a JH probe (pJ11) (Figure 2D). A 7.9 kb fragment was observed for 3H9H/GL in addition to the 6.4 kb wild-type germline band (Figures 2B, 2C, and 2F). Targeted ES cells were injected into C57Bl/6 blastocysts. Chimeric mice were crossed to C57Bl/6 mice to screen for germline transmission. The tg status of PCR-positive agouti mice was confirmed by DH1.5/neo PCR (Figure 2G) and DNA sequencing of leader (LD)/CDR3 amplicons from tail DNA. sd-tg mice were backcrossed to the BALB/c background.

The mice used in this study were 10–12 weeks old and at the second backcross generation for the 3H9H/GL model and the ninth backcross generation for the 3H9H models on the BALB/c background. Mouse genotypes were determined by PCR analysis of genomic DNA. Mice used to test the expression of receptor allotypes were offspring of 3H9H/GL and 3H9H crossed onto the allotype-congenic, CB17 (The Jackson Lab, Bar Harbor, ME).

FACS Analysis

Single-cell suspensions from spleen and bone marrow were prepared from 10- to 12-week-old mice. Red blood cells were lysed for 5 min on ice using a 0.15 M NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM EDTA solution. Most samples were stained for the pan-B cell marker, B220 using anti-B220-APC (clone RA3-6B2, Pharmingen, San Diego, CA). Cells were stained for surface IgM^a with a FITC-conjugated antibody (clone RS3.1, Molecular Probes, Eugene, OR) and IgM^b with a PE-conjugated antibody (clone AF6, Pharmingen, San Diego, CA).

Hybridoma Production

Splenic B cells were isolated from 3H9H/GL and 3H9H sd-tg mice on the BALB/c background at 3 months of age using the protocols described previously (Chen et al., 1997). All splenic B cells were stimulated by LPS (20 μg/ml, #L2630, Sigma) for 3 days before the fusion with Sp2/0 myeloma cells (Kohler, 1980) using established procedures (Chen et al., 1997). Only hybrids growing as a single colony per well were subjected to further studies.

PCR

All PCRs were carried out in 1× buffer II (Perkin Elmer Life Sciences, Inc., Boston, MA) with a final concentration of 200 μM of each dNTP, 50 pmol of each primer, 1.5 mM $MgCl_2$, and 1 unit of AmpliTaq Gold (Perkin Elmer Life Sciences, Inc., Boston, MA). All PCR amplifications were performed using a Peltier-type thermal cycler (Hybaid Instruments, Franlin, MA). Primers and conditions used for H and L chain PCR assays have been described previously (Brard et al., 1999; Chen et al., 1995b; Li et al., 2001; Luning Prak et al., 1994; Luning Prak and Weigert, 1995). For PCR detection of the sd-tg H chains, a primer homologous to the leader sequence (LD, MW114) and a sd-tg-specific primer in the CDR3 region (MW162) of the 3H9H gene were used. PCRs to detect the rearranged κ genes were performed as described previously (Luning Prak et al., 1994). To detect the specific Vκ editor rearrangements, the forward primers of Vκ12-46, Vκ21-4, Vκ38c, and Vκ20 (bt20 and bw20) (Li et al., 2001) were used in combination with the standard reverse primers (Jκ2, Jκ5). In our experience, the degenerate 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 the hybridomas. The L5 primer detects multiple Vκ genes but not the Vκ/Jκ2 rearrangement of Sp2/0 (Luning Prak and Weigert, 1995). Neither the VκS nor the L5 primer detects the Vκ38c gene. The above PCRs also provide information on Jκ usage and rearrangement configuration. For example, an inverted VκS/Jκ1 rearrangement types positive by VκS/Jκ1 PCR and negative by VκS/Jκ2 PCR. Cμ deletion was detected using VκS as a forward primer and RS-101 as a reverse primer (Retter and Nemazee, 1998). The RS-101 primer is homologous to the recombination signal sequence located downstream of Cμ. The other type of Cμ 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 rearranged Vκ genes were confirmed by sequencing the VκS or L5/Jκ1, Jκ2, or Jκ5 PCR products. Amplicons were band purified from a 1.5% agarose gel (Ultra Pure Agarose, GIBCO BRL, Rockville, ML) using QIAquick gel extraction (Qiagen Inc., Valencia, CA). Sequencing was carried out as described previously (Li et al., 2001).

ELISA, dsDNA, and PS Binding Assays

Ig heavy chain isotypes and κ or λ chain usage were determined by ELISA as described previously (Li et al., 2001). Double-stranded DNA (ds-DNA) binding was determined by liquid phase ELISA using biotinylated ds-DNA as described previously (Li et al., 2001; Radic et al., 1993a). PS binding assays were carried out according to Cocca et al. (2001). Immulon 2 microtiter plates (#3455, Dynex Technologies, Inc., Chantilly, VA) were coated with 50 μl of DOPS (#P-1060, Sigma, St. Louis, MO) at 10 μg/ml in 100% ethanol and dried under a tissue culture hood for 30 min. Plates were blocked with 50 μl of PBS containing 0.5% gelatin and 10% FCS at 37°C for 1.5 hr. Supernatants (50 μl) were incubated at 37°C for 1.5 hr. Unbound molecules were removed by washing with PBS. Bound anti-PS Abs were detected with alkaline phosphatase-conjugated goat anti-mouse κ (Southern Biotechnology Associates, Inc., Birmingham, AL) and para-nitrophenyl phosphate (#104-105, Sigma, St. Louis, MO). Absorbance was measured at 405 nm. The OD_{405} of PS binding was normalized based on the Ig concentration of each supernatant.

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