B Cell Deletion, Anergy, and Receptor Editing in "Knock In" Mice Targeted with a Germline-Encoded or Somatically Mutated Anti-DNA Heavy Chain

Yael Pewzner-Jung, Dinorah Friedmann, Eiichiro Sonoda, Steffen Jung, Klaus Rajewsky and Dan Eilat

*J Immunol* 1998; 161:4634-4645; ;
http://www.jimmunol.org/content/161/9/4634

**Why The JI?**

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**  This article cites 72 articles, 37 of which you can access for free at:
http://www.jimmunol.org/content/161/9/4634.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
B Cell Deletion, Anergy, and Receptor Editing in “Knock In” Mice Targeted with a Germline-Encoded or Somatically Mutated Anti-DNA Heavy Chain

Yael Pewzner-Jung,* Dinorah Friedmann,* Eiichiro Sonoda,† Steffen Jung,* Klaus Rajewsky,† and Dan Eilat2*

To study the relative contributions of clonal deletion, clonal anergy, and receptor editing to tolerance induction in autoreactive B cells and their dependence on B cell receptor affinity, we have constructed “knock in” mice in which germline encoded or somatically mutated, rearranged anti-DNA heavy (H) chains were targeted to the H chain locus of the mouse. The targeted H chains were expressed on the vast majority of bone marrow (BM) and splenic B cells and were capable of Ig class switching and the acquisition of somatic mutations. A quantitative analysis of B cell populations in the BM as well as of Jk utilization and DNA binding of hybridoma Abs suggested that immature B cell deletion and light (L) chain editing were the major mechanisms affecting tolerance. Unexpectedly, these mechanisms were less effective in targeted mice expressing the somatically mutated, anti-DNA H chain than in mice expressing the germline-encoded H chain, possibly due to the greater abundance of high affinity, anti-DNA immature B cells in the BM. Consequently, autoreactive B cells that showed features of clonal anergy could be recovered in the periphery of these mice. Our results suggest that clonal deletion and receptor editing are interrelated mechanisms that act in concert to eliminate autoreactive B cells from the immune system. Clonal anergy may serve as a back-up mechanism for central tolerance, or it may represent an intermediate step in clonal deletion. The Journal of Immunology, 1998, 161: 4634–4645.

Anti-DNA autoantibodies (1–3) are the hallmark of human systemic lupus erythematosus (SLE)3 and of mouse models (NZB/NZW F1, MRL/lpr) of this prototypic autoimmune disease (4, 5). The extensively studied anti-DNA Abs resemble secondary response Igs in experimental animals mostly comprising somatically mutated IgG with high affinity for DNA (6–8). In contrast, mostly low affinity, germline encoded, IgM “natural” anti-DNA Abs can be found in the sera of healthy individuals (9, 10). This difference has been attributed by most investigators to mechanisms of self tolerance, leading to negative selection of autoreactive B and T cells (11, 12). Several recent studies have suggested, at least for murine SLE, that intrinsic B cell defects may bear the primary responsibility for the loss of self tolerance and for the development of autoimmune disease (13–16). Therefore, the mechanisms of B cell tolerance, particularly those involving clonal deletion of autoreactive cells (17, 18) and their functional inactivation (clonal anergy) (19), have become the focus of intensive investigation.

1 Anti-DNA autoantibodies (1–3) are the hallmark of human systemic lupus erythematosus (SLE)3 and of mouse models (NZB/NZW F1, MRL/lpr) of this prototypic autoimmune disease (4, 5). The extensively studied anti-DNA Abs resemble secondary response Igs in experimental animals mostly comprising somatically mutated IgG with high affinity for DNA (6–8). In contrast, mostly low affinity, germline encoded, IgM “natural” anti-DNA Abs can be found in the sera of healthy individuals (9, 10). This difference has been attributed by most investigators to mechanisms of self tolerance, leading to negative selection of autoreactive B and T cells (11, 12). Several recent studies have suggested, at least for murine SLE, that intrinsic B cell defects may bear the primary responsibility for the loss of self tolerance and for the development of autoimmune disease (13–16). Therefore, the mechanisms of B cell tolerance, particularly those involving clonal deletion of autoreactive cells (17, 18) and their functional inactivation (clonal anergy) (19), have become the focus of intensive investigation.

Received for publication February 6, 1998. Accepted for publication June 29, 1998. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This study was supported by the German-Israeli Foundation for Scientific Research and Development (GIF), the United States-Israel Binational Science Foundation (BSF), the Israel Science Foundation, the European Union with Grants BI02-CT94-2005 and BI04-CT96-0077, and the Deutsche Forschungsgemeinschaft through SFB 243.

2 Address correspondence and reprint requests to Dr. Dan Eilat, Division of Medicine, Hadassah University Hospital, P.O. Box 12000, Jerusalem 01120, Israel. E-mail address: eilat@md2.huji.ac.il

3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; BM, bone marrow; wt, wild type; LH, long homology; SH, short homology; PE, phycoerythrin; HEL, hen egg lysozyme; H, heavy; L, light; ES, embryonic stem.

Copyright © 1998 by The American Association of Immunologists

0022-1767/98/$02.00

Downloaded from http://www.jimmunol.org/ by guest on November 17, 2017
In the study reported here, we have utilized the recently developed technique of targeted insertion of a rearranged variable region gene into the mouse Ig H chain locus (36), to obtain transgenic “knock in” mice, carrying anti-DNA H chains with different affinity for DNA. Autoreactive H chain-only transgenic mice have been used by several groups of investigators (26, 31, 33, 37) and provide a useful tool for studying populations of B cells that are restricted by a common transgenic H chain but possess a wide variety of endogenous L chains from the entire mouse repertoire. The inserted H chains in this study were derived from the anti-DNA hybridoma D42 (38–40) that binds both ssDNA and native dsDNA. This hybridoma represents a group of mouse anti-DNA autoantibodies (about 5% of NZB/NZW anti-DNA; Ref. 7) whose H chains are encoded by the V_{H}11 (SI07) gene segment. Two separate mouse lines were constructed: one carrying the unmutated, germline-encoded V_{H}11 gene and the other carrying the original D42 H chain containing two replacement somatic mutations, a Ser to Asn at position 35 in CDR1 and an Asn to Tyr at position 53 of CDR2. In both cases the V_{H}11 gene is rearranged to a D segment encoding an arginine-rich CDR3 (39, 40) that was found to be essential for DNA binding (40). The difference in DNA affinity between the two H chains, in combination with the D42 L chain was about 12-fold (40). Our results suggest that the major mechanisms of tolerance that prevent B cell autoreactivity in these transgenic mice are clonal deletion and L chain editing; however, the extent of these processes is not correlated with receptor affinity in a simple way. Additionally, anti-DNA B cells that escape deletion and receptor editing do not undergo class switching or somatic mutation and show several features of clonal anergy.

Materials and Methods

Generation of targeted mice

The targeting vector pVHL2neoR(B6SH)(g)D42H was constructed by replacing the short homology (SH) region (0.8-kb I_{P}Cla intron fragment) of pVHL2neoR vector (41) with the corresponding fragment derived from C57BL/6 genomic DNA. The gD42H and D42H cassettes, including promoter and rearranged VDJ genes, were excised from the gD42 or D42 expression vector, respectively (40), and cloned into the SalI/ClaI site of pVHL2neoR(B6SH) to generate the targeting vector. NotI-linearized target constructs (80–100 pg) were transfected by electroporation into 4 × 10^7 B6III ES cells (42). The transfected cells were selected with G418 (300 μg/ml) and Gancyclovir (2 μM). Double-resistant clones were identified by PCR using a V_{H}11 sense primer and an antisense primer located 3′ to the IgH intron enhancer. Putative targeted transfectants, positive for a 1.3-kb PCR fragment, were further analyzed by Southern blotting with a probe spanning the EcoRI-HindII region, 3′ to the IgH intron enhancer. Homologous recombinants were identified by a 6.1-kb band, corresponding to the gD42- or D42-targeted allele, in addition to the 2.3-kb band representing the wt allele. To delete the neo gene, 1 × 10^7 targeted ES cells were transfected by electroporation with 40 μg of the circular Cre-encoding plasmid pGCre-4 (obtained from Dr. H. Gu, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Twinbrook, MD). Targeted clones lacking the neo gene were identified by having a 5.1-kb band in addition to the 2.3-kb wt band. ES cell clones bearing the rearranged D42VDJ gene or the gD42VDJ gene (with or without the neo gene) were injected into blastocytes of CB.20 mice and transplanted into the uteri of C57BL/6 × BALB/c(F) foster mothers. Chimeric mice were mated to C57BL/6 mice.

Flow cytometric analysis

Single cell suspensions from BM, spleen, and peripheral blood were three-color stained with monoclonal or polyclonal Abs and analyzed by FACScan (Becton Dickinson, San Jose, CA) using “Lytes II” program. The following Abs were used: FITC-, PE- or biotin-conjugated MB86 anti-μ (43), FITC-, PE- or biotin-conjugated RS3.1 anti-μ (44), PE-conjugated RA3-6B2 anti-CD45R/B220 (45), FITC-conjugated CJO-1 (anti-Thy1.2) (46), FITC-conjugated S7 anti-CD43 (47), and RF-81, a rabbit anti-D42H Id, primarily directed to V_{H}11 epitopes (R. Fischel and D. Eilat, unpublished observations). Biotin conjugates were revealed by Cy-chrome-, PE- or FITC-conjugated streptavidin (PharMingen, San Diego, CA). RF-81 was revealed by FITC- conjugated F(ab’2) goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL).

Mouse immunizations

Hybridomas were produced by fusion of BALB/c NOE myeloma cells with spleen cells from wt or mutant mice following 3 days incubation with 40 μg/ml LPS (Sigma). To analyze the targeted locus genotype of IgM expression clones, PCR was performed on genomic DNA using a sense D42 leader primer (5′-CCCGCTCTAGAGGCTGCAG(GC)TTCAGTGGCAGTGG(AG) and an antisense D42 primer (5′-GCCATTTTCTGATGTTGCTAGCTGTC-3′) and an antisense J_{H}1 primer (5′-GCCATTTTCTGATGTTGCTAGCTGTC-3′). When the 0.5-kb targeted band was not present in the PCR product, an additional PCR was performed using a sense Dsp2 primer (5′-ACGAATCTAAGGCGAGAATATG-3′) with both containing an antisense D42CDR3 primer (5′-CTAAGACATCTCCCTGGCTAGAAC-3′) (containing an XbaI site) for the detection of secondary rearrangements in the targeted locus. DNA from selected PCR bands (~0.7 kb) was cloned into a pBluescript plasmid and sequenced with a T7 Sequenase 2.0 Kit (Amersham, Arlington Heights, IL).

To detect somatic mutations on the targeted D42 H chain, PCR was performed on hybridoma genomic DNA. PCR primers were sense D42 leader and antisense J_{H}1. The reaction products were run on a 0.7% low melting agarose gel (SeaPlaque GTG, FMC, Vallensbaek Strand, Denmark), and a 0.5-kb band was cut out from the gel to perform “in-gel” direct sequencing (49), using D42CDR3 or D42CDR2 (5′-CTCGTTGAATTCCATAGTGCTG-3′) primers. To analyze Je usage of hybridoma clones, total RNA was prepared from ~3 × 10^7 hybridoma cells using TRI Reagent (Molecular Research Center, Cincinnati, OH). RT-PCR was performed using κ constant-region primers and a degenerate Vκ primer 5′-CCCGCTCTAGAGGCTGCAG(GC)TTCAGTGGCAGTGG(AG) TC(A)/AC-3′. The PCR products were subjected to direct sequencing (49).

LPS activation of splenocytes

Splenocytes (1.5 × 10^6) were cultured in RPMI 1640 medium containing 10% FCS and different concentrations of LPS, in the presence or absence of 25 μg/ml DNAse I (Boehringer Mannheim, Indianapolis, IN). Additional DNsase I (25 μg/ml) was added daily to the cultures. This concentration had been calibrated for efficient degradation of ^3H-labeled DNA in a preliminary experiment. On day 5, cultured cells were collected, and live cells
were counted. Analysis of supernatants for IgM- and anti-DNA IgM- secreted Abs was performed by ELISA. Ficoll gradients were performed to enrich for live cells, and the percentage of IgM- and IgM-expressing cells was determined by FACSscan as described above.

Results

Generation of targeted V_{H11} “knock in” mice

Two lines of Ig H chain transgenic mice were constructed by target- ed replacement of the J_{H} locus in C57BL/6 ES cells with the rearranged V_{H} gene segment of the D42 anti-DNA Ab, derived from a diseased NZB/NZW F_{1} female mouse (38). One mouse strain, designated glD42i (neo), carries the unmutated V_{H11} germ-line sequence, combined with an arginine-rich Dsp2.3 J_{C3} CD3 (39). A variant of this mouse line was generated (glD42i) that lacks the selection marker gene, following Cre-recombinase mediated deletion (50). We have not found significant differences between the glD42i (neo) and glD42i mouse strains in any subsequent studies, and data of the two glD42i variants can be regarded as interchangeable. The second mouse line, designated D42i (neo), carries the original, somatically mutated D42i V_{H} region.

The construction of H chain-only targeted mice was based on two assumptions: the first was that the V_{H}11 H chain would combine with a large variety (but not all; Ref. 40) of L chains to give DNA binding B cell receptors, ranging from very low to high affinity. This was based on previous transfection experiments (40), as well as on the large number of Vk gene families, including Vk1 (6, 7, 51, 52), Vk2 (52, 53), Vk4/5 (52, 54), Vk8 (54), Vk10 (7, 52), Vk12/13 (51), Vk20 (51), and Vk21 (7), whose representatives have been reported in the literature to bind DNA by association with the V_{H}11 (S107) H chain. Moreover, since most of these Vk groups comprise several gene members (55, 56) and different members of the same group (e.g., Vk1, Ref. 52; Vk4/5, Ref. 53; and our unpublished transfection experiments) have been shown to combine with V_{H}11 to give DNA specificity, it is reasonable to predict (based on the complexity of each gene family) that about 80% of the presumed 140 mouse Vk gene segments would combine with the transgenex(s) to generate some affinity for DNA.

The second assumption was that the dominance of the H chain in determining affinity for DNA (40) would result in higher affinity anti-DNA B cell receptors for most H/L combinations containing the mutated V_{H}11 H chain, as compared with those having the unmutated H chain. This is supported by the finding that the Ser to Asn mutation at position 35 of V_{H}CDR1 is the most consistent somatic mutation found in V_{H}11-encoded anti-DNA autoantibodies (7, 39, 53, 54). Apparently, Asn at this position in the H chain is very instrumental in DNA binding and may be found also in J558 V_{H}14 genes, such as 3H9 (2) and B/W DNA-16 (3, 8) that consistently appear in high affinity anti-DNA Abs. This assumption was also supported by preliminary transfection experiments of mutated and unmutated D42 with V_{H}42 (40), Vk4 and Vk8 (Y. Yarkoni, N. Yachimovich and D. Eliat, unpublished results) in which the mutated H chain induced higher anti-DNA affinity in all of the H/L combinations.

The targeting of the rearranged VDJ genes into the DQ52-J_{H} region of the C57BL/6 mouse Ig H chain locus (36) is illustrated in Figure 1A. Recombinant ES clones were injected into C2B2 blastocysts to generate chimeric mice that were bred to C57BL/6 mice and gave rise to heterozygous transgenic mice (Fig. 1B). To distinguish between transgenic and endogenous H chains by allotype specific Abs, the targeted C57BL/6 mice were further crossed with unmanipulated BALB/c mice, and the heterozygous transgenic F1 hybrids were selected for all subsequent studies.

Cells expressing the targeted H chain were analyzed by flow cytometry, using antiiallotypic and antiidiotype reagents (Fig. 2).

A FACS analysis of peripheral blood lymphocytes shows (Fig. 2A) that the majority of B cells in the two independently constructed transgenic mice expressed the D42 Id. This Id surface expression was restricted to B cells carrying the transgenic IgM- allotype (Fig. 2B). Staining of splenocytes with allotype-specific reagents (Fig. 2C) shows, in agreement with previous results (41), that allotypic exclusion at the individual B cell level was maintained in the mutant mice and was similar to that observed in wt F_{1} mice. However, a small but significant population (3–14%) of transgenic B cells expressed the endogenous IgM- allele exclusively. This population, presumably arising as a result of transgene inactivation, was always 1.5- to 2-fold greater in the D42i mice than in glD42i mice.

Serum Abs of V_{H11}-targeted mice

Measurements of serum IgM and IgM anti-DNA in wt and targeted mice are shown in Figure 3. The concentrations of IgM- Igs were reduced to about 20% of normal levels in glD42i and were almost undetectable in D42i mice (Fig. 3A). One explanation for this finding is that the D42 H chain in combination with endogenous L chains does not provide the required B cell Ag receptor (BCR) repertoire for the immune response to environmental Ags. This is unlikely, however, since the V_{H}11 (S107) gene, unlike certain other anti-DNA V_{H}11 genes (8), has been reported to participate in several different natural and experimental immune responses (57–59) (see also Figure 4). A second explanation fulfills our initial prediction, namely, that the great majority of transgenic B cells would have H/L combinations with some affinity for DNA and, therefore, would be subject to regulatory mechanisms of immune tolerance. The serum IgG2a levels of wt and transgenic mice showed a similar pattern to that of IgM (results not shown). Interestingly, the IgM- levels in wt and mutant mice were similar, although only about 10% of transgenic B cells expressed the endogenous IgM- allotype. This suggests some type of homeostatic control of serum Abs, as suggested by Cascalho et al. (60).

Serum anti-DNA activity of IgM- Abs as measured by ELISA was almost undetectable in the targeted glD42i and D42i mice as compared with low affinity anti-DNA IgM Abs secreted by wt B cells and by cells expressing the endogenous IgM- allele in the mutant mice (Fig. 3B). The latter anti-DNA activity is attributed to spontaneously secreted natural Abs, present in normal mouse sera (9, 10), since the same sera did not bind radioactive DNA in the more discriminatory nitrocellulose filter solution assay (8, 40, 61) that measures affinities of 10^{6} M^{-1} bp or greater (Fig. 3C). It follows that both high and low affinity anti-DNA B cells representing the whole range of H/L receptor combinations in the two targeted mouse lines were secreting neither high affinity (typical of autoimmune mice) nor low affinity (typical of normal mice) anti-DNA Abs.

Active immunization of targeted mice

To test whether or not the transgenic B cells can be activated by exogenously administered Ags, wt and transgenic mice were immunized with influenza virus or with an immunogenic complex of Fus1 peptide/DNA that are known to elicit strong humoral immune responses to influenza hemagglutinin and DNA, respectively, with the concomitant production of V_{H}11-encoded Abs (Refs. 8 and 57; and T. Marion, unpublished observations). As shown in Figure 4A, immunization of mutant mice with live influenza virus resulted in an IgG antiinfluenza immune response of both a and b allotype, indicating that the targeted B cells could be activated by an exogenous Ag. The titer of allotype b IgG2a was much lower than that of allotype a since the arginine-rich CDR3 may not be optimal for binding the influenza hemagglutinin. This may also apply to the somatic mutations in the anti-DNA H chain, which would make the immune response of D42i mice weaker than that of glD42i.
mice (Fig. 4A). Indeed, the sequence of the targeted V_H11 gene from an antiinfluenza IgG2a hybridoma derived from an immunized D42i mouse (Fig. 4B) shows, in addition to multiple somatic mutations, a reversal of the mutation in CDR2 from Tyr to the original germline Asn. This experiment further demonstrates that the targeted H chain was capable of physiologic class-switching and of the acquisition of somatic mutations. Immunization of targeted mice with Fus1/DNA complex in CFA has led to a minimal IgMb immune response in glD42i (data not shown) and no response in D42i mice (Fig. 4C). In contrast, B cells expressing the endogenous a allele showed a vigorous response to peptide/DNA immunization. This difference is striking, since a large number of targeted B cells was expected to be uniquely suitable for binding DNA due to the promoting features (CDR3, somatic mutations) of the H chain. These results suggest that the potentially reactive B cells are either absent from the mouse peripheral immune system, or that their activation by immunogenic DNA is inhibited by regulatory mechanisms of self tolerance.

Analysis of BM and spleen cells from targeted mice

The absence of anti-DNA Abs in the serum of glD42i- and D42i-targeted mice could result from deletion and/or functional inactivation of autoreactive B cells. To address these possibilities, we have measured the size of the different cellular compartments in the BM of targeted mice, reflecting the developmentally ordered B cell subpopulations on the basis of cell surface markers (62). The results are summarized in Table I. They show twofold reduction in the absolute numbers of total BM cells and total lymphocytes in all mutant mice compared with their wt, nontransgenic littermates. This difference is also seen in the pro-B and pre-B cell
populations; however, these compartments have similar relative sizes in wt and mutant mice (5% and 50% of total lymphocytes for pro B and pre B cells, respectively). This may suggest that the decrease in total cell numbers results from some differences in developmental patterns at early stages of B cell maturation, due to the introduction of a rearranged H chain to the mouse germline (41). In contrast, a twofold reduction was observed in IgM+ immature B cells from all of the mutant mouse lines (8.8–9.8% of total lymphocytes, numbers underlined in Table I), compared with wt mice (17.5% of total lymphocytes). A similar deletion of immature B cells was also noted when glD42i B cells were compared with B cells expressing nonautoimmune anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) Abs in identically prepared H chain-targeted mice (41). Moreover, a recently constructed double transgenic V\text{H}11/Vk4 anti-DNA mice had further reduced the relative number of their immature B cells to one third of that compartment in Vk4-only targeted mice (N. Yachimovich and D. Eilat, unpublished observations). These results, also supported by preliminary Annexin staining data that showed increased apoptosis of glD42i and D42i immature B cells compared with wt immature B cells, suggest that immature B cells in V\text{H}11-targeted mice are subject to negative selection by clonal deletion, probably due to their anti-DNA autoreactivity, at the pre-B to immature B stage or just after expression of the surface Ig. Similar results were previously obtained by Weigert and coworkers, who studied a different anti-DNA transgenic H chain, 3H9 (26). However, in their study, B cell deletion in the BM was observed only after the affinity of the anti-DNA H chain was increased by directed mutagenesis. In contrast, we observe a substantial deletion of B cells, targeted with the germline-encoded V\text{H}11 and only a minor increase in the extent of deletion of presumably higher affinity B cells, expressing the somatically mutated anti-DNA H chain (Table I).

With respect to mature BM B cells (Table I) and splenic B lymphocytes (not shown), the number of targeted cells was still reduced, compared with wt cells (this study) and with targeted cells expressing nonautoimmune H chain specificities such as (4-hydroxy-3-nitrophenyl)acetyl (NP) (41), but a substantial recovery of the B cell compartment was observed (10–12.7% mature B cells in mutant mice, compared with 15.8% in wt mice; Table I). The fraction of B cells expressing endogenous IgM+ H chains was fairly constant in immature and mature BM B cells (Table I), as well as in splenic B cells (not shown), and accounted for 3 to 7% in glD42i mice and for 6 to 14% in D42i mice. These proportions did not change with increasing age of the mutant mice (data not shown).

**Production of B cell hybridomas from V\text{H}11-targeted mice**

Fusions of splenocytes from the two targeted mouse lines were conducted with NSO myeloma cells, following in vitro activation with LPS (Table II), to analyze the transgenic B cells at the single cell level. Hybridomas were first examined for the expressed H chain and then selected for the presence of anti-DNA Abs. The B cell hybridomas were further screened for the presence of anti-DNA Abs, and the hybridomas producing Abs with the highest affinity were selected for further analysis.

**FIGURE 2.** Representative flow cytometric analysis of B cells from (C57BL/6 × BALB/c)F\text{1} wt and transgenic mice. A and B, Peripheral blood B cells were stained with rabbit anti-mouse D42 Id Ab, then with FITC-F(ab\text{\prime})\text{2} goat anti-rabbit IgG for detection of Id-positive cells. PE-RA3–6B2 and PE-MB86 Ab conjugates were used to detect B220- and IgM+ positive cells, respectively. C, Splenocytes were stained with FITC-RS3.1 for detection of IgM+ and with PE-SAv/biotin-conjugated-MB86 for detection of IgMa Abs. Percentages are calculated from total lymphocytes as defined by forward/sideward scatter.
Features of B cell anergy in D42i mice

The finding that a significant fraction (~30%) of D42i IgM<sup>a</sup> hybridomas secreted anti-DNA Abs contrasted with our inability to measure anti-DNA activity in the sera of these mice (Fig. 3). This could result from a functional silencing (anergy) of the relevant B cells through a continuous B cell antigen receptor (BCR) engagement with DNA; alternatively, the cells could be unresponsive to DNA in vivo due to their low affinity for the autoantigen. The first clue to B cell anergy came from the significant increase in the ratio of IgM<sup>a</sup>- to IgM<sup>b</sup>-expressing hybridomas of D42i as compared with glD42i mice, following LPS activation (Table II). This suggested that the activation of IgM<sup>a</sup>-expressing cells may be restricted due to their DNA reactivity. To dissociate the effects of LPS stimulation and fusion with myeloma cells, in vitro B cell cultures were set up with increasing concentrations of LPS in the presence or absence of DNase I. It had been shown previously (11, 21) that a functional recovery from the anergic state could be achieved by LPS in vitro; however, differentiation into Ab-secreting plasma cells was inhibited by the continued binding of autoantigen to surface Ig receptors.

The results on day 5 of LPS stimulation of wt and mutant splenocytes are shown in Figure 5. The stimulation of transgenic glD42i B cells by LPS was essentially unaffected by the presence of DNase I, which had been calibrated to remove all traces of DNA from the cell cultures. In contrast, LPS stimulation was low in D42i cells and could reach the levels of glD42i stimulation only if DNA was removed from the cell cultures. Since the stimulation of wt B cells was not dependent on the presence of DNase (Fig. 5A), the ratio of IgM<sup>a</sup>- to IgM<sup>b</sup>-expressing cells was increased in D42i-stimulated cells, in agreement with the hybridoma data (Table II). The analysis of cell supernatants for DNA binding on day 5 of LPS stimulation (Fig. 5B) shows that not only proliferation of anti-DNA IgM<sup>a</sup> cells but also their LPS-induced differentiation into Ab-secreting cells was affected by DNase I, since elevated levels of IgM<sup>a</sup> anti-DNA Abs were observed upon removal of DNA by the enzyme. The finding that the state of anergy is dependent on the continuous engagement of B cells by DNA in vitro suggests that anergic cells may also be controlled by permanent Ag encounter in vivo.
It has been previously shown by Goodnow and his coworkers in the transgenic anti-HEL experimental system (21), that B cell anergy was accompanied by down-modulation of surface IgM receptors. This was not initially found in the A6.1 (27) and 3H9 (23) anti-DNA transgenic mouse models; however, a recent analysis of anergic VH 3H9/VK 8 transgenic B cells has shown a two- to five-fold decrease in total surface Ig density (64). We have tested the splenic IgMα-expressing B cell population from wt and mutant mice for the intensity of surface IgM (and IgD) expression by flow cytometric analysis. As shown in Figure 5C, the major population of IgMα-positive cells in the two transgenic lines had surface IgM densities that were significantly lower than those observed for wt IgMα-expressing cells (mean fluorescence of 190). However, while gld42i mice still contained a minor population of cells expressing wt IgM densities (mean fluorescence of 465), these cells were essentially absent in D42i mice. Instead, a prominent population of low density IgM-expressing B cells was evident in D42i mice (mean fluorescence of 57). This population may represent the anergic B cells with anti-DNA autoreactivity. The fluorescence intensity of IgD did not differ between gld42i and D42i B cells (not shown).

Secondary rearrangements and receptor editing in VH11-targeted mice

The presence in the targeted mice of B cells expressing the endogenous IgMα allele (Fig. 2C and Table I) and the G418 sensitivity of most IgMα hybridoma suggested that the transgene had
Table I. Characterization of IgM-secretory hybridoma cells derived from (C57BL/6 × BALB/c)F1 transgenic mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>No. of IgM- Secreting Clones</th>
<th>No. of IgMa Clones</th>
<th>No. of IgMb Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>glD42(neo)</td>
<td>46</td>
<td>1 (2%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>45 (98%)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anti-DNA specificity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>3 (7%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>22 (50%)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>G418 sensitivity</td>
<td>85</td>
<td>4 (5%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>81 (95%)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anti-DNA specificity</td>
<td>1</td>
<td>1 (1.2%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>D42i&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87</td>
<td>18 (21%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>69 (79%)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Percentage of total IgM hybridoma clones.</td>
<td>3 (16%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>25 (36%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>G418 sensitivity</td>
<td>16 (89%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3 (2%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Anti-DNA specificity</td>
<td>145</td>
<td>49 (34%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>96 (66%)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>G418 sensitivity</td>
<td>2 (4%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>22 (23%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>47 (96%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2 (2%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Fusions of splenocytes were performed on day 3 after LPS activation.
<sup>b</sup> Specificity for DNA was determined by ELISA. OD values 10 times greater than background or higher were considered positive. The nitrocellulose filter DNA binding of all ELISA-positive hybridomas was not significantly different from that of ELISA-negative hybridomas.
<sup>c</sup> Percentage of total IgM hybridoma clones.
<sup>d</sup> Percentage of IgMa clones.
<sup>e</sup> Percentage of IgMb clones.

been inactivated by secondary rearrangement(s) in these cells. Indeed, Southern blot analysis of IgM<sup>b</sup> hybridoma DNA showed that the 6.1-kb band representing the intact targeted allele (Fig. 1B) was replaced by a different band in each individual clone (data not shown). Additionally, PCR analysis of IgM<sup>b</sup> hybridoma DNA using D42 leader and J<sub>411</sub> primers confirmed that all IgM<sup>b</sup>-expressing cells had lost their D42 leader sequence (data not shown). To further the nature of these secondary rearrangements, DNA from IgM<sup>b</sup>-expressing clones was amplified with Dsp2-D42CDDR3 and universal V<sub>4-11</sub>CD42CDDR3 primers, and the resulting PCR products were subjected to DNA sequencing (Fig. 6). The sequences revealed that, similarly to previous analysis (63), two hypermutative sequences were identified in all joints. Secondary rearrangements that led to a functional replacement of the targeted gene by an upstream V<sub>4-11</sub> gene (33) were apparently very rare in the D42i mice, since only one IgM<sup>b</sup> hybridoma was identified that fulfilled the two criteria of G418 sensitivity and the absence of the D42 leader sequence. The relevance of H chain secondary rearrangements to receptor editing is discussed below.

The molecular organization of the κ L chain locus allows for secondary rearrangements of upstream Vk gene segments to downstream Jκ elements, which result in deletion or inactivation (by inversion) of primary VKJκ combinations. Secondary rearrangements of endogenous L chains in V<sub>κ</sub><sub>1</sub>-targeted B cells were indirectly inferred by a shift in Jκ usage from the V-proximal Jκ1 plus Jκ2 to the distal Jκ5, as previously suggested by Weigert and his associates (31, 65–67). Table III summarizes the frequency of Jκ gene expression in the different categories of mutant hybridomas (described in Table II), as compared with hybridomas derived from wt mouse littermates. The distribution of Jκ usage in wt mouse hybridomas resembled the reported frequencies in the literature (34, 35, 66), in that about 80% of Vk gene segments were rearranged to Jκ1 or Jκ2 and only 17% used Jκ5. In a sharp contrast, 60 to 70% of non-DNA binding, IgM<sup>b</sup>-expressing transgenic B cells (p < 0.002) had their Vk genes rearranged to Jκ5 (Table
III). This striking shift in Jκ utilization is best explained by editing of autoreactive B cell receptors through secondary Vk rearrangements, since hybridomas expressing endogenous IgMκ alleles did not differ in Jκ5 usage from wt hybridomas. Additionally, the pattern of Jκ distribution in wt and targeted mice is suggestive of a stepwise editing mechanism (no rearrangements to Jκ1 remain in transgenic κ-chains), in which successive rounds of L chain editing have taken place. This could result from the dominance of the anti-DNA H chain that would retain DNA binding in many H/L combinations. Interestingly, the group of low affinity IgMκ-expressing anti-DNA hybridomas, described in Table II, had an intermediate distribution of Jκ usage between wt and mutant hybridomas (Table III), suggesting a partial failure of the deletion/editing mechanism in D42i mice.

Discussion

In this study, we have examined the contribution of several mechanisms of immunologic tolerance, i.e., clonal deletion, clonal anergy, and receptor editing to the prevention of self reactivity with a prototypic autoantigen (DNA) in nonautoimmune animals. The immune response to this Ag is likely to be strictly regulated to protect the organism against the development of autoimmunity and the serious consequences of SLE. As shown above, the construction of H chain-targeted mice enabled us to study these mechanisms under nearly physiologic conditions, since the inserted H chain was able to switch Ig class and to accumulate somatic mutations, as was demonstrated for anti-influenza Abs (this report) and for anti-DNA autoantibodies in H chain transgenic mice bred to an autoimmune background (D. Friedmann and D. Eilat, unpublished results).

Clonal deletion of anti-DNA B cells

Negative selection by clonal deletion is an effective means for preventing anti-DNA B cell autoimmunity. This mechanism has been previously demonstrated for B cells reactive with membrane-bound Ags (20, 68) as well as for DNA (26, 65). It is remarkable that, within the range of affinities dictated by the two anti-DNA H chain transgenes, there was little difference in the extent of deletion between glD42i and D42i B cells. These results suggest a low threshold affinity for anti-DNA B cell deletion and differ from those obtained with conventional anti-DNA transgenic mice (26, 65); however, they agree with the recent results of Lang et al. (28), in which immature B cells were shown to be exquisitely sensitive to central tolerance and receptor editing by low affinity, membrane-bound Ag. Indeed, the DNA Ag apparently belongs to the class of densely arranged and repetitive Ags (like those of viruses,
bacteria, and parasites) that may interact directly with B cells to induce deletion (12, 65).

Contrary to our expectation, deletion of DNA-reactive B cells in the BM was essentially complete for the lower affinity glD42i transgenic B cells (Table II) but incomplete for the higher affinity D42i cells. In the latter case, a significant fraction of the autoreactive B cell population has found its way to peripheral lymphoid tissue (spleen) and could be induced to secrete DNA-binding Abs, albeit with low affinity. This binding may be explained in several different ways: i) the transgenic B cells are negatively selected in the BM by an autoantigen that is different from DNA (52), and, in this case, the somatically mutated H chain may decrease rather than increase the affinity for the deleting Ag; ii) the L chain repertoire may give rise to H/L receptor combinations with noncontiguous distribution of DNA binding affinities. Thus, it is conceivable that L chains producing intermediate affinity with D42H and low affinity with glD42 H chain would be missing from the repertoire. In that case, one would expect to find B cells expressing low affinity Abs (and escaping deletion) in D42i but not in glD42i mice, as is indeed observed. iii) More likely, clonal deletion is not regarded either as a back-up mechanism of functional inactivation for B cells that fail to undergo editing and deletion, or as an intermediate step in clonal deletion that fails to be completed (19).

Editing of transgenic B cell receptors

H and L chain editing of autoreactive B cells (29–33) may be accomplished in several ways, including i) the inactivation of the expressed allele and subsequent rearrangement of the second allele, and ii) replacement of rearranged variable gene segments in cis with an upstream VH or Vk genes. We regularly observed that a small fraction of the targeted B cells (Fig. 1 and Tables I and II) had inactivated their transgenic IgMb allele and expressed the endogenous IgMa allele. Sequence analysis of several inactivated IgMb alleles revealed secondary rearrangements of upstream D or VD segments into the leader intron of the targeted VDJ gene (Fig. 6). We suspect that these secondary rearrangements may have occurred spontaneously and may not be due to autoreactivity for the following reasons: i) most of these rearrangements involved upstream D segments that are not present in normal, rearranged H chain alleles, and the nonphysiologic presence of D elements upstream of the VDJ complex may result in varying degrees of locus instability depending on the structure of the inserted VDJ gene segment (41); ii) most rearrangements involved N-sequence additions, typically introduced by TdT enzyme in pro B cells that are negative for surface Ig, suggesting that they may have occurred early in B cell development and irrespective of autoreactivity; and (receptor editing) that give rise to B cell receptors with reduced affinity. When a multitude of high affinity autoreactive B cells is present in the BM, as is the case in transgenic mice or in mice prone to autoimmunity, deletion may become less efficient because of insufficient time (before B cells exit the BM) or due to the scarcity of Ag (as may be the case for memory B cell selection in germinal centers (70)); consequently, low affinity, autoreactive B cells would leak to peripheral organs. In this view, the anergic state in which these cells are found in the spleen (Table II) would be regarded either as a back-up mechanism of functional inactivation for B cells, or as an intermediate step in clonal deletion that fails to be completed (19).

Table III. Jk expression in hybridoma cells derived from wt and mutant (C57BL/6 × BALB/c)F1 mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Expressed H Chain</th>
<th>Anti-DNA Specificity*</th>
<th>No. of Sequenced Hybridoma Clones</th>
<th>Jc1 (%)</th>
<th>Jc2 (%)</th>
<th>Jc4 (%)</th>
<th>Jc5 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>IgM</td>
<td>−</td>
<td>24</td>
<td>29</td>
<td>50</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>glD42i</td>
<td>IgMb</td>
<td>−</td>
<td>21</td>
<td>24</td>
<td>5</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>D42i</td>
<td>IgMb</td>
<td>−</td>
<td>19</td>
<td>32</td>
<td>5</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgMb</td>
<td>+</td>
<td>21</td>
<td>10</td>
<td>43</td>
<td>9</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>IgMa</td>
<td>−</td>
<td>26</td>
<td>27</td>
<td>38</td>
<td>19</td>
<td>15</td>
</tr>
</tbody>
</table>

* Anti-DNA specificity was determined by ELISA.
iii) similar inactivating secondary rearrangements have been demonstrated by targeted H chains with no apparent autoreactivity, such as T15 (36, 63), and many of these rearrangements occurred in the early stages of B cell development, before the expression of surface Ig (63). These arguments notwithstanding, there remains a possibility that transgene inactivation was induced or promoted by contact with the autoantigen.

The second route of receptor editing, via V-gene replacement did not occur with the targeted V_{4\text{HL}}-encoded H chains, since no example of V or VD recombinant with the heptamer embedded at the 3' end of most V_{4Ig} genes (33) was found, although both this heptamer and a putative nonamer with the appropriate spacer length are present in the V_{4Ig}11 gene. In contrast, however, L chain editing seemed to represent a major component of B cell tolerance in V_{4HL}-targeted mice (Table III), as judged by the dramatic shift in Jk utilization. Jk usage appears to be a reliable parameter of L chain editing for the following reasons: i) the frequencies of individual Jk expression were found to be relatively constant (34, 35); and, ii) unlike the biased distribution of Vk gene segments in receptors of tolerant B cells (that largely depends on Ag binding preferences), the choice of Jk is relatively independent of DNA binding (40). Like clonal deletion, the mechanism of L chain editing was found to be at least as efficient in glD42i as in D42i transgenic mice. This finding is again in agreement with the recent data of Lang et al. (28), using anti-class I transgenic mice; it also supports the view that clonal deletion and receptor editing are interrelated processes that occur in the immature B cell population (26, 71) at roughly the same time and have similar requirements with respect to Ag specificity and affinity. Indeed, an unknown fraction of the deleted immature B cells may be accounted for by autoreactive cells that failed the process of receptor editing, due to nonfunctional secondary rearrangements.

The close relationship between B cell deletion and receptor editing would also predict that clonal deletion be severely hampered when receptor editing fails to reduce or eliminate autoreactivity by L chain secondary rearrangements. This would further suggest that, if targeted L chains capable of secondary rearrangements or H chain-only mice were to be employed in the anti-lysozyme experiments of Goodnow et al. (21), or in the anti-DNA experiments of Nguyen et al. (64), then more B cell deletion/editing and less B cell anergy could be observed.

**Clonal anergy in anti-DNA D42i B cells**

A significant fraction of D42i, but not glD42i, splenic IgM<sup>+</sup>-expressing B cells could bind DNA with low affinity, following LPS stimulation and fusion with myeloma cells. These B cells were shown to be anergic by three independent criteria: their surface IgM receptors were down modulated, they proliferated poorly and secreted little Ab upon mitogenic stimulation in the presence of the autoantigen, and they were poorly responsive to immunization with a strongly immunogenic peptide/DNA complex in CFA.

These low affinity B cells could be regulated by a distinct, peripheral tolerance mechanism that would render them anergic; alternatively, as discussed above, they could be the fugitives of an incomplete process of clonal deletion and/or receptor editing. Indeed, the Jk distribution of the anergic cells (Table III) was suggestive of an intermediate stage of clonal deletion/receptor editing.

The existence of intermediate stages in B cell clonal deletion that give rise to reversible maturation arrest and functional inactivation has been previously demonstrated by Hartley et al. (69) in anti-HEL transgenic mice. The arrested B cells had decreased surface IgM and were triggered to die after 1 to 3 days exposure to membrane-bound HEL. However, they could be rescued by removal of Ag and were able to secrete Ab upon reactivation with LPS, as was found to be the case with the anergic anti-DNA population in this study. A shortened life span of transgenic anti-HEL B cells was also found by Fulcher et al. (72), who concluded that anergy is a form of delayed deletion and that the distinction between B cell anergy and deletion may be more relative than absolute.

**Acknowledgments**

We thank Ms. Angela Egert for her assistance in the injection of the targeted ES cells into blastocysts and the generation of chimeric mice, Drs. Arie Ben-Yehuda and Zichria Rones for their assistance in the influenza virus immunizations, Dr. Gustavo Mostoslavsky for the preparation and testing of ssDNA and dsDNA, Dr. Ruth Fischel for providing the anti-D42 Id Abs, Dr. Marko Radic for the Vl4 and Vk8 expression vectors used in transfection experiments, and Dr. Tony Marion for providing the Fus1 peptide.

**References**


