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Persistence of functionally compromised anti-double-stranded DNA B cells in the periphery of non-autoimmune mice

Jessica H. Roark, Anh Bui, Kim-Anh T. Nguyen, Laura Mandik and **Jan Erikson**

The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

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Abstract

Both anti-single-stranded (ss) and anti-double-stranded (ds) DNA antibodies are associated with the autoimmune disease systemic lupus erythematosus (SLE), but only anti-dsDNA antibodies are considered one of the diagnostic criteria. Using Ig transgenes coding for anti-DNA we have determined the fate of anti-dsDNA B cells in a non-autoimmune environment. In a Rag-2 wild-type background, B cells expressing the anti-dsDNA Ig transgenes are present in the spleen but dsDNA specificity is disrupted due to expression of endogenous L chains. In a Rag-2-deficient background where co-expression of endogenous Ig is blocked, splenic B cells expressing only the anti-dsDNA transgene Ig are present, indicating that endogenous Ig expression is not required for bone marrow export. The anti-dsDNA B cells that persist are profoundly crippled in that they are unable to proliferate to lipopolysaccharide or anti-Ig stimulation. Furthermore, these anti-dsDNA Ig transgene B cells show a decreased lifespan relative to non-transgene BALB/c B cells. Persistence of anti-dsDNA B cells in the periphery of non-autoimmune mice raises the possibility that their appearance in the context of SLE is due to their reactivation by T cell help.

Introduction

The autoimmune disease systemic lupus erythematosus (SLE) is characterized by the presence of serum autoantibodies, specifically anti-DNA antibodies (1). Although anti-single-strand (ss) DNA antibodies are present in the sera of >70% of SLE patients (2), they are not specific for SLE (2,3). In contrast, anti-double-strand (ds) DNA antibodies are specific for SLE (2,3) and rising titers of anti-dsDNA antibodies are often predictive of disease flare (3). To understand how autoantibodies are normally regulated, we have generated mice that have transgenes coding for an anti-dsDNA antibody. The use of these transgenes addresses whether anti-DNA B cells are regulated, presumably by antigen, in normal (non-autoimmune) mice and, if so, whether B cell tolerance to dsDNA is achieved by deletion, anergy or other mechanisms.

Manifestations of B cell tolerance using Ig transgenes to self were first demonstrated by comparing the expression of B cells transgenic for anti-H-2K^k or anti-hen egg lysozyme (HEL), within or without the context of self antigen (reviewed in 4,5). Without self antigen, the transgenes are expressed on B cells and in serum. In the presence of self antigen, the transgene-expressing B cells are deleted in the case of the anti-H-2K^k transgene (4) or are functionally restricted (anergic) in the case of the anti-HEL transgene (5). Additional studies that varied the form of the antigen have addressed factors which dictate the opposing outcomes in these model systems (reviewed in 5). In an effort to extend these studies to the regulation of B cells with disease-associated specificities that arise in autoimmunity, we have established a transgene model to study the regulation of anti-DNA B cells (6). For these studies we have relied on a particular H chain, VH3H9, that was used repeatedly with a variety of different L chains in anti-DNA antibodies isolated from MRL-*lpr/lpr* mice (7,8). As a transgene, VH3H9 has been shown to generate both anti-ssDNA and anti-dsDNA Ig with many endogenous L chains; however, the VH3H9 transgene has also revealed a number of L chains that disrupt DNA binding (7–9). In the absence of regulation, we expected to find both anti-ssDNA and anti-dsDNA binding cells in the VH3H9 transgenic mice. Indeed, in VH3H9 transgene MRL-*lpr/lpr* mice anti-ssDNA and anti-dsDNA B cells are readily recoverable as hybridomas, and anti-ssDNA as well as anti-dsDNA Ig is detected in the serum (8).

Correspondence to: J. Erikson

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In contrast to the results obtained with the sera of VH3H9 transgene MRL-*lpr/lpr* mice, only background levels of anti-ssDNA or anti-dsDNA serum Ig are detected when the VH3H9 transgene is expressed in a non-autoimmune (BALB/c) background, suggesting that both anti-ssDNA and anti-dsDNA B cells are regulated (6,8). Further examination of VH3H9 transgene BALB/c mice provided evidence that anti-DNA B cells may be differentially regulated depending on their specificity for ssDNA versus dsDNA. B cells expressing anti-ssDNA are present at high frequencies and anti-ssDNA hybridomas are readily retrieved from VH3H9 transgene BALB/c mice, yet serum anti-DNA levels are no higher than those of non-transgenic mice. We have interpreted these results to mean that anti-ssDNA B cells are inactivated (6). We have gone on to show that transgene anti-ssDNA B cells also fail to secrete their Ig *in vitro* after a variety of stimuli, suggesting that they are blocked in their ability to differentiate into antibody-secreting cells (10). Anti-dsDNA B cells, on the other hand, have not been retrieved from hybridoma panels of VH3H9 transgene BALB/c mice nor is their Ig evident in the serum (6,8,9). However, we were unable to distinguish from these studies whether their absence is due to deletion in the bone marrow or to their exclusion from the fusable population in the periphery. This distinction is not trivial to our goal of understanding what accounts for the expression of anti-dsDNA B cells in SLE: conditions required to spare a B cell from deletion in the bone marrow may be quite different from those that reactivate B cells in the periphery.

Other anti-dsDNA transgene models have reported varying fates of anti-dsDNA B cells. One study reported deletion of anti-dsDNA B cells in the bone marrow at the pre-B to immature B cell stage (11). Other studies have found that anti-dsDNA transgene B cells are present in the periphery but their specificity is not expressed in the serum (12–14). In two of these models the absence of anti-dsDNA B cells has been attributed to endogenous Ig expression disrupting DNA binding (12,13) and has led to the suggestion that autoreactive B cells escape negative selection by receptor editing, a process by which the specificity of the B cell Ig is modified by further Ig gene rearrangement (9,12,15).

Given the clear demonstration that the presence of additional L chains can alter specificity (12,13), we sought to establish a transgene system that would allow us to follow the fate of anti-dsDNA B cells in situations where the expression of endogenous Ig was or was not possible. Our approach was to identify a λ L chain that would pair with VH3H9 to make an anti-dsDNA Ig. The advantage of using λ L chains is that, contrary to κ L chains, reagents exist that cannot only differentiate λ L chains from κ L chains, but that can distinguish different λ L chains as well. Thus the use of anti-λ and anti-κ reagents allows us to assess both if a λ L chain introduced as a transgene is expressed and if endogenous L chains are also co-expressed. We evaluated the contribution of L chains to DNA binding in conjunction with the VH3H9 heavy chain and identified a mutated λ2 as being compatible with both ssDNA and dsDNA binding. The use of a VH3H9/λ H + L Ig transgene system allows us to eliminate the presence of endogenous Ig altogether by introduction of an inactivated Rag-2 gene. By blocking endogenous Ig expression, whether it be due to receptor editing or to the inefficiency of particular transgenes to shut off endogenous rearrangement, we are able to track B cells that exclusively express the transgene anti-dsDNA Ig.

Methods

Transfectant

The H chain loss line of MOPC315 was generously provided by Dr Gillian Wu (University of Toronto, Toronto, Canada). This myeloma produces a mutated λ2 L chain (designated λ2*) (16). The VH3H9/V_λ2* transfectant, MOPC315γT9, was generated by standard procedures as detailed previously (7).

Mice

The generation of VH3H9 transgenic mice using a construct consisting of the MRL-*lpr/lpr* -derived 3H9 V region combined with the BALB/c C_μ region has been described previously (6). V_λ2* transgenic mice were generously provided by Dr Ursula Storb (University of Chicago, Chicago, IL) (17). V_λ2* mice were bred to Rag-2–/– mice (18) and to VH3H9 transgene BALB/c mice. Rag-2–/– mice were generously provided by Dr Fred Alt (Children's Hospital, Boston, MA). Mice in which both alleles of the Rag-2 gene have been inactivated were generated by intercrossing mice heterozygous for the Rag-2 inactivation and carrying the VH3H9 transgene or the V_λ2* transgene. The mice examined in this study represent an intercross mating of backcross matings 1 or 2 onto the BALB/c background. Non-transgene BALB/cJ mice were purchased from Charles River (Wilmington, MA). VH3H9 transgenic mice were also backcrossed to the MRL-*lpr/lpr* strain. The animal used to generate hybridomas was a backcross 9 on MRL-*lpr/lpr*. All mice analyzed were 8–16 weeks of age.

Phenotyping of mice

The VH3H9 transgene was identified by PCR analysis as described previously (6). V_λ2* transgene and Rag-2 targeted mice were identified by Southern blot analysis. DNA was prepared from mouse tails using proteinase K digestion followed by phenol extraction. DNA was then digested, size separated and blotted onto Zeta-probe blotting membrane (BioRad, Richmond, CA). Mice carrying the V_λ2* transgene were identified by probing *Eco*RI-digested genomic tail DNA with the pJ11 insert (19). pJ11 detects the μ enhancer which was used in the V_λ2* construct (17) and appears as an ~3 kb fragment after *Eco*RI digestion. Transgenic mice bearing the targeted Rag-2 gene were identified by probing *Eco*RI–*Xba*I double digested genomic DNA using the E9 insert (plasmid originally provided by Dr Fred Alt). E9 detects the wild-type Rag-2 gene as a 6 kb fragment, and the targeted gene as a doublet of 3.5 and 3.4 kb (18).

B cell hybridoma production

Spleen cells from MRL4, a 5-week-old VH3H9 transgenic MRL-*lpr/lpr*, were fused without further manipulation to the Ig^- myeloma Sp2/0 by standard procedures (20). Cells were plated at limiting dilution and only wells bearing single colonies were expanded for analysis. Spleen cells from V_λ2* transgenic and VH3H9/V_λ2* H + L transgenic 3-month-old mice, backcrossed onto BALB/c for three generations, were first

stimulated with 50 μg lipopolysaccharide (LPS) for 2 days prior to fusion with Sp2/0 cells.

Sequence analysis

Sequence analysis was carried out as described previously (8) using an RT-PCR-based approach. Subgroup and germline status of λ L chain genes was determined by comparison to sequences reported in Kabat and Wu (21).

ELISA assays

Isotype and Ig concentration of both serum and culture supernatants were determined using indirect solid-phase ELISA assays, as described previously (9). Isotype was determined using anti-κ or anti-λ L chain antibodies (Southern Biotechnology Associates, Birmingham, AL) as the primary antibody, then developing with alkaline phosphatase labeled anti-IgM, -IgG1, -IgG2a, -IgG2b, -IgG3 or -IgA antibodies (Southern Biotechnology Associates). Ig concentration was determined by comparing samples to a titrated isotype-matched standards.

Anti-DNA ELISA assays

Binding of antibody-containing supernatants or purified antibodies to ssDNA was detected as for Ig, except that boiled and snap-chilled sonicated salmon sperm DNA (Sigma, St Louis, MO) at 14 μg/ml in PBS was used in place of primary antibodies, as described previously (6). To distinguish ssDNA from dsDNA, biotinylated DNA was prepared and DNA binding was assayed as described (7).

Antinuclear antibody (ANA) detection

The presence of ANA in sera was detected using permeabilized HEP-2 cells as the substrate (Antibodies Incorporated, Davis, CA) according to the manufacturer's instructions. Antibody binding was detected using FITC-labeled goat anti-mouse Ig (Southern Biotechnology Associates).

Flow cytometry

Single-cell suspensions were prepared by disruption of the organ of interest. Red blood cells were lysed when necessary with red cell lysis buffer (Sigma). Total live nucleated cell numbers were determined by counting cells which excluded Trypan blue. Depending upon the number of cells recovered, $1–2.5\times10^5$ cells were seeded into 96-well round-bottom plates. Cells were washed once with FACS Buffer (RPMI without phenol red or biotin, 2% FCS, 0.1% sodium azide) to remove any biotin from the culture medium, then stained on ice. Staining consisted of adding primary antibodies for 15 min on ice, washing cells twice with FACS buffer, adding secondary antibodies for 10 min on ice, washing cells twice with FACS buffer, then resuspending cells in FACS buffer for analysis. Either 10,000 or 15,000 live lymphocytes as determined by forward and side scatter were collected on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Data were analyzed using CellQuest 1.2 software (Becton Dickinson) on a FACStation. Primary antibodies used conjugated to either biotin, FITC or phycoerythrin were the following: anti-B220 (RA3-6B2; ATCC, Rockville, MD), anti-CD5 (53-7.3; PharMingen, San Diego, CA), anti-IgM (DS-1; PharMingen), anti-κ and anti-λ (187.1 and JC5.1; both generous gifts of Dr J. Kearney, University of Alabama, Birmingham), anti-HSA (M1/69; PharMingen), and anti-BrdU (B44; Becton Dickinson, San Jose, CA). For analyses, samples were first gated on live lymphocytes based on forward and side scatter, then on the relevant surface markers before presentation in histogram form to show fluorescence intensity on a logarithmic scale.

BrdU labeling of cells in vivo

Mice were labeled *in vivo* by i.p. injection with 0.2 ml of 3 mg/ml BrdU in PBS every 12 h. At the end of the labeling period mice were euthanized, organs removed and cell preparations generated for FACS analysis as described (22). At least 20,000 events falling within a live lymphocyte gate were collected for BrdU analysis. Injection of BrdU did not affect B cell numbers or surface phenotype so data from BrdU-labeled mice were included in both the cell number and phenotype analyses.

B cell proliferation

Splenic B cells were prepared by depletion of T cells by complement-mediated lysis after incubation with the following cocktail of mAb from hybridoma supernatants: anti-CD4 (GK1.5), anti-CD8 (3.168.8) and anti-Thy-1 (J1j). Splenic B cells were then cultured at 1×10^6 cells/ml in 100 μl total volume in microtiter plates in increasing concentrations of either LPS (Sigma) or goat anti-mouse IgM $F(ab')_2$ fragments (Jackson, Bar Harbour, ME). Cells were pulsed with $[^3H]$thymidine at the time point indicated in the text (usually 48 h) then triplicate wells read 16 h later.

Results

Strategy for generating anti-dsDNA Ig transgenic mice

To extend our survey of the specificity of VH3H9 paired with λ L chains, we generated an additional panel of hybridomas from a VH3H9 transgene MRL-*lpr/lpr* mouse. Table 1 summarizes these results. In addition to the previously observed VH3H9/$V_\lambda1$, other λ-expressing hybridomas were recovered allowing assignment of the VH3H9 transgene/λ gene segment use to specificities (Table 1). Paired with the VH3H9 transgene, neither germline λ2 nor λX results in an anti-DNA Ig. However, when $V_\lambda2$ is rearranged to $J_\lambda1$ ($C_\lambda1$) the resulting Ig is positive in the anti-DNA and ANA assays. Rearrangements of this type are highly unusual (23,24), presumably due to the fact that $V_\lambda2$ is separated from the $J_\lambda1$–$C_\lambda1$ cluster by >190 kb (25). This $V_\lambda2$–$J_\lambda1C_\lambda1$ hybridoma is particularly interesting because of the information it provides with regard to the contribution of individual amino acids to specificity. Fig. 1 compares the amino acid sequences of the different λ L chains and their specificity when paired with VH3H9. Although relatively conservative, the change to tryptophan (W) from phenylalanine (F) at the V–J junctional-encoded amino acid or the more conservative valine (V) to leucine (L) change nearby in FW4 appear to be responsible for the fact that the VH3H9 transgenes paired with $V_\lambda2J_\lambda1$ and $V_\lambda1J_\lambda1$ Ig are anti-DNA while, in contrast, $V_\lambda2J_\lambda2$ hybridomas are non-DNA.

Besides analyzing the endogenous germline λ genes found paired with the VH3H9 transgene in hybridomas from the

Table 1. Gene use and specificity of λ-expressing hybridomas

Hybridoma	Light chains				Heavy chains		Specificity	
	λ1	λ2	λX	κ	VH3H9	Endogenous	Anti-DNA	ANA
MRL4–140	+	–	–	–	+	–	+	+
MRL4–23	–	+	–	–	+	–	–	–
MRL4–159	–	+[a]	–	–	+	–	+	+
MRL4–175	–	+	–	+	+	–	+	–
MRL4–79	–	–	+	+	+	–	–	–
MRL4–174	–	–	+	–	+	–	–	–

[a]This hybridoma expresses a $V_\lambda 2$ recombined to $J_\lambda 1$.

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Lambda                FW1                          CDR1            FW2
V1J1 (MRL4-140)       QAVVTQDSALTTSPGETVTLTC RSSTGAVTTSNYAN WVQEKPDHLFTGLIG
V2J1 (MRL4-159)       ----------------G--I--- -------------- ----------------
V2J2 (MRL4-23)        ----------------G--I--- -------------- ----------------
V2J2* (MOPC315)       ----------------G--I--- -------------- -I--------------

CDR2          FW3                                CDR3      FW4        DNA  ANA
GTNNRAP GVPARFSGSLIGDKAALTITGAQTEDEAIYFC ALWYSNHWV FGGGTKLTVL  +    +
--S---- ---V----------------------D-M--- -----T--- ----------  +    +
--S---- ---V----------------------D-M--- -----T-F- ------V---  -    -
--S---- ---V----------------------D-M--- ---FRN-F- ------V---  +    +
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Fig. 1. Limited amino acid changes lead to alteration in VH3H9 transgene/λ specificity. Sequence and specificity were determined as described (8). Sequence comparison of the different λ L chains, including the germline $V_\lambda 2$ (26) and mutated $V_\lambda 2$ light chain from MOPC315 (16), reveals a limited number of differences that must account for the altered specificity. The MRL4 mouse is a 5-week-old VH3H9 transgene MRL-*lpr/lpr* mouse which has low titers of anti-HN antibody in the serum.

VH3H9 transgene MRL-*lpr/lpr* mouse, the MOPC315 myeloma, which retained a mutated form of the λ2 L chain (denoted as λ2*), was transfected with the VH3H9 H chain resulting in a VH3H9/λ2* Ig that bound both ssDNA and dsDNA (Fig. 2). A comparison of the sequence of germline λ2 (26) and the mutated MOPC315 λ2* (16) reveals a limited number of mutations that could account for the anti-DNA specificity of the VH3H9/$V_\lambda 2$* antibody (Fig. 1). The most striking mutations include changes to arginine (R) and asparagine (N) in CDR3 (Fig. 1) that have been previously implicated in binding to DNA (27). Since both germline λ1 and the mutated λ2* pair with VH3H9 to generate an anti-dsDNA Ig, we chose to focus on λ2* to generate H + L chain transgenes because the mutations provide a means to distinguish the transgene from endogenous λ2.

Anti-DNA Ig are heterogeneous in terms of their reactivity with various forms of DNA and DNA–protein complexes, and this is apparent from the heterogeneous ANA patterns they generate (7). For example, while VH3H9/$V_\kappa 4$, VH3H9/$V_\lambda 1$ and VH3H9/$V_\lambda 2$* Ig are all anti-dsDNA as defined by their binding in a solution-phase dsDNA ELISA (7,8) (Fig. 2), in the ANA assay, VH3H9/$V_\lambda 2$* has a fine speckled ANA pattern while VH3H9/$V_\kappa 4$ and VH3H9/$V_\lambda 1$ have a homogeneous nuclear ANA pattern. Nevertheless all three antibodies stain mitotic figures (7) (Fig. 3). The ANA(+) patterns of the anti-dsDNA antibodies are in contrast to the ANA(–) pattern of the anti-ssDNA antibody generated by the pairing of the VH3H9 H chain with the $V_\kappa 8$ L chain (VH3H9/$V_\kappa 8$) (Fig. 3).

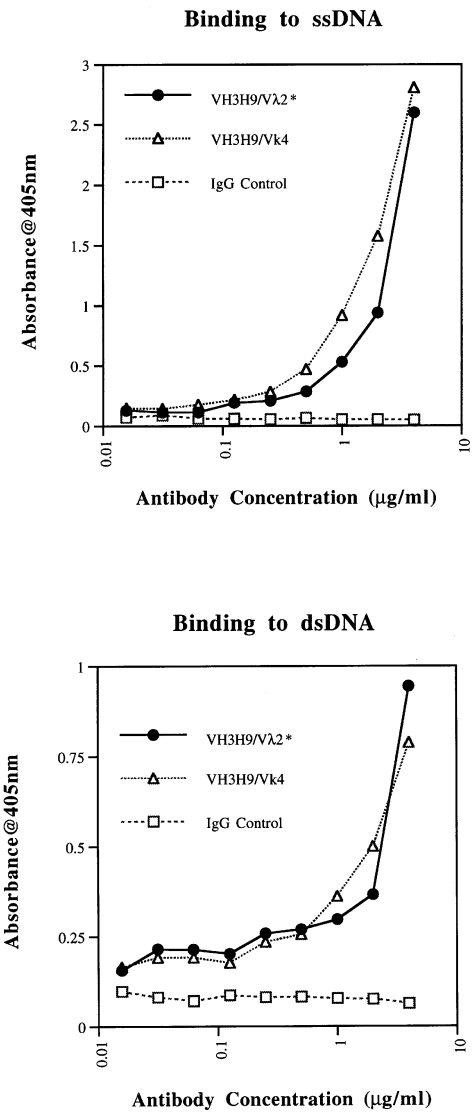

Fig. 2. Binding of VH3H9 H chain-expressing antibodies to ssDNA versus dsDNA is dependent upon the L chain expressed. Binding to ssDNA versus dsDNA in an ELISA assay was determined for MOPCγT9 (VH3H9/$V_\lambda 2$*)- and 3H9 (VH3H9/$V_\kappa 4$)-derived antibodies. The non-DNA binding 1.209 antibody is presented as a control.

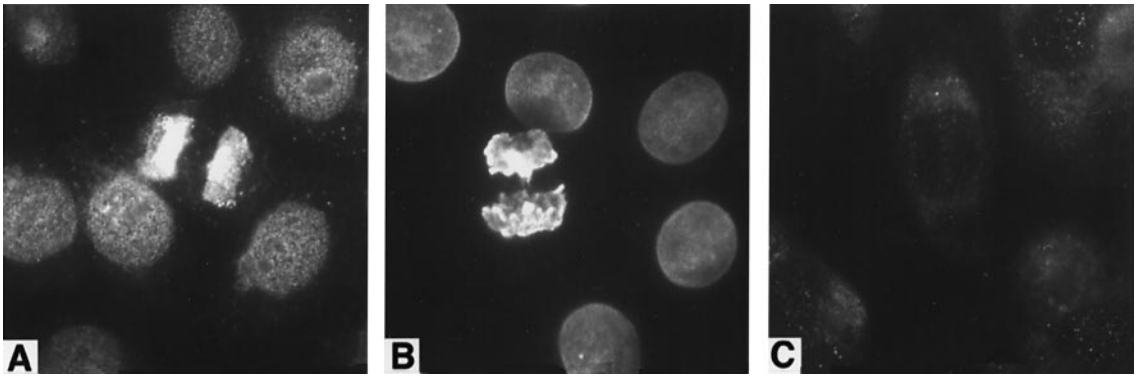

Fig. 3. ANA patterns of three VH3H9 H chain-expressing antibodies. The pairing of the VH3H9 H chain with the $V_\lambda2$* light chain of the MOPC315 cell line results in an anti-dsDNA Ig with an ANA pattern that stains interphase nuclei and mitotic figures (first panel), but that is distinct from the HN pattern produce by the MRL-*lpr/lpr*-derived anti-dsDNA Ig 3H9 (VH3H9/$V_\kappa4$) (second panel). In contrast to the anti-dsDNA antibodies, the anti-ssDNA VH3H9/$V_\kappa8$ antibody is ANA$^-$ (third panel). Antibody binding was detected using FITC-labeled goat anti-mouse Ig.

Table 2. Most hybridomas derived from $V_\lambda2$* and VH3H9/$V_\lambda2$* transgenic mice co-express κ and λ proteins

Isotype	$V_\lambda2$ transgene		VH3H9/$V_\lambda2$ transgene	
κλ	33	(73%)	70	(91%)
λ	12	(27%)	5	(6%)
κ	0		2	(3%)

Splenic hybridomas were generated from VH3H9/$V_\lambda2$* transgene and $V_\lambda2$* transgenic mice. The presence of κ and λ protein was determined by ELISA on hybridoma supernatant. None of the λ only hybridomas exhibited the VH3H9/$V_\lambda2$* transgene specificity, but the mechanism for this has not been determined.

Most VH3H9/$V_\lambda2$ B cells co-express κ L chains*

To determine the fate of VH3H9/$V_\lambda2$* B cells in non-autoimmune mice we have intercrossed mice transgenic for the VH3H9 H chain with mice carrying the MOPC315 $V_\lambda2$* gene as a transgene (here called VH3H9/$V_\lambda2$* transgenic mice) (17). These mice have the potential to fix the B cell repertoire to anti-dsDNA, and provide a source of B cells that can be purified and analyzed functionally. Furthermore, the fates of anti-DNA Ig with different properties can be determined by comparing B cells bearing anti-dsDNA Ig which differ in their ANA patterns (VH3H9/$V_\lambda2$* and VH3H9/$V_\kappa4$), as well as anti-ssDNA B cells (VH3H9/$V_\kappa8$). Finally, the availability of the same H chain transgene paired with different L chain transgenes that generate Ig with particular specificities allows us to distinguish the effects of selection from potential transgene artifacts.

The VH3H9 transgene is an efficient excluder of endogenous H chain gene rearrangement in a non-autoimmune background (6,8). The $V_\lambda2$*-only transgenic mice, however, have been previously reported, and confirmed here, to be poor excluders of endogenous L chain expression (17) (Table 2). Flow cytometry reveals that this is also the case in the context of the VH3H9 transgene since most splenic B cells from VH3H9/$V_\lambda2$* transgenic mice express both λ and endogenous κ L chains (Fig. 4). Likewise the majority of B cell hybridomas generated from VH3H9/$V_\lambda2$* transgenic mice also produce both λ and κ proteins by ELISA (Table 2).

Elimination of an autoreactive H + L transgene specificity by the co-expression of an endogenous L chain has been observed in other transgene systems (12,15) and referred to as receptor editing (9,12,15). In our studies, however, the co-expression of κ L chains in the $V_\lambda2$*-only transgenic mice (17) (Table 2) makes it difficult to distinguish the inherent inability of the $V_\lambda2$* transgene to inhibit endogenous L chain rearrangement from an attempt to edit the L chain transgene. Both scenarios could lead to the elimination of anti-dsDNA specificity. Therefore, we chose to block endogenous L chain gene (as well as H chain and TCR) rearrangement by breeding VH3H9/$V_\lambda2$* H + L transgenic mice to Rag-2-deficient (Rag-2–/–) mice in which both alleles of the Rag-2 gene are inactivated through gene targeting (18). The resulting VH3H9/$V_\lambda2$*-Rag-2–/– mice generate a monospecific VH3H9/$V_\lambda2$* anti-dsDNA B cell repertoire. One complication of this approach could be that T cells are required for B cell tolerance. Precedent exists, however, that T cells are not required for the deletion of B cells or their emigration to the periphery: in Rag-1-deficient anti-H-2^k Ig transgenic mice that do not express H-2^k the anti-H-2^k B cells are present in the bone marrow and periphery, while in mice expressing H-2^k the anti-H-2^k B cells are deleted (28).

Anti-dsDNA VH3H9/$V_\lambda2$ B cells are present in the bone marrow and spleen of VH3H9/$V_\lambda2$*-Rag-2–/– mice*

We analyzed the bone marrow, spleen, lymph nodes and peritoneum of VH3H9/$V_\lambda2$*-Rag-2–/– mice to determine if κ co-expression is required for the exit of VH3H9/$V_\lambda2$*-expressing B cells to the periphery. Cells positive for the B cell marker B220 are found not only in the bone marrow (Fig. 5A) but also in the spleen (Fig. 5B), lymph nodes (data not shown) and peritoneum (data not shown) of VH3H9/$V_\lambda2$*-Rag-2–/– mice. The absence of κ L chain-expressing B cells (data not shown) and of T cells (CD5$^+$, B220$^-$ cells, Fig. 5) in the VH3H9/$V_\lambda2$*-Rag-2–/– mice is consistent with their Rag-2-deficient phenotype. In addition, the B cells that are present appear to be conventional B-2 cells in that they are CD5$^-$ (Fig. 5). These results clearly establish that in non-autoimmune prone mice there are anti-dsDNA (VH3H9/$V_\lambda2$*) B cells that are not regulated by deletion in the bone marrow. Furthermore,

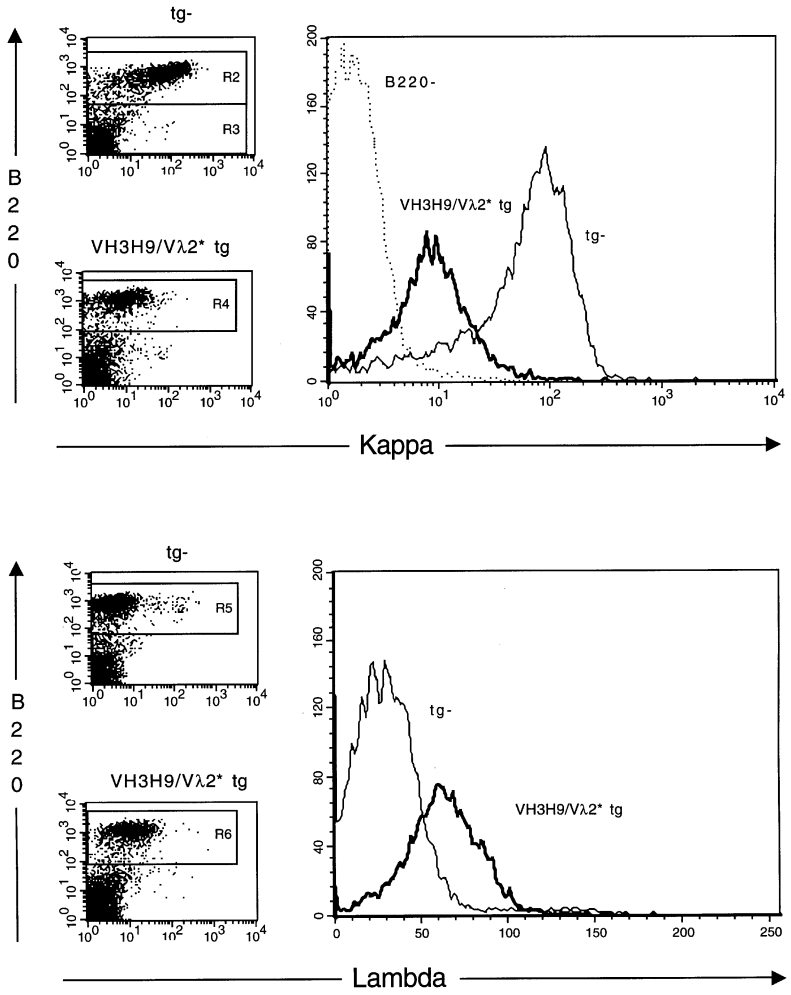

Fig. 4. B cells from VH3H9/V_λ2* mice express both λ and κ. Splenic B cells were stained with the pan-B cell marker B220 and with an anti-κ (top panel) or anti-λ (bottom panel) reagent. The λ density on $B220^+$ cells from VH3H9/V_λ2* mice is reduced ~10-fold compared to the few λ-expressing cells found in BALB/c mice. Likewise the κ density on $B220^+$ cells from VH3H9/V_λ2* mice is also reduced compared to BALB/c $B220^+$ cells. Nonetheless, most $B220^+$ cells from VH3H9/V_λ2* mice do express some amount of κ as can be seen by comparison to the background staining of $B220^-$ spleen cells.

editing of the L chain transgene as has been observed in VH3H9/V_κ4 transgenic mice (12) is not required for emigration of VH3H9/V_λ2* B cells to the spleen.

B cell numbers are decreased in the spleen of VH3H9/V_λ2-Rag-2–/– mice*

Total B cell numbers ($B220^+$) in the bone marrow of VH3H9/V_λ2*-Rag-2–/– mice are similar to BALB/c mice (Table 3). An increase in IgM^+ cells is observed in transgene versus BALB/c mice, however (Table 3), possibly due to early expression of the transgene during bone marrow development. In contrast to this relatively normal number of B cells in the bone marrow, VH3H9/V_λ2*-Rag-2–/– mice were found to have on average a 5-fold reduction in splenic B cell number (IgM^+ cells) (Table 3). In VH3H9/V_λ2*-Rag-2 wild-type mice, however, splenic B cell numbers are reduced $<$2-fold (Table 3), presumably due to the presence of B cells expressing endogenous κ. The reduced splenic B cell numbers in VH3H9/V_λ2*-Rag-2–/– mice is in contrast to Rag-2-deficient mice bearing the VH3H9 transgene paired with the V_κ8 transgene: VH3H9/V_κ8-Rag-2–/– mice have normal splenic B cell numbers (Table 3). Since the VH3H9/V_κ8-Rag-2–/– mice utilize the same H chain transgene as VH3H9/V_λ2*-Rag-2–/– mice but are anti-ssDNA monospecific due to the presence of the V_κ8 L chain transgene, this argues that the reduced B cell numbers in VH3H9/V_λ2*-Rag-2–/– mice is a consequence of the VH3H9/V_λ2* B cells' specificity for dsDNA and not simply due to transgene expression or the absence of T cells.

Cell surface phenotype of anti-dsDNA B cells in VH3H9/V_λ2-Rag-2–/– mice*

B cells in bone marrow (Fig. 5A) and spleen (Fig. 5B) of VH3H9/V_λ2*-Rag-2–/– mice have a 10-fold reduced level of IgM compared to BALB/c B cells. [It should be noted that mature BALB/c B cells also bear IgD, so while surface IgM density is heterogeneous the total surface Ig is more uniform. VH3H9/V_λ2* Rag-2–/– B cells express only the IgM of the μ-only transgene construct (6).] This is a much lower IgM

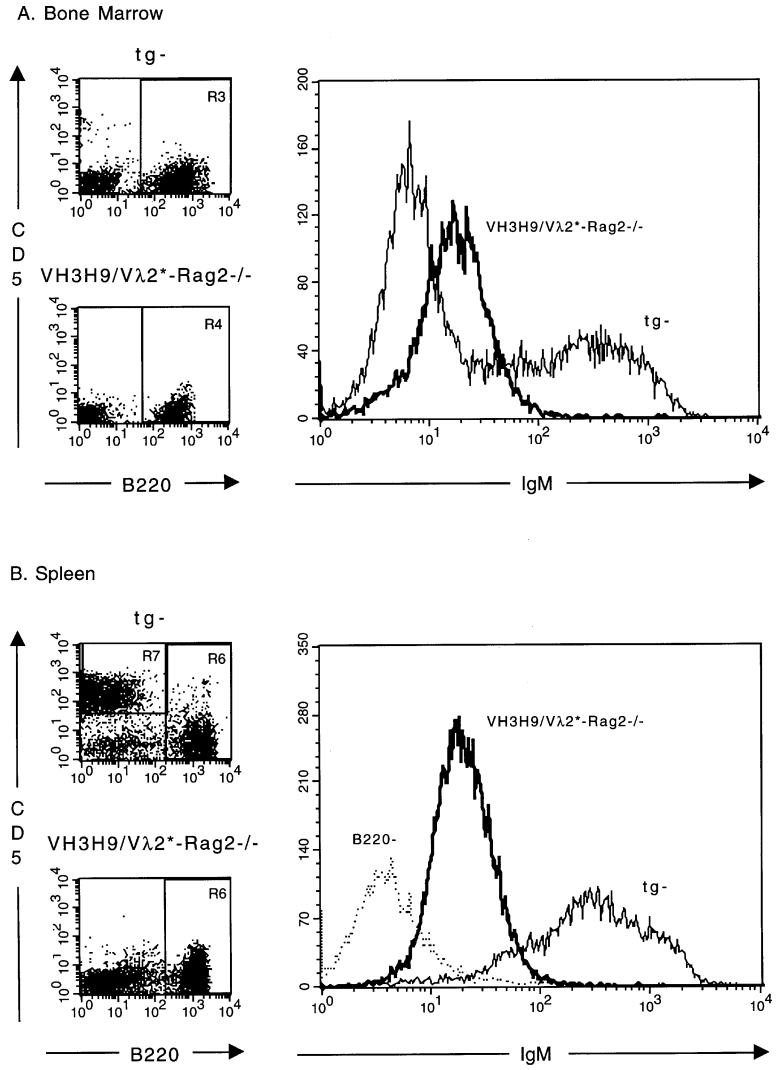

Fig. 5. Anti-dsDNA B cells with reduced levels of surface IgM are present in the bone marrow and spleen of VH3H9/V_λ2*-Rag-2–/– mice. Bone marrow (A) and spleen (B) cells were stained with anti-B220 to detect B cells and anti-CD5 to detect T cells. The level of surface IgM on gated $B220^+$ cells was then determined using anti-IgM. FACS profiles are representative of seven experiments.

Table 3. B cell numbers in VH3H9/V_λ2*-Rag-2–/– mice versus controls[a]

		BALB/c	VH3H9/V_λ2* Rag-2+/–	VH3H9/V_λ2* Rag-2–/–	VH3H9/V_κ8 Rag-2–/–[b]
Spleen					
IgM^+	($\times 10^6$)[c]	33	18	7	53
	(range)	(23–43)	(14–28)	(2–17)	(38–74)
Bone marrow					
$B220^+$	($\times 10^6$)[c]	5.9	3.0	3.3	2.1
	(range)	(1.2–12.2)	(1.8–4.9)	(0.8–5.0)	(1.1–2.9)
IgM^+	($\times 10^6$)[c]	1.9	3.0	3.3	ND
	(range)	(0.2–4.2)	(1.8–4.9)	(0.8–5.0)	

[a]Numbers of mice examined were the following: BALB/c $N = 8$, VH3H9/V_λ2* transgenic mice $N = 5$, VH3H9/V_λ2*-Rag-2–/– mice $N = 7$, VH3H9/V_κ8-Rag-2–/– mice $N = 3$.

[b]Data for VH3H9/V_κ8-Rag-2–/– mice from (10).

[c]Nucleated cell numbers were determined by counting the number of nucleated cells which excluded Trypan blue. The B cell number was then calculated by multiplying the percent of $B220^+$ or IgM^+ cells in a live lymphoid gate by flow cytometry times the total number of nucleated cells. Since all of the transgenic bone marrow B cells express IgM, the values are the same for both $B220^+$ and IgM^+ cell numbers.

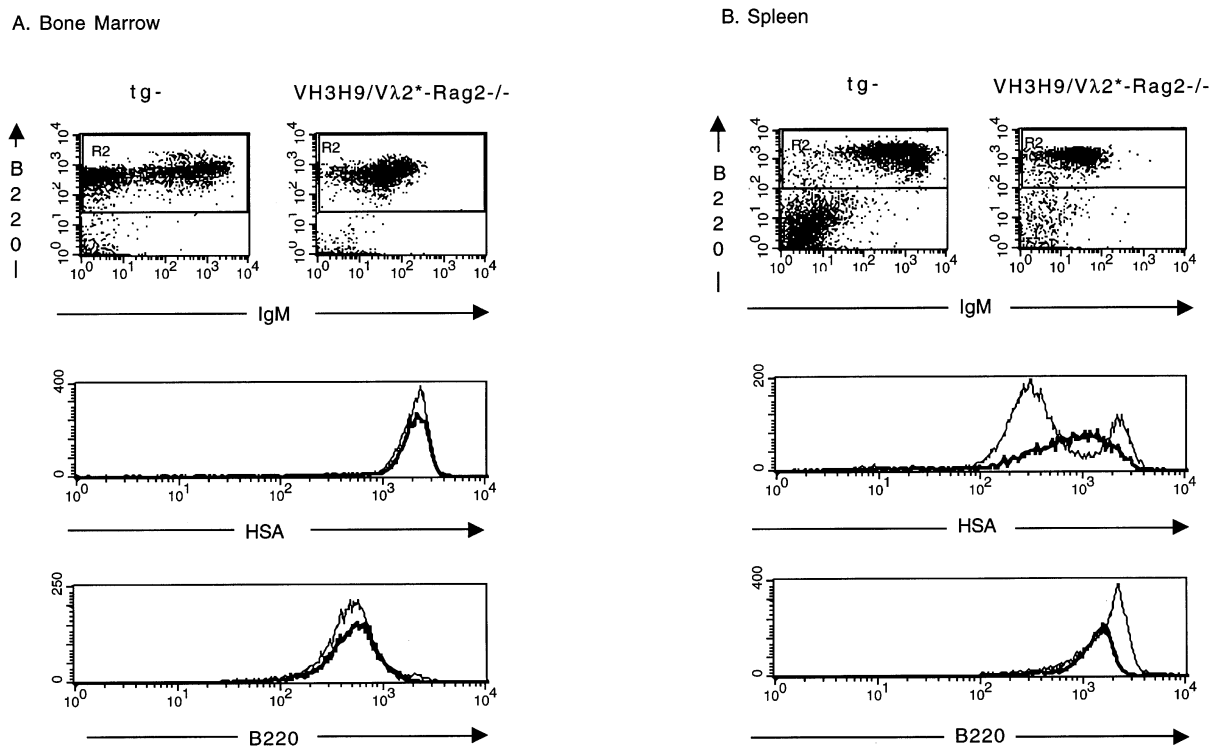

Fig. 6. Surface phenotype of VH3H9/V_λ2*-Rag-2–/– B cells. Expression of the developmental markers B220 and IgM is presented as two-color dot plots then the relative levels of HSA ($N = 5$) and B220 ($N = 6$) compared in histograms. Representative bone marrow (A) and splenic (B) profiles from a BALB/c mouse (light line) and a VH3H9/V_λ2*-Rag-2–/– mouse (dark line) are compared.

expression than anti-ssDNA specific VH3H9/V_κ8 H + L transgene B cells which do not show a decrease in IgM levels regardless of Rag-2 status (10). IgM levels in VH3H9/V_λ2*-Rag-2–/– mice are also reduced in lymph node and peritoneal B cells (data not shown).

The phenotype of B cells present in VH3H9/V_λ2*-Rag-2–/– mice was examined further for the expression of heat stable antigen (HSA) and B220, two markers that have been used both to delineate bone marrow subsets (29 and summarized in 30) and distinguish recent bone marrow emigrants to the spleen. Newly emerging B cells are $B220^{lo}HSA^{hi}$, while more mature splenic B cells are $B220^{hi}HSA^{lo}$ (31). Bone marrow VH3H9/V_λ2*-Rag-2–/– B cell HSA levels (Fig. 6a, HSA histogram) were similar to those found on total BALB/c bone marrow B cells. B220 levels on bone marrow VH3H9/V_λ2*-Rag-2–/– B cells (Fig. 6a, B220 histogram) are slightly elevated compared to B220 levels on total BALB/c bone marrow B cells. The increased bone marrow B220 levels may be due to the expression of the H and L chain transgenes causing B cells to by-pass the $B220^{lo}$ stages of bone marrow development by virtue of the expression of surface IgM (30,32); compare the B220 versus HSA profiles in the bone marrow in Fig. 6(A).

Splenic VH3H9/V_λ2*-Rag-2–/– B cells fail to clearly subset into newly emerging and mature B cells (Fig. 6B). The splenic HSA profile of $B220^+$ cells was found to be unique in VH3H9/V_λ2*-Rag-2–/– B cells: in five of six mice the median density of HSA observed was intermediate to the lower levels of HSA on mature splenic B cells and the higher levels of HSA found on newly emerging splenic B cells (Fig. 6b, HSA histogram). The level of B220 on VH3H9/V_λ2*-Rag-2–/– B cells is less than that present on the majority of BALB/c splenic B cells (Fig. 6b, B220 histogram). The phenotype of splenic VH3H9/V_λ2*-Rag-2–/– B cells is distinct from anergic anti-ssDNA VH3H9/V_κ8 B cells which do progress to the $B220^{hi}HSA^{lo}$ stage of development characteristic of mature splenic B cells (10).

Splenic B cells from VH3H9/V_λ2-Rag-2–/– mice fail to proliferate to LPS*

The presence of autoreactive B cells in the periphery with reduced levels of surface IgM is reminiscent of anergic B cells described in mice transgenic for anti-HEL Ig in the context of soluble hen egg lysozyme (sHEL) as a neo-self antigen (reviewed in 5). Although anergic B cells from the soluble HEL/anti-HEL mice can proliferate in response to LPS (but not anti-IgM F(ab′)$_2$, they are unable to differentiate into antibody-secreting cells (reviewed in 5). Similar to these systems, culturing of splenic VH3H9/V_λ2* B cells from Rag–/– mice with either LPS or anti-IgM F(ab′)$_2$ failed to induce antibody secretion (data not shown). Surprisingly, however, splenic B cells from VH3H9/V_λ2*-Rag-2–/– mice fail to proliferate not only to anti-IgM F(ab′)$_2$ (Fig. 7A) but also to LPS (Fig. 7B), indicating that they are severely crippled functionally. The low levels of serum IgM in VH3H9/V_λ2*-Rag-2–/– mice compared to control BALB/c mice (an average of 4.5 μg/ml in six VH3H9/V_λ2*-Rag-2–/– mice versus 866 μg/ml in BALB/c controls) indicates that antibody secretion is also

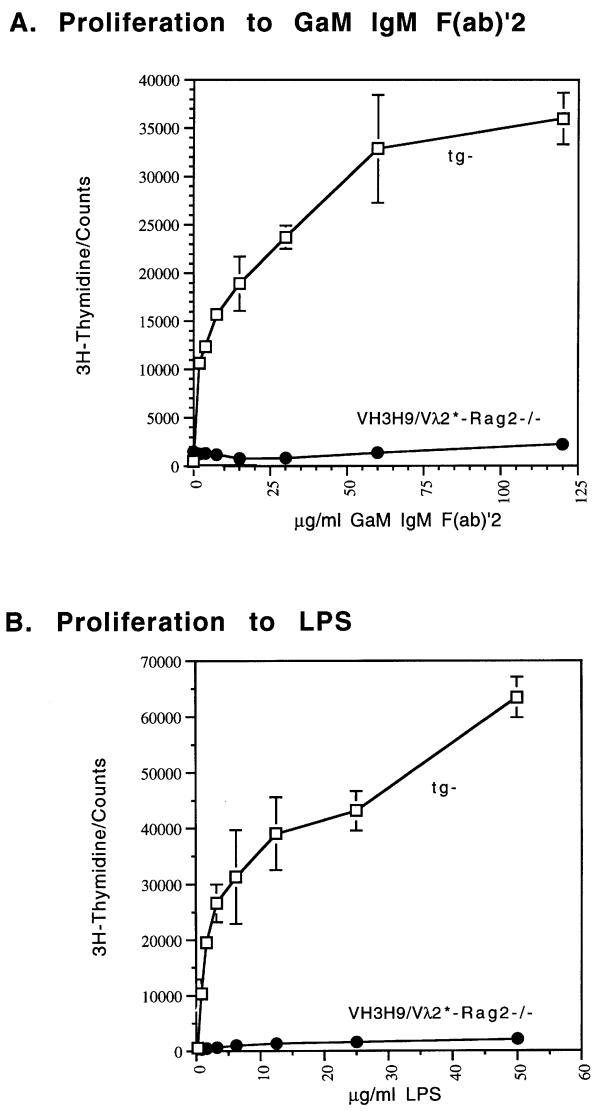

Fig. 7. Splenic VH3H9/V_λ2*-Rag-2–/– B cells do not proliferate in response to polyclonal *in vitro* stimulation. Splenic cells from VH3H9/V_λ2*-Rag-2–/– mice (N = 11, circles) were compared to T cell-depleted BALB/c (squares) spleen cells for their ability to respond to LPS and goat anti-mouse IgM F(ab′)$_2$. Proliferation was determined after 48 h of stimulation with a 16 h pulse of [^{3}H]thymidine. These results were not changed by adding the [^{3}H]thymidine at the zero time-point of culture (data not shown).

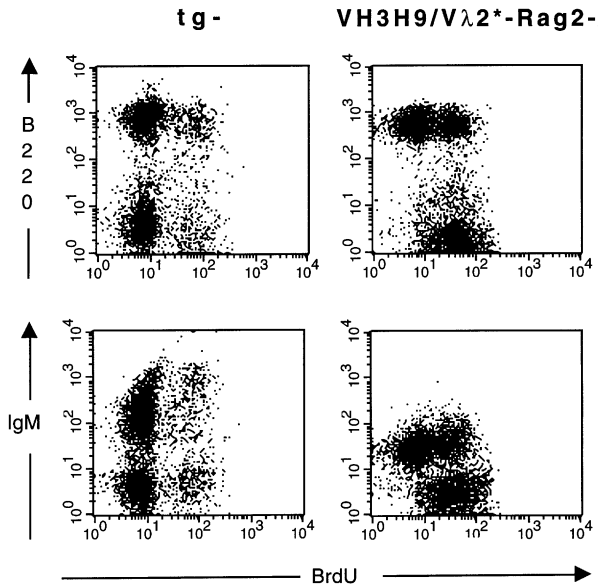

Fig. 8. Splenic VH3H9/V_λ2*-Rag-2–/– B cells rapidly incorporate BrdU. Mice were labeled with BrdU for 5 days then spleen cells examined for incorporation of BrdU by FACS. Double staining for BrdU versus B220 and BrdU versus IgM is presented and the percentage of B220$^+$ cells summarized in Table 4.

Table 4. Frequency of BrdU-labeled cells in VH3H9/V_λ2*-Rag-2–/– and BALB/c mice

	B220$^+$ cells labeled with BrdU (%)		
	5 days	9 days	15 days
Spleen			
BALB/c	4.8, 12.6	16.8, 17.3	21.2, 26.2, 31
VH3H9/V_λ2*-Rag-2–/–	31.3, 56.3	54.4, 76.3	ND
VH3H9/V_κ8-Rag-2–/–[a]	ND	ND	27.1
Bone marrow			
BALB/c	73.0, 94.3	90.9, 93.5	ND
VH3H9/V_λ2*-Rag-2–/–	90.0, 94.6	84.0, 95.6	ND
VH3H9/V_κ8-Rag-2–/–[a]	ND	ND	ND

[a]Data for VH3H9/V_κ8-Rag-2–/– mice from (10).

drastically inhibited *in vivo*. It seems unlikely that the low level of serum Ig in these mice is due to the absence of T cells since in another study Rag-2-deficient mice reconstituted with non-autoreactive H + L chain transgenes have serum Ig levels similar to Rag wild-type controls (33), indicating that non-autoreactive transgene B cells can secrete Ig *in vivo* in the absence of T cells.

B cell turnover rate in VH3H9/V_λ2-Rag-2–/– mice*

To determine if VH3H9/V_λ2*-Rag-2–/– B cells have a reduced lifespan *in vivo* which could account for their reduced B cell numbers, VH3H9/V_λ2*-Rag-2–/– and BALB/c mice were continuously labeled with BrdU for either 5 or 9 days. In the bone marrow similar frequencies of B220$^+$ cells were labeled in VH3H9/V_λ2*-Rag-2–/– and BALB/c mice at both the 5 and 9 day time-points (Table 4). In the spleen, however, VH3H9/V_λ2*-Rag-2–/– mice showed dramatically higher frequencies of BrdU-labeled B220$^+$ cells compared to BALB/c mice (Table 4). This was most striking at the 9 day time-point but was readily detectable at the 5 day time-point as well (Fig. 8). Although a limited number of mice have been examined, it is apparent that VH3H9/V_λ2*-Rag-2–/– B cells turnover much more rapidly in the periphery than do BALB/c B cells. The reduced lifespan of VH3H9/V_λ2*-Rag-2–/– B cells is not simply due to the transgenic nature of the B cells or the lack of T cells in these mice since anti-ssDNA VH3H9/V_κ8 transgene B cells have a normal half-life regardless of Rag-2 expression

[Table 4 and (10)]. Likewise the normal half-life of VH3H9/V_κ8-Rag-2–/– B cells argues that the absence of T cells does not account for the reduced lifespan of VH3H9/V_λ2*-Rag-2–/– B cells. Rather, the reduced lifespan is most likely due to the autospecificity of the B cells.

Discussion

We set out to design and examine Ig transgenic mice coding for anti-dsDNA antibodies to determine if B cell tolerance to DNA manifests itself in similar ways to that described for non-disease-associated antigens. To this end we identified, using hybridomas from VH3H9 transgene MRL-*lpr/lpr* mice and transfection into L chain-only lines, λ L chains that formed anti-dsDNA Ig in association with VH3H9. λ L chain transgenes allow us to easily follow both the transgene and endogenous L chain expression such that we are able to evaluate the contribution of additional L chains (κ) to B cell fate. One of the λs identified that paired with VH3H9 resulting in an anti-dsDNA Ig is a mutated λ2* L chain. Mutations in the λ2* L chain include CDR3 changes from serine and threonine to arginine and asparagine (16), which likely explain the observation that germline λ2 paired with VH3H9 does not bind DNA. Asparagine and arginine are thought to be able to interact with DNA, and have been shown to be present at an unusually high frequency in V_H regions of anti-DNA antibodies (27). Studying mice transgenic for the mutated λ2* (17) in combination with the VH3H9 H chain transgene has allowed us to follow the fate of anti-dsDNA B cells in a non-autoimmune background. In a Rag-2 wild-type background the majority B cells in the periphery expressed the anti-dsDNA transgenes as well as endogenous κ chains and were not reactive to dsDNA. However, when Rag-2 expression was disrupted and endogenous Ig rearrangement therefore blocked, B cells exclusively expressing the transgene anti-dsDNA Ig were now evident in the periphery, but in a much compromised state.

Although VH3H9/V_λ2*-Rag-2–/– B cells are present in the periphery they are profoundly crippled: they fail to proliferate or secrete antibody to either LPS or anti-IgM stimulation and have an increased *in vivo* turnover rate. These data suggest, in agreement with others, that T cells are not required for some manifestations of B cell tolerance (28). It has been proposed in the HEL transgene model that the exclusion of anergic B cells from follicles by non-autoreactive B cells leads to their shortened lifespan (34). In the absence of competition by non-HEL binding B cells, anti-HEL B cells enter the follicles and are longer lived even though they are described as anergic (34). Anti-dsDNA VH3H9/V_λ2*-Rag-2–/– B cells have a reduced lifespan even in the absence of competition; thus, for anti-dsDNA VH3H9/V_λ2*-Rag-2–/– B cells, competition is not a requisite for a shortened lifespan. Furthermore, the phenotype of anti-dsDNA VH3H9/V_λ2*-Rag-2–/– B cells is distinct from that of other anergized B cells that have been examined in detail. Anti-HEL B cells are able to proliferate in response to LPS (reviewed in 5) even though they have a reduced lifespan (35). Anti-ssDNA VH3H9/V_κ8 transgene B cells also can proliferate to LPS and have a normal lifespan relative to non-transgene B cells, in both Rag-2-sufficient and -deficient mice (10).

The presence of VH3H9/V_λ2* B cells in the periphery of VH3H9/V_λ2*-Rag-2–/– mice clearly shows that VH3H9/V_λ2* B cells are not deleted in the bone marrow. Previous studies using the VH3H9 H chain transgene have noted an absence of anti-dsDNA B cells in hybridoma panels generated from LPS-activated spleen cells (6,8,9,36). The fact that the VH3H9/V_λ2* dsDNA B cells are refractory to LPS may account for the absence of this specificity when the B cell repertoire of VH3H9 H chain transgenic mice is sampled via LPS hybridomas. In contrast to data presented here, data obtained from a different anti-dsDNA transgene model suggested that anti-dsDNA B cells are eliminated at the pre-B to immature B stage of development (11). In the later case, receptor editing was blocked by backcrossing the Ig transgenes onto the J_H–/– mice as opposed to our approach of using the Rag-2–/– mice. It is unlikely that the absence of bone marrow deletion in the VH3H9/V_λ2*-Rag-2–/– mice is due to the absence of T cells or other consequences of the Rag-2 deficiency given the previous work from Nemazee's laboratory showing appropriate deletion of autoreactive B cells in Rag-1-deficient mice (28).

A more interesting possibility for the apparent contradiction raised by different anti-dsDNA B cell transgene models is that the Ig coded for by the different transgenes are heterogeneous with respect to their specificity for nucleic acid/protein complexes. Thus the different fates of these various anti-dsDNA antibodies may be due to differences in their specificity for *bona fide* self antigens *in vivo*. One clear way of seeing this heterogeneity is by the diverse ANA patterns of the different anti-dsDNA Ig (7) (Fig. 3). Likewise, anti-DNA B cells isolated from MRL-*lpr/lpr* mice with spontaneous SLE are also heterogeneous in terms of the ANA patterns they produce (7). Since it is far from clear which anti-dsDNA Ig are pathogenic it is important to analyze the regulation of a range of anti-dsDNA B cells.

In conclusion, we have established an Ig transgene model to follow the fate of anti-dsDNA B cells in a non-autoimmune genetic background. We have found that B cells bearing anti-dsDNA Ig transgenes are actively regulated in healthy animals and that the manifestations of this regulation reflect the ANA specificity of the anti-dsDNA Ig. Our results do not fit precisely any of the current views of B cell tolerance that recognize several fates for a tolerized autoreactive B cell including their rapid elimination in the bone marrow (4), a 'delayed death' which involves a shortened lifespan (reviewed in 5) or escape from deletion through receptor editing (9,12,15). Anti-dsDNA B cells from the VH3H9/V_λ2*-Rag-2–/– mice have a unique phenotype in that they are present in the periphery with an accelerated turnover rate, but are refractory to LPS and anti-IgM F(ab′)$_2$ stimuli. It is clear from our studies, therefore, as well as those in the literature, that 'anergy' is not a well-defined state. The functional status of anergized cells and their lifespan vary depending on the model system, or in some cases the approaches taken to study them. B cell tolerance to disease-associated antigens such as dsDNA may not follow the rules emerging for tolerance to neo-self antigens. Moreover, the anti-dsDNA B cells examined here do not fit clearly into any known subset of peripheral B cells: although the VH3H9/V_λ2*-Rag-2–/– B cells are most similar to transitional B cells in terms of their phenotype and turnover

rate (37), transitional B cells are responsive to LPS (31), whereas VH3H9/V_λ2*-Rag-2–/– B cells are not. The demonstration that B cells with a disease-associated specificity, anti-dsDNA, can persist in non-autoimmune animals leaves open the question of whether these B cells can be reactivated *in vivo*.

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Abbreviations

ANA	anti-nuclear antibody
HEL	hen egg lysozyme
HN	homogeneous nuclear
HSA	heat stable antigen
LPS	lipopolysaccharide
RAG	recombination activating gene
SLE	systemic lupus erythematosus

References

1 Tan, E. M. 1989. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv. Immunol.* 44:93.

2 Tan, E. M., Chan, E. K. L., Sullivan, K. F. and Rubin, R. L. 1988. Antinuclear antibodies (ANAs): diagnostically specific immune markers and clues toward the understanding of systemic autoimmunity. *Clin. Immunol. Immunopathol.* 47:121.

3 Condemi, J. J. 1992. The autoimmune diseases. *J. Am. Med. Ass.* 268:2882.

4 Nemazee, D., Russell, D., Arnold, B., Haemmerling, G., Allison, J., Miller, J. F. A. P., Morahan, G. and Buerki, K. 1991. Clonal deletion of autospecific B lymphocytes. *Immunol. Rev.* 122:117.

5 Goodnow, C. C., Cyster, J. G., Hartley, S. B., Bell, S. E., Cooke, M. P., Healy, J. I., Akkaraju, S., Rathmell, J. C., Pogue, S. L. and Shokat, K. P. 1995. Self-tolerance checkpoints in B lymphocyte development. *Adv. Immunol.* 59:279.

6 Erikson, J., Radic, M. Z., Camper, S. A., Hardy, R. R., Carmack, C. and Weigert, M. 1991. Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. *Nature* 349:331.

7 Radic, M. Z., Mascelli, M. A., Erikson, J., Shan, H. and Weigert, M. 1991. Ig H and L chain contributions to autoimmune specificities. *J. Immunol.* 146:176.

8 Roark, J. H., Kuntz, C. L., Nguyen, K.-A., Caton, A. J. and Erikson, J. 1995. Breakdown of B cell tolerance in a mouse model of SLE. *J. Exp. Med.* 181:1157.

9 Radic, M. Z., Erikson, J., Litwin, S. and Weigert, M. 1993. B lymphocytes may escape tolerance by revising their antigen receptors. *J. Exp. Med.* 177:1165.

10 Nguyen, K.-A. T., Mandik, L., Bui, A., Kavaler, J., Norvell, A., Monroe, J. G., Roark, J. H. and Erikson, J. 1997. Characterization of anti-ssDNA B cells in a non-autoimmune background. *J. Immunol.*, in press.

11 Chen, C., Nagy, Z., Radic, M. Z., Hardy, R. R., Huszar, D., Camper, S. A. and Weigert, M. 1995. The site and stage of anti-DNA B cell deletion. *Nature* 373:252.

12 Gay, D., Saunders, T., Camper, S. and Weigert, M. 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J. Exp. Med.* 177:999.

13 Iliev, A., Spatz, L., Ray, S. and Diamond, B. 1994. Lack of allelic exclusion permits autoreactive B cells to escape deletion. *J. Immunol.* 153:3551.

14 Tsao, B. P., Chow, A., Cheroutre, H., Song, Y. W., McGrath, M. E. and Kroneberg, M. 1993. B cells are anergic in transgenic mice that express IgM anti-DNA antibodies. *Eur. J. Immunol.* 23:2332.

15 Tiegs, S. L., Russell, D. M. and Nemazee, D. 1993. Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med.* 177:1009.

16 Wu, G. E., Govindji, N., Hozumi, N. and Murialdo, H. 1982. Nucleotide sequence of a chromosomal rearranged λ2 immunoglobulin gene of mouse. *Nucleic Acids Res.* 10:3831.

17 Hagman, J., Lo, D., Doglio, L. T., John Hackett, J., Rudin, C. M., Haasch, D., Brinster, R. and Storb, U. 1989. Inhibition of immunoglobulin gene rearrangement by the expression of a λ2 transgene. *J. Exp. Med.* 169:1911.

18 Shinkai, Y., Rathbun, G., Lam, K. P., Oltz, E., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. and Alt, F. 1992. Rag-2 deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68:855.

19 Marcu, K. B., Banerji, J., Penncavage, N. A., Lang, R. and Arheim, N. 1980. 5′ flanking region of immunoglobulin heavy chain constant region genes displays length heterogeneity in germlines of inbred mouse strains. *Cell* 22:187.

20 Kohler, G. 1980. Immunoglobulin chain loss in hybridoma lines. *Proc. Natl Acad. Sci. USA* 77:2197.

21 Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. and Foeller, C. 1991. *Sequences of Proteins of Immunological Interest*, 5th edn. US Department of Health and Human Services, Washington, DC.

22 Allman, D. M., Ferguson, S. E., Lentz, V. M. and Cancro, M. P. 1993. Peripheral B cell maturation. II. Heat-stable antigen^{hi} splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. *J. Immunol.* 151:4431.

23 Reilly, E. B., Blomberg, B., Imanishi-Kari, T., Tonegawa, S. and Eisen, H. N. 1984. Restricted association of V and J–C gene segments for mouse λ light chains. *Proc. Natl Acad. Sci. USA* 81:2484.

24 Sanchez, P., Nadel, B. and Cazenave, P.-A. 1991. V_λ–J_λ rearrangements are restricted within a V–J–C recombination unit in the mouse. *Eur. J. Immunol.* 21:907.

25 Storb, U., Haasch, D., Arp, B., Sanchez, P., Cazenave, P.-A. and Miller, J. 1989. Physical linkage of mouse l genes by pulse-field gel electrophoresis suggests that the rearrangement process favors proximal target sequences. *Mol. Cell. Biol.* 9:711.

26 Tonegawa, S., Maxam, A. M., Tizard, R., Bernard, O. and Gilbert, W. 1978. Sequences of a mouse germ-line gene for a variable region of an immunoglobulin light chain. *Proc. Natl Acad. Sci. USA* 75:1485.

27 Radic, M. Z. and Weigert, M. 1994. Genetic and structural evidence for antigen selection of anti-DNA antibodies. *Annu. Rev. Immunol.* 12:487.

28 Spanopoulou, E., Roman, C. A. J., Corcoran, L. M., Schlissel, M. S., Silver, D. P., Nemazee, D., Nussenzweig, M. C., Shinton, S., Hardy, R. R. and Baltimore, D. 1994. Functional immunoglobulin transgenes guide ordered B-cell differentiation in Rag-1-deficient mice. *Genes Dev.* 8:1030.

29 Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D. and Hayakawa, K. 1991. Resolution and characterization of pro-B and pre-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213.

30 Loffert, D., Schaal, S., Ehlich, A., Hardy, R. R., Zou, Y.-R., Muller, W. and Rajewsky, K. 1994. Early B-cell development in the mouse: Insights from mutations introduced by gene targeting. *Immunol. Rev.* 137:135.

31 Allman, D. M., Ferguson, S. E. and Cancro, M. P. 1992. Peripheral B cell maturation. I. Immature peripheral B cells in adults are heat-stable antigen hi and exhibit unique signaling characterisitcs. *J. Immunol.* 149:2533.
32 Chen, J., Trounstine, M., Kurahara, C., Young, F., Kuo, C.-C., Xu, Y., Loring, J. F., Alt, F. W. and Huszar, D. H. 1993. B cell development in mice that lack one or both immunoglobulin κ light chain genes. *EMBO J.* 12:821.
33 Young, F., Ardman, B., Shinkai, Y., Lansford, R., Blackwell, T. K., Mendelsohn, M., Rolink, A., Melchers, F. and Alt, F. W. 1994. Influence of immunoglobulin heavy-and light chain expression on B-cell differentiation. *Genes Dev.* 8:1043.
34 Cyster, J. G. and Goodnow, C. C. 1995. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. *Immunity* 3:691.
35 Fulcher, D. A. and Basten, A. 1994. Reduced life span of anergic self-reactive B cells in a double-transgenic model. *J. Exp. Med.* 179:125.
36 Prak, E. L., Trounstine, M., Huszar, D. and Weigert, M. 1994. Light chain editing in κ-deficient animals: a potential mechanism of B cell tolerance. *J. Exp. Med.* 180:1805.
37 Carsetti, R., Kohler, G. and Lamers, M. C. 1995. Transitional B cells are the target of negative selection in the B cell compartment. *J. Exp. Med.* 181:2129.