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Secondary Heavy Chain Rearrangement: A Mechanism for Generating Anti–double-stranded DNA B Cells

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Abstract

The chronic graft-versus-host (cGVH) reaction results in a syndrome that closely resembles systemic lupus erythematosus (SLE). It is induced in nonautoimmune mice by the transfer of alloreactive T cells. The availability of anti-DNA transgenes allows us to study the genetic origins of autoantibodies in this model. We induced cGVH in two anti-DNA H chain site-directed transgenic mouse strains. This resulted in clonal expansion and selection of specific mutations in the anti–double-stranded (ds) DNA B cell population. These data, together with a high frequency of anti-dsDNA B cell clones recovered as hybridomas, suggested that anti-dsDNAs are the product of an antigen-driven immune response. Genetic analysis associated this response with the generation of anti-dsDNA B cells through secondary rearrangements that replaced the site-directed transgene (sd-tg) with endogenous VH genes.

Key words: SLE • autoimmunity • anti-dsDNA • B lymphocytes • graft vs. host

Introduction

SLE is an autoimmune disease characterized by lesions in multiple organs and formation of autoantibodies. Among these, anti–double-stranded (ds)* DNA Abs are particularly specific. Their rising titers can correlate with disease activity, and they are implicated in kidney pathology (1). Thus, understanding what causes their production in SLE should provide insight into the basic pathogenic mechanisms of the disease itself. Previous studies on anti-DNA B cells have demonstrated that in nonautoimmune mice these cells are actively regulated (2–8). How tolerance loss occurs in SLE is not yet clear.

The chronic graft-versus-host (cGVH) model of SLE is produced by the transfer of allogeneic T cells into normal mice. Donor helper T cells react against incompatible host class II structures and generate T help. This T help then activates the self-reactive host B cells that produce the autoantibodies characteristic of SLE (9–17). In our experiments cGVH is induced by the transfer of alloreactive spleen cells from "nonautoimmune" bm12 mice into coisogenic, "nonautoimmune" C57BL/6 (B6) recipients (bm12→B6). As donor and recipient cells differ by only three amino acids on their class II molecule, this difference in the MHC can be identified as the factor responsible for the cGVH reaction (9–11).

We induced cGVH in B6 mice carrying two different H chain Ig site-directed transgenes (sd-tg), the 3H9 knock-in (KI; reference 6), and the 3H9/56R.KI (18), which possess an increased frequency of anti-DNA B cells. Previous studies have shown that the 3H9 H chain plays a key role in determining DNA specificity. When paired with most but not all L chains, it gives rise to either anti-single stranded (ss) DNA or anti-dsDNA Abs (19). Thus, these 3H9.KI mice enabled us to focus on the particular anti-dsDNA B cell population transitioning from tolerance to autoimmunity, and to do so in the context of a broad repertoire. The 3H9/56R sd-tg has an additional arginine in the CDR2 of the 3H9 H chain, which heightens its affinity for dsDNA (18) and generates a more restricted repertoire. Here, with few exceptions, the pairing of L chains with the 3H9/56R H chain gives rise to anti-dsDNA Abs (5, 18). Affinity of the B cell receptor for self-Ag could help determine the selective breakdown of tolerance in SLE. Thus, by comparing these site-directed transgenic models, we were able to determine how both the restricted repertoire and the increased dsDNA affinity affects tolerance.

Our data indicate that the mechanisms of tolerance loss appear to be similar in both mouse models, although the resultant phenotype is more pronounced in the cGVH.3H9/56R mice. We found that the allo-T help of

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*Abbreviations used in this paper: B6, C57BL/6; cGVH, chronic graft-versus-host; ds, double-stranded; FWR, framework; KI, knock in; sd-tg, site-directed transgene; ss, single-stranded; tg, transgene.

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cGVH induces two stages of activation: a nonspecific stage that affects most splenic B cells (20), and an Ag-specific stage associated with the production of SLE-Abs. This latter, receptor-driven stage is specifically marked by oligoclonal B cell expansion, a high frequency of mutations and a high degree of selection, both positive and negative, on the anti-dsDNA B cell population. Most importantly, however, genetic analysis associated these responses with the generation of anti-dsDNA B cells through secondary rearrangements.

Materials and Methods

Mice

The generation of 3H9 KI and 3H9/56R.KI (or site-directed transgenic) mice has been described previously (6, 18). The sd-tg has been backcrossed onto the nonautoimmune B6 background for at least seven generations (unless otherwise stated) to engender 3H9.KI.B6 (3H9(+)) or 3H9/56R.KI.B6 (56R(+)). The presence of the 3H9 or 3H9/56R sd-tg was determined by PCR amplification of tail DNA (3).

B6 and coisogenic B6.C-H2^{bm12}/KhEg (bm12) mice were originally obtained from The Jackson Laboratory. Strains B6 and bm12 differ only by three amino acids in the β-chain of the I-A molecule.

All mice were bred and maintained in our mouse colony at the University of Pennsylvania Medical Center.

Experimental cGVH Disease Protocol

Mice were 2 to 5 mo at the time of cGVH initiation. In all cases, age-matched B6 mice or 3H9/56R.KI negative littermates (56R(−)) were used as controls. cGVH disease was induced as described previously (9). Briefly, recipient mice on a B6 background were injected intraperitoneally with single cell suspensions of 10^8 class II incompatible bm12 donor splenocytes, prepared by pressing donor spleens through a wire mesh screen in HBSS.

Characterization of the Autoimmune Disease

Follow-up of Mice. Blood samples were obtained from experimental mice before the induction of cGVH disease and at 2- to 4-wk intervals thereafter. Sera were stored at −20°C for later analysis.

Detection of Autoantibodies in Sera ELISA. Expression of anti-DNA Abs was determined via a solid-phase ELISA in assays similar to those described previously (21, 22). Plates were coated with optimal concentrations of autoantigen, and sera were added at 1:500 dilution. Biotinylated goat anti–mouse IgG (pFc' specific; Jackson ImmunoResearch Laboratories) was added as a secondary Ab. The following autoantigens were used: (a) ssDNA: calf thymus DNA (Sigma-Aldrich) was heated to 97°C for 10 min and cooled on ice quickly. (b) dsDNA:calf thymus DNA was extracted with chloroform, precipitated by addition of ethanol, and then treated with S1 nuclease for 45 min at 37°C, to remove ss regions.

For the ELISA for dsDNA, plates were first coated with poly-L-lysine, followed by an incubation with the purified calf thymus. Before the usual BSA/Tween blocking step, a preblock with poly-L-glutamine was performed.

For reference, a standard serum from a diseased MRL/*lpr* mouse with high titer of autoantibodies was tested at serial two-fold dilutions from 1:250 to 1:128,000.

Allotype-specific Anti-dsDNA. The allotypes of IgG2a anti-dsDNA Abs were tested by assays similar to those for anti-dsDNA, except that the sera were diluted 1/250. The assays were developed with rabbit anti–mouse preabsorbed allotype reagents (anti-IgG2a^a or anti-IgG2a^b; Nordic Immunology) and detected with alkaline phosphate anti–rabbit IgG Ab (Jackson ImmunoResearch Laboratories).

Generation of Hybridomas

To induce cGVH, a 3H9.KI and a 3H9/56R.KI mouse (backcrossed three times onto the B6 background) were injected with bm12 spleens as described above. The presence of anti-DNA Abs in sera was corroborated 10-wk-postinjection, and hybridomas from the cGVH mouse were generated as described (5). In brief, spleen cells from the immunized mice were fused without further manipulation to Sp2/0 myeloma. All hybridomas were plated at limiting dilution, and wells bearing single colonies on 96-well plates were expanded for further analysis.

ELISA Assays on Hybridomas

Ig Secretion in Supernatants. Isotype and Ig concentration in culture supernatants were determined using a solid phase ELISA assays, as described (23). In brief, to determine the isotype, plates were coated with optimal concentrations of unconjugated anti-total Ig, and serial dilutions of supernatants from individual hybrids were added. Binding was detected through further incubation with anti-IgM, or anti-IgG coupled to alkaline phosphatase and developed with alkaline phosphatase substrate. Ig concentration was determined by comparing samples to a standard curve generated by a titrated isotype-matched Ab.

DNA Binding. Binding to dsDNA was measured by a two step fluid-phase ELISA, as described previously (2, 24). The Ab concentration was normalized for each individual sample. Appropriate concentrations of Ab and biotinylated dsDNA were mixed, incubated, and transferred to avidin-coated microtiter plates. Bound DNA-Ab complexes were detected with alkaline phosphatase-conjugated anti–mouse isotype-specific Ab.

Allotype-specific Anti-dsDNA. The allotypes of IgG2a anti-dsDNA monoclonal Abs were tested by assays similar to those for allotype-specific anti-dsDNA in sera, except that in this case hybridoma supernatants were added.

PCR Assays on Hybridoma DNA

Genomic DNA was purified from individual hybrids. 100 ng of DNA were used in each reaction. Primers and conditions for H and L chain PCR assays have been detailed previously (6, 23, 25).

H Chain PCR Assay. The presence of 3H9 or 3H9/56R H chain sd-tg was identified by PCR amplification using primers complementary to the 3H9 H chain leader exon (the LD3H9) and the CDR3 sequence (3).

κ L Chain PCR Assay. For the Jκ typing PCR assays, Vs (Schlissel; reference 26) or L5 forward Vκ primers were used with Jκ2, or Jκ5 reverse-Jκ primers (27). The Vs PCR primer should amplify 80–90% of Vκ genes. The size of the PCR product corresponds to the Jκ segment participating in the rearrangement event and has been described previously (25).

λ L Chain PCR Assays. The PCR conditions for detecting rearrangement of the λ genes, λ1 and λx, were performed as described previously (23).

Vκ38c and Vκ32a. To detect Jκ usage of the editor rearrangements, Vκ-specific forward primers (Vκ38c and Vκ32a) were used in combination with the standard reverse primers (Jκ2, Jκ5). The primers and conditions have been described previously (18).

Sequence Analysis of H Chain Genomic DNA

The H chain V regions were sequenced from DNA according to protocols described (28). In brief, DNA was isolated and amplified using constant region-specific primers. The PCR product was gel purified and sequenced using an automated sequence analyser (ABI). The 5′ primers used were the LD3H9, that binds V genes with leader sequences similar to those used by the 3H9 sd-tg, in combination with a primer located in the J_H-C_H intron. This PCR is not as specific as the LD/CDR3 PCR and detects 3H9 or 3H9/56R-sd-tgs with mutations in CDR3 region, as well as V_H replacements where the invading V_H gene uses a 3H9-like leader sequence (which includes all members of the J558 family, that correspond to 80% of all VH genes).

Statistics

Statistical significance was determined using chi-square for hybridoma analysis; and *t* test for analysis of ELISAs.

Online Supplemental Material

L chain rearrangement of the clones is presented in online supplemental Tables S1 to S3. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20020737/DC1.

Results

Our previous work began to define the mechanisms by which the allo-T-help of cGVH provokes a loss of tolerance in mice carrying the anti-DNA H chain sd-tg, 3H9.KI (20). To determine whether this process is affected by the use of a different anti-DNA H chain, we induced cGVH in mice carrying a related sd-tg, the 3H9/56R.KI. This sd-tg differs from the 3H9 by a single mutation that substitutes an arginine for a histidine at position 56 in the CDR2 region. This results in higher affinity for dsDNA, a more restricted repertoire of L chains capable of vetoing DNA binding, and an increased frequency of VH replacement (5, 18). In this paper we compare the results obtained from inducing cGVH in 3H9/56R.KI on a B6 background to those obtained from inducing cGVH in 3H9.KI mice on the same background.

The experimental group consisted of unirradiated 3H9/56R.KI recipients injected with bm12 spleen cells from mice of the same sex (bm12→56R(+)). Negative control groups consisted of 3H9/56R.KI mice and 3H9/56R negative littermates that had received syngeneic B6 spleen cells (B6→56R(+) and B6→56R(−), respectively). 3H9/56R negative littermates and normal B6 mice that had received bm12 spleen cells (bm12→56R(−) and bm12→B6, respectively) were also included as a comparison group.

Induction of Anti-DNA after cGVH. All mice undergoing cGVH were bled monthly, and the sera tested by ELISA for the presence of anti-ssDNA and anti-dsDNA IgG Abs. The 3H9/56R.KI.B6 mice (56R(+)) produced both anti-ssDNA and anti-dsDNA IgGs, even before the transfer of bm12 cells (Fig. 1). This was unexpected, as BALB/c mice carrying a conventional 3H9/56R transgene did not produce anti-DNA Abs (5), and 3H9/56R.KI.BALB/c mice produce only modest amounts of IgM anti-dsDNA (unpublished data). Thus, the spontaneous breakdown in tolerance to DNA was influenced by the background genes of the mice, the placement of the transgene (tg) in the genome, and the affinity of the Ig H chain.

After cGVH induction, higher levels of both anti-ssDNA ($P \leq 0.05$) and anti-dsDNA ($P \leq 0.05$) Abs were found in 3H9/56R.KI mice (compare 56R(+) vs. bm12→56R(+)). Thus, allogeneic T cell help increased anti-DNA Abs in 3H9/56R.KI mice (Fig. 1).

In bm12→56R(+) mice, anti-ssDNA IgGs peaked at a time and at levels similar to those in our nontransgenic cGVH groups (bm12→56R(−), $P \geq 0.05$; and bm12→B6, $P \geq 0.05$; unpublished data). By contrast, anti-dsDNA IgGs peaked by 4 wk and at levels much higher than those found in nontransgenic cGVH ($P \leq 0.05$) and bm12→3H9(+) mice ($P < 0.05$; unpublished data). As in

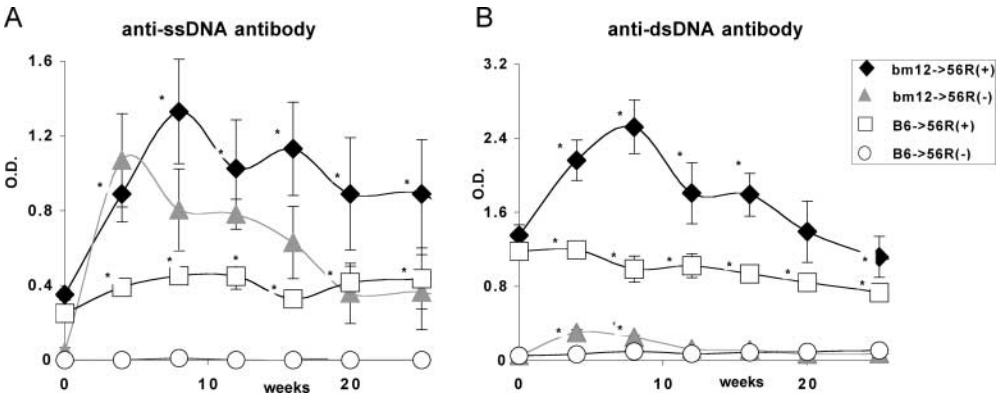

Figure 1. Anti-DNA Abs in 3H9/56R.KI mice after cGVH. Groups tested: $n = 4$–5 mice per group. Experimental GVH group: bm12 spleen cells injected into 3H9/56R.KI recipients (bm12→56R(+), ◆). Positive GVH control: bm12 spleen cells injected into 3H9/56R.KI negative littermates (bm12→56R(−), gray triangle). Negative controls: syngeneic B6 spleen cells injected into 3H9/56R.KI positive mice (B6→56R(+), □); B6 spleen cells injected into 3H9/56R.KI negative littermates (B6→56R(−), ○). Mice were bled at 4-wk intervals. The presence of anti-DNA Abs in sera was tested by ELISA. Results represent means ± SEM. (A) anti-ssDNA IgG. (B) anti-dsDNA IgG. Titers of anti-dsDNA in bm12→56R(+) differed from the positive control bm12→56R(−) at all weeks ($P \leq 0.05$), while for anti-ssDNA, the difference was only at the prebleed. Titers of both anti-ssDNA and anti-dsDNA from B6→56R(+) mice differed significantly from those of B6→56R(−) mice at all time points. Anti-dsDNA titers from bm12→56R(+) were significantly higher than those from 3H9(+) at 4 and 8 wk after cGVH ($P \leq 0.05$) (unpublished data). MRL/*lpr* sera tested at the same concentration gave an OD of 0.9 for anti-dsDNA. * represents statistical difference ($P \leq 0.05$) from the appropriate negative control.

3H9.KI after cGVH, this difference in response between anti-ssDNA and anti-dsDNA suggests that the presence of the anti-DNA Ig sd-tg focused allogeneic T cell help on anti-dsDNA B cells (20; see Fig. 1).

Comparison of Hybridomas and mAbs from 3H9.KI and 3H9/56R.KI Mice after cGVH. To study the nature of autoantibodies in more detail, we compared a hybridoma panel from a bm12→3H9(+) mouse to one from a bm12→56R(+) mouse. Both panels were generated 10 wk after cGVH was induced, a time when anti-dsDNA titers were still high.

Results from the bm12→3H9(+) and bm12→56R(+) hybridomas support the conclusion that B cells from mice undergoing cGVH were activated in vivo. We observed a consistently high percentage (~70%) of hybridomas that were anti-dsDNA$^+$ (see anti-dsDNA$^+$ group; Table I). As most anti-dsDNA$^+$ clones in the bm12→56R(+) set were not using the 3H9/56R sd-tg (see below), this frequency cannot be directly attributed to the presence of our anti-DNA H chain. Rather, the high frequency of anti-dsDNAs suggests that the self-Ag(s) involved was selectively activating these cells in vivo. It is in fact possible that the initial 56R B cells acted as APCs, presenting DNA associated molecules and therefore stimulating more anti-DNA B cells to mount a response. Also, isotype usage in the hybridomas indicated Ag activation: 42% of anti-dsDNA$^+$ clones from the bm12→3H9(+) mouse, and 68% of those from the bm12→56R(+) secreted IgG. This activation may explain why hybridomas were formed without in vitro stimulation. These results provide strong evidence for activation by endogenous self-Ag(s).

Ig H Chain Gene Usage in cGVH Mice: Loss of the sd-tg. To determine the contribution of the sd-tg to the anti-dsDNA specificity in cGVH, we analyzed the rearranged Ig genes in our hybridoma panels. We first assessed the status of the 3H9 and 3H9/56R construct by PCR. The test used primers complementary to the 3H9 and 3H9/56R H chain leader, and to the CDR3 sequence specific for the sd-tg. Successful amplification by these primers indicated the presence of the complete sd-tg.

DNA sequence analyses further confirmed the presence of this unmodified sd-tg in all PCR positive bands for both 3H9 and 3H9/56R (unpublished data). On this basis, we defined those hybridomas that had retained the sd-tg as tg positive (3H9tg$^+$ and 56Rtg$^+$; Table I).

By contrast, we defined a tg$^-$ B cell (3H9tg$^-$ or 56Rtg$^-$; Table I) as one whose DNA had failed to amplify the 3H9 VH with primers complementary to the 3H9 CDR3 and leader (LD). There are several ways that a tg$^-$ B cell might arise. One is mutation in CDR3 that destroys complementarity between the 3H9VH and the CDR3 PCR primer. We have ruled out this case by showing that the tg$^-$ hybridomas also failed to amplify with the LD-JHCH primer combination (unpublished data). JHCH binds to a site between JH4 and CH (28). tg$^-$ B cells might arise by replacement of 3H9VH by a VH (with a different LD sequence) or DH invasion of 3H9VH (in which case there is no accompanying LD sequence). Both examples have been described (6). In addition, there are trivial ways of generating a tg$^-$ such as segregation of the tg$^-$ bearing chromosome after fusion. To test for loss of the locus we amplified hybridoma DNA with a 3H9 CDR3-reverse primer (the complement of the 3H9 CDR3 primer described above) and the JHCH primer. This primer should amplify truncated or replaced 3H9VH genes because both occur at the embedded heptamer 5′ of CDR3. About 2/3 of VH and DH replacement is usually at a site just 5′ of CDR3 and such cases are amplified by this primer combination (unpublished data), the rest of replacements nibble into CDR3

Table I. *Comparison of dsDNA Binding and H Chain Usage in 3H9.KI and 3H9/56R.KI Hybridomas after cGVH Induction*

	bm12→3H9(+)			bm12→56R(+)		
	Total	3H9tg$^+$[a]	3H9tg$^-$	Total	56Rtg$^+$	56Rtg$^-$
DNA binding[b]						
dsDNA$^+$	**26 (68%)**	17 (65%)	9 (35%)	**56 (72%)**	16 (29%)	40 (71%)
dsDNA$^-$	**12 (31%)**	3 (25%)	9 (75%)	**22 (28%)**	2 (9%)	20 (91%)
Isotype[c]						
IgM	**20 (53%)**	12 (60%)	8 (44%)	**22 (28%)**	18 (100%)	4 (7%)
IgG	**18 (47%)**	8 (40%)	10 (55%)	**56 (72%)**	0	56 (93%)
Total		**20 (53%)**	**18 (47%)**		**18 (23%)**	**60 (77%)**

Monoclonal hybridomas were derived from unmanipulated spleen cells from either 3H9.KI or 3H9/56R.KI mice 10 wk after cGVH induction. Ig-secreting hybridomas were assayed for dsDNA binding, Ig isotype, and persistence of the H chain sd-tg (3H9 or 3H9/56R). Some of the 3H9.KI results have already been published (reference 20).

[a]Retention of the 3H9 or 3H9/56R H chain sd-tg was determined by specific PCR assays. Positive clones are grouped in the column under 3H9tg$^+$ or 56Rtg$^+$. Negative clones, with disrupted sd-tgs, are grouped in the column under 3H9tg$^-$ or 56Rtg$^-$.

[b]dsDNA binding was determined by a fluid phase ELISA, and confirmed by either a solid phase ELISA or crithidia assay.

[c]Ig isotype was assessed with a solid phase ELISA.

and are unlikely to be amplified. We tested 30 tg^- hybridomas by this PCR and found that 18 gave a PCR product. Thus, we believe that the tg^- category in Table I is mainly the result of 3H9VH truncation or VH replacement.

The dominant role of the 3H9 H chain in DNA binding (24) was evident in the anti-dsDNA$^+$ clones in bm12→3H9(+), where most anti-dsDNA mAbs used the 3H9 sd-tg. Nevertheless, in the same fusion, 35% of the anti-dsDNA$^+$ cells actually lost the 3H9 sd-tg. This tendency was even more marked in the bm12→56R(+) fusion, where the majority (72%) of anti-dsDNA$^+$ clones did not use the sd-tg. Strikingly, of the anti-dsDNA$^+$IgG clones, 19% from bm12→3H9(+), and 100% of those derived from bm12→56R(+) were tg^-. This suggests that isotype switching (or the stage of Ag driven activation) can be linked with the disruption of the sd-tg under cGVH.

Sd-tg Replacement in bm12→3H9(+) and bm12→56R(+) Mice. The sd-tg can be replaced by another V region from the same chromosome (VH replacement; reference 6), or the other allele can be rearranged after sd-tg inactivation. We were able to determine which mechanism had been used by defining the allotype of the B cell receptor: the transgenic allele is of the a allotype, and the untargeted allele is of the b allotype. Of the IgG anti-dsDNA$^+$3H9tg^- hybridomas, 50% were IgG2a^b. Of the anti-dsDNA$^+$IgG+ hybridomas from the bm12→56R(+), 79% expressed IgG2a^b, while only 5% used the IgG2a^a from the 3H9/56R transgenic allele (Table II). None of the 3H9/56R IgG clones expressed the sd-tg itself. Therefore, in both fusions, secondary rearrangements at the H chain took place in a significant percentage of the population, resulting in the expression of the untargeted allele.

This distribution of Igh^a and Igh^b mAbs parallels serum IgG expression. After cGVH induction, both 3H9.KI and 3H9/56R.KI mice produced anti-dsDNA Abs from both the a (transgenic) and the b (nontransgenic) allele (Fig. 2). The serum data from the 3H9/56R experiment provided additional in vivo information: anti-dsDNA Abs in the (pre-cGVH) 3H9/56R.KI.B6 mice were of the a-allele. As this is the site-directed transgenic allele, it is likely that the mechanism of central tolerance failed to eliminate B cells carrying the 3H9/56R H chain (29). Significantly, however, anti-dsDNA Abs expressing the b (nontransgenic) allele were produced only under cGVH.

H Chain Usage. To determine why the allotype switched after cGVH, we analyzed VH usage. V region nucleotide sequences were assigned to VH germlines by comparison to established sequences, from GenBank and from Haines et al. (30). The latter offered a more complete delineation of the b allotype of the B6 strain. Out of the 12 IgG2a^b hybrids sequenced, 7 used the J558.B3 gene (accession no. AF296420), 2 the J558.B4 (AF296421), 1 the MVARG2 and 2 the J558.B18 (AF296435). All 3 IgG2a^a (transgenic allele) hybrids used the 45.21.1 gene.

Table II. *Allotype Analysis of IgG Hybridomas*

	IgG2a^a	IgG2a^b	Non-IgG2a
bm12→3H9(+)			
dsDNA^{+a} IgG = 10			
3H9tg^+ = 6 (60%)	4 (67%)	0	2 (33%)
3H9tg^- = 4 (40%)	0	2 (50%)	2 (50%)
dsDNA$^-$ IgG = 7			
3H9tg^+ = 2 (29%)	0	0	2 (100%)
3H9tg^- = 5 (71%)	0	0	5 (100%)
bm12→56R(+)			
dsDNA$^+$ IgG = 38			
56Rtg^+ = 0			
56Rtg^- = 38 (100%)	2 (5%)	30 (79%)	6 (16%)
dsDNA$^-$ IgG = 18			
56Rtg^+ = 0 (0%)			
56Rtg^- = 18 (100%)	1 (5%)	10 (55%)	7 (39%)

The use of the sd-tg allotype (a) or the non-sd-tg allotype (b) was determined in supernatants of IgG2a hybridomas by ELISA. Clones that were neither IgG2a^a nor IgG2a^b were presumably using another isotype.
[a]Transgene typing as in Table I.

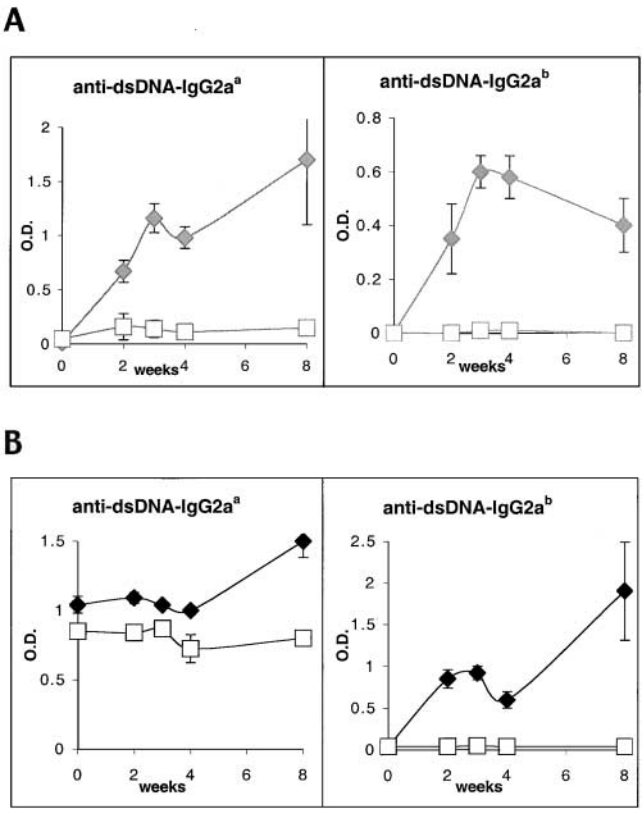

Figure 2. Allotype analysis of IgG2a anti-dsDNA Abs in 3H9.KI and 3H9/56R.KI mice after cGVH. The use of the sd-tg (a) allotype (left hand graphs) or the nonsd-tg (b) allotype (right hand graphs) in IgG2a anti-dsDNA Abs was determined by ELISA in sera. (A) 3H9(+), □, and bm12→3H9(+), gray diamond, mice. (B) 56R(+), □, and bm12→56R(+), ◆, mice.

Properties of the H Chain Used by Anti-dsDNA$^+$ Cells: Biased VH Usage. In the specific anti-dsDNA$^+$ response, germline genes J558.B3, J558.B4, and 45.21.1, are overrepresented (see Fig. 3 and Table III). This over-representation can be explained in part by clonal expansion (see below). Nevertheless, the representation of these genes is higher than would be expected, and likely indicates a process of Ag (DNA) selection. Indeed, these germline sequences do share a cluster of codons rich in asparagine residues. Located at positions 39, 41, and 42 in the CDR2

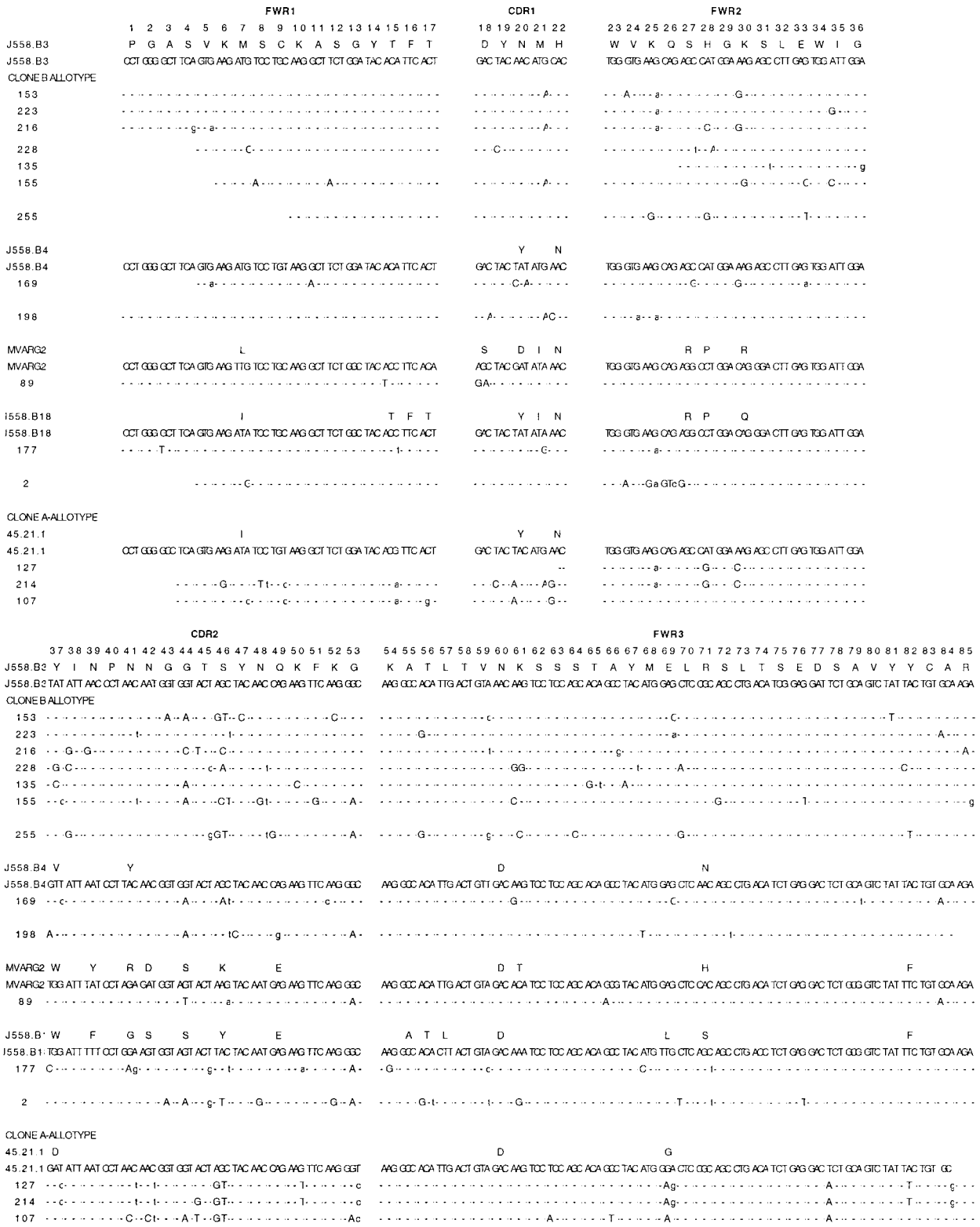

Table III. *Summary of Sequence Characteristics of tg⁻ Hybridomas for bm12→56R(+) Fusion*

Cell	Clone	Isotype	VH[a]	DH[b]	JH	CD3 length[c]	Vκ[a]	Jκ[d]	dsDNA binding
153	1	IgG2a[b]	J558.B3	FL16.1/Q52	4	13		NF	+
223	1	IgG2a[b]	J558.B3	FL16.1/Q52	4	13		2	+
216	1	IgG2a[b]	J558.B3	FL16.1/Q52	4	13		2	+
228	1	IgG2a[b]	J558.B3	FL16.1/Q52	4	13		NF	+
135	1	IgG2a[b]	J558.B3	FL16.1/Q52	4	13		2	+
155	1	IgG2a[b]	J558.B3	FL16.1/Q52	4	13		2	+
255	3	IgG2a[b]	J558.B3	FL16.1/FL16.1E/Q52	4	13		2	+
169	4	IgG2a[b]	J558.B4	FL16.1/ST4-C	4	9		NF	−
198	5	IgG2a[b]	J558.B4	ST4/FL16.2-C	4	7		NF	+
89	6	IgG2a[b]	MVARG2	SP2.9/Q52	4	9	ae4	5	+
177	7	IgG2a[b]	J558.B18	ST4/FL16.1E/Q52-C	4	10		2	−
2	8	IgG2a[b]	J558.B18	ST4/FL16.1E/SPE.8	4	10		Vk38c-Jk2	−
127	2	IgG2a[a]	45.21.1	FL16.1/ST4/FL16.1-C	4	13	bb1	4	+
214	2	IgG2a[a]	45.21.1	FL16.1/ST4/FL16.1-C	4	13	bb1	4	+
107	2	IgG2a[a]	45.21.1	FL16.1/ST4/FL16.1-C	4	13	bb1	4	−

Hybrids were assigned to clones according to the criteria outlined in the text. Clone names are given for all hybrids.
[a]VH and Vκ assignments were made based on the closest sequence homology to known germline genes.
[b]DH designations were made by homology to the closest DH gene.
[c]The CDR3 length is defined by the number of amino acids present between the last amino acid encoded by VH genes (the invariant arginine residue), and the residue encoded by all JH genes at position 7.
[d]Jκ genes are numbered 1–5 in the order 5′ → 3′.

region, these asparagines constitute a pocket of positive charges that might strongly attract the negatively charged dsDNA backbone. The presence of aspartate residues in the CDR1 might neutralize the positive charge of this CDR2 pocket; and such aspartates are present in other germlines such as J558.B1 and J558.B2 from B6. However, germlines J558.B3 and J558.B4 lack such negatively charged clusters in the CDR1. Therefore, J558.B3 and J558.B4 are different from most other VH genes by the combination of the concentrated area of positive charges in the CDR2 and the lack of a strong negatively charged pocket in the CDR1 region (30). Since, according to the current database, these germlines are unique to B6 mice, the VH.B6 allotype may be especially amenable for generating anti-dsDNA Abs.

Thus, CDR2 appears to be a major contributor to DNA binding. Analysis of mutations supports this hypothesis because replacement to arginines occurred in several of the codons in the nearby framework region (FWR 2). For example, replacements to arginine are found between positions 25 and 30 of the FWR2 region in anti-DNA$^+$ hybrids such as nos. 155, 255, 127, 214, and 89. As stated above, and shown in Fig. 3, the FWR2 and CDR2 regions are close together in the H chain structure. The inserted positive arginines occupy the space adjacent to the previously mentioned pocket of asparagines, and the mutations to arginine may increase the tendency of this region to bind dsDNA. This analysis suggests that the combination of inherited asparagine residues and mutations to arginine contribute to DNA binding.

Clonal Relatedness. Clonal relatedness is shown by identity or near identity of the CDR3s of the Ab (31). Table III summarizes the genes used by each hybrid. Of the 12 IgG2a[b] hybridomas, six shared both the J558.B3 VH gene and a CDR3 region consisting of 3 N nucleotides, a D-D fusion (composed of an FL16.1 and a Q52 gene), and

Figure 3. Nucleotide and deduced amino acid sequences of hybridoma H chains from the bm12→56R(+) mouse after the induction of cGVH. The panel shows nucleotide sequences of the H chains used by 56Rtg⁻ hybridomas derived from the bm12→56R(+) mouse. The sequences for each hybrid were determined by using the LD-JHCH PCR, as described in Materials and Methods. For each hybridoma, sequences were analyzed for homology to published VH gene segments using both GenBank/EMBL/DDBJ databases and B6 VH gene segments determined by Haines et al. (reference 30). Established germline codons and their corresponding single letter amino acids are shown in the top rows. Sequences from hybridomas using the same H chain are grouped together below these codons. Hybridomas 153, 223, 216, 228, 135, and 155 represent potential members of the same clone. Hybridomas 127, 214, and 107 represent potential members of a second clone. Clone assignments were made as described in Results. Dashes represent bases identical to those of the germline. Upper case letters indicate replacement mutations; silent mutations are in lower case. The J558.B3, J558.B4, and J558.B18 sequences described in this paper are available from GenBank/EMBL/DDBJ under the following accession nos.: AF296429, AF296421, AF296435, respectively. CDR, complementarity-determining regions.

a JH4 segment. This makes it highly probable that these clones were derived from the same in vivo founder, the VHJ558.B3/DFL16.1/DQ52/JH4 clonotype (hereafter clone 1). Similarly, the 3 IgG2a^a hybrids shared the 45.21.1 VH gene, as well as a CDR3 region consisting of 4 N nucleotides, a D FL16.1, a D ST4, an inverted D FL16.1 gene and a JH4 segment (see also Fig. 4). Thus, in this mouse, B cells with common, anti-dsDNA autoantibody specificities provide evidence of clonal expansion. From these data, we conclude that the six anti-dsDNA-producing hybridomas derived from one B cell precursor (clone 1), and the three IgG2a^a hybrids from another (clone 2). Additional analysis showed that the CDR3 regions are relatively long; in clone 1 and 2 the length is 13 amino acids. A high frequency of D-D fusions and JH4 usage, as reported by Monestier, was also seen. (32, 33).

High Frequency of Mutations in cGVH B Cells. By comparing these VH sequences to germline V, D and J sequences (reference 30, and GenBank), the presence and frequency of somatic mutations could be determined (Figs. 3 and 4). In all VH regions of the IgG Abs sequenced, this comparison revealed a high frequency of mutation, ranging from 14 to 33 per sequence, with an average of 24. The frequency of mutations in this cGVH model was more than twice as high as that seen during secondary immune response to foreign Ags (average lower than 10 mutations per Ab; reference 34). It was also notably higher than in autoantibodies from the MRL/*lpr* mice (35). This high frequency of mutations most likely reflects a lengthy period of activation during cGVH, and suggests that hypermutation and clonal expansion are essential to the generation of autoreactivity in this syndrome.

As expected, some mutations were shared among clonal relatives. In clone 1, all hybrids shared a T to C substitution in JH4, three shared one replacement mutation, while two hybrids shared four other mutations. In clone 2, eleven mutations were shared. The individual hybrids derived from clone 1 differed from each other by 11 to 24 mutations; in clone 2, 18.5 different mutations were found among the two main branches of the clonal tree (Fig. 5). These unique mutations suggested extensive expansion of the clones late in the response.

L Chain Rearrangement in bm12→3H9(+) and bm12→56R(+) Clones. To determine the contribution of L chain editing to the self-reactive phenotype of these cells, we assayed the rearrangement status of the κ and λ genes in hybridomas from both mice, using a series of PCR assays (online supplemental Tables S1–S3). As described in our previous paper (20), both Jκ5 and λ1 usage was high in anti-dsDNA$^+$3H9tg$^+$ hybridomas. As compared with their usual frequency (36, 37), this increased usage of downstream genes supports the idea that anti-

	FWR3			>	<	CDR3														
	30	31	32	VH	N	D 1	D 2	D3	N	JH4 1	JH4 2	JH4 3	JH4 4	JH4 5	JH4 6	JH4 7	> VH	D
J558.B3	TGT	GCA	AGA		GL	(TTT ATT ACT ACG GTA GTA GCT AC)	(GGAC)			JH4	TAC	TAT	GCT	ATG	GAC	TAC	J558.B3	FL16.1/Q52
CLONE B ALLOTYPE																		
153 (dsDNA+)	---	---	---	GAG GGA AAA		(ATT ACT ACG TCA GTG CCT AC)	(G GAC)					--C	-T-	---	---	---	J558.B3	FL16.1/Q52
223 (dsDNA+)	---	A--	---	AAG GGG GAA		(ATT ACT CCG GTA GTA GGT AC)	(G GAC)					--C	-G-	---	---	---	J558.B3	FL16.1/Q52
216 (dsDNA+)	---	---	-A-	GAG GGG GAG		G(TT ACT TCG GTA GTG ACT AC)	(G GAC)					-GC	A--	G--	---	---	J558.B3	FL16.1/Q52
228 (dsDNA+)	---	---	---	GAG GGG GAA		(ACT ACT TCC GTT GTA CCT AC)	(G GAC)					--C	--C	---	---	---	J558.B3	FL16.1/Q52
135 (dsDNA+)	---	---	---	GAG GGG GGA		(ATT ACA ACG GTA GTA GTT AC)	(G GAC)					--C	---	---	---	---	J558.B3	FL16.1/Q52
155 (dsDNA+)	---	---	--G	GAG AGG GAG		(ATC ACT ACG TTA GTA GCT AC)	(G GGC)					--C	---	---	---	---	J558.B3	FL16.1/Q52
					GL	(TTT ATT ACT ACG GTA G)	(ACCACC)	GGAC										
255 (dsDNA+)	---	---	---	GAG GGG G	CG	(ATT ACT GCG GTT G)	A(A CCT CC)	(G GAC)				---	A--	A--	-T-	---	J558.B3	FL16.1/FL16.1E-C/Q52
J558.B4	TGT	GCA	AGA		GL	(TAC GGT AGT AG)	(TGTC)											
169 (dsDNA-)	---	A--	---			(TAC GGT GGT AG)	(T GTC)		AAT TC		-	---	-T-	---	---	--T	J558.B4	FL16.1/ST4-C
					GL	(AGC TCG GGC TA)	TAGCC											
198 (dsDNA+)	---	--			T	(AGA TCG GGT TA)	(T GGC C)				--	---	---	---	---	---	J558.B4	ST4/FL16.2-C
MVARG2	TGT	GCA	AGA		GL	(GAT GGT TAC)	(CT GGG)			JH4			GCT	ATG	GAC	TAC		
89 (dsDNA+)	---	A--	---	GAG GGG	CGG	(GAT GAT TAC)	T(CT GGG)						---	---	---	---		SP2.9/Q52
J558.B18	TGT	GCA	AGA		GL	(AGC TCG GGC TAC)	(GTG G)	(AGT T)										
177 (dsDNA-)	---	---	---		TCG AAT	(AGT TCA GGC TAC)	(GTG G)	AG (AGT T)					--	---	---	---		ST4/16.1E/Q52-C
					GL	(C AGC TCG GGC TAC)		(AG TAT GG)										
2 (dsDNA-)	---	---	---		TCG AG	(C AGC TCA GTC TAC)	(GTG G)	(AG AAT GG)					-	---	---	---		ST4/16.1E/SP2.8
CLONE A-ALLOTYPE																		
45.21.1	TGT	GC			GL	(AT TAC TAC GG)	(A CAG) CTC GG	(AAT AAA)	JH4-TG		TCC	TAT	GTT	ATG	GAC	TAC		
127 (dsDNA+)	---	--G	---		CGG A	(AT GAC TAC GG)	(A GCG) ACA	(AAT GAA)		TAC	---	---	---	--A	--T	-T-		FL16.1/ST4/FL16.1-C
214 (dsDNA+)	---	--G	---		CGGA	(AT GAC TAC GG)	(AGCG)ACA	(AAT GAA)		TAC	---	---	---	--A	--T	-T-		FL16.1/ST4/FL16.1-C
107 (dsDNA-)	---	---	---		CGG A	(AT TCC TAC GG)	(A CAG) AAC	(ATT AAA)		TAT	---	-T-'	---	---	---	---		FL16.1/ST4/DLF16.1-C

Figure 4. Nucleotide sequences of the recombinational junctions of hybridoma H chains from the bm12→56R(+) fusion. Hybridomas in this figure are the same ones depicted in Fig. 3, and are presented in the same order. The top line of each group represents the presumed V, D, and J germline (GL) genes used. Sequence identities with the germline VH or JH are denoted by dashes. The DH segments are indicated on the right. DH sequences are determined by identifying at least 4 nucleotide identities to a known DH segment. These, as well as the original DH sequences, are indicated in parentheses. The non-DH, non-VH sequences are assumed to be N sequences. As in Fig. 3, the VH sequences are assigned to the known VH germline from the GenBank or Haines et al. (reference 30). Nucleotides that differ from germline are underlined.

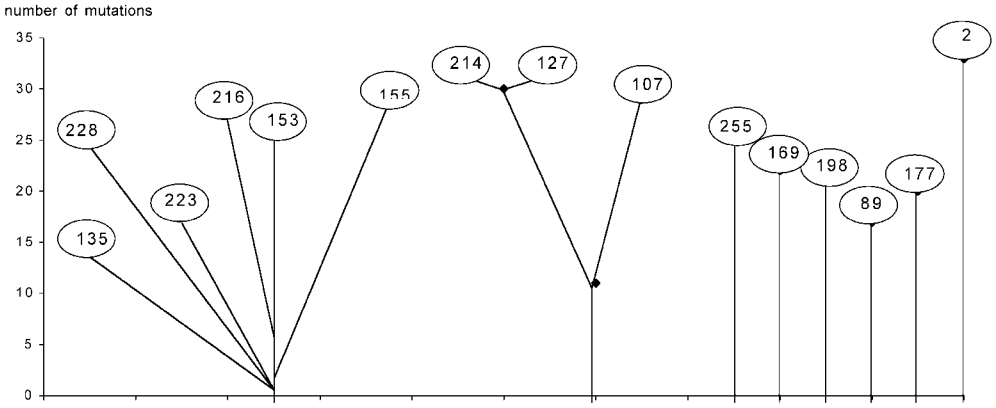

Figure 5. Genealogical trees generated from hybridoma sequences, demonstrating ongoing somatic VH region diversification (Hybridomas are those analyzed in Figs. 3 and 4). The individual clones sequenced were assigned to a particular genealogical tree on the basis of shared and unique VH mutations. Although we interpret most shared mutations as representing single events, we did observe independent parallel mutations (presumed hot spots). Multiple hybridomas with identical nucleotide sequences share a line. The height of each line shows the number of mutations recorded. For clarity, the hybridomas are identified by number within the ovals. Note the high number of mutations carried by each of the sequences analyzed. All hybrids except 169, 177, 2, and 107 bind dsDNA.

dsDNA B cells have undergone secondary L chain rearrangements. In the present study we tested the same set of clones for rearrangement to L chains that veto dsDNA binding in the context of the 3H9 or 3H9/56R sd-tg (Vκ38c, Vκ32a, and λx). Some clones indeed had rearranged Vκ38c and Vκ32a, but they also appear to be expressing another Vκ chain simultaneously (dual rearrangement is represented by a superscript a in online supplemental Table S1).

Of the anti-dsDNA hybridomas from the 3H9/56R fusion, 16 (30%) retained the H chain sd-tg. L chain data from these hybridomas demonstrated a biased Vκ repertoire: 10 (62%) used Vκ38c (online supplemental Table S2). A biased distribution of Jκ gene segments was also observed among these $56Rtg^+$ hybridomas: 5 (32%) arranged to Jκ5, a frequency higher than that found in conventional mice. Only one clone used both Vκ38c and Jκ5. All these data point to secondary rearrangements of the L chains in anti-$dsDNA^+56Rtg^+$ hybridomas. It is intriguing that Vκ38c, when paired with the 3H9/56R sd-tg, did not appear to veto dsDNA binding. This might be explained by rearrangement of the other allele in three of the ten clones, similar to what was seen in the 3H9 clones. Indeed, we showed by sequence analysis that at least one of these second L chains could be functional (unpublished data). In five other clones, we observed triplet insertions and mutations in the Vκ38c gene at its junction with the Jκ gene (unpublished data). These mutations may have altered the editing potential of the L chain.

Discussion

Mice undergoing cGVH develop a lupus disease similar to mice with spontaneous lupus. cGVH mice develop the same spectrum of autoantibodies and similar kidney pathology. We have extended the comparison between these models to include the genetic control and etiology of anti-DNAs. The analogy remains compelling: the mouse models of SLE such as MRL/*lpr* have two phases of autoantibody induction. The first phase is characterized by polyclonal, unmutated IgM Abs (38) and appears to result from nonspecific activation. The second phase is characterized by oligoclonal, IgG Abs and resembles a typical, Ag-driven immune response. Members of clones have somatic mutations that yield amino acids such as arginine and asparagines that play a role in DNA–protein interaction (39).

cGVH in the anti-DNA site directed transgenic mice also undergoes both types of activation. All B cells have increased levels of activation markers in 3H9(+) after cGVH, even though only a subset of B cells are anti-DNA (20). A similar phenotype was found in preliminary data from bm12→56(+) (excepting CD23). This indicates a nonspecific activation phase (20). This study also shows that the IgM B cells are highly diverse i.e., polyclonal. Most, but not all, IgM hybridomas bind DNA, and the anti-DNAs have different avidities. In addition, most of these B cells are independently derived. Even though the majority express the sd-tg and the Vκ38c L chain, they can be shown to be of independent origin by several criteria: they utilize different Jκ segments or they have unique Vκ-Jκ junctions. Within the entire subset of IgM anti-dsDNA hybridomas, at most 4/18 (22%) might be clonally related as compared with 8/11, or 73%, in the set of IgG anti-DNA (see below).

That cGVH also causes an Ag-driven immune response was first suggested by the narrow spectrum of the specificities of the IgG autoantibodies expressed in these mice (12, 13). If autoimmunity in GVH were entirely due to nonspecific activation, then a much broader spectrum would be expected and would include many other autoantibodies seen in other murine models of autoimmunity. This is not the case and instead just autoantibodies typical of SLE are expressed. The transgenic models allows us to reach a similar conclusion: whereas nonspecific activation should yield edited anti-DNAs or anti-ssDNA Abs, the majority of monoclonal IgGs bind dsDNA. Serum anti-DNA titers also show that anti-dsDNA increases disproportionately to anti-ssDNA (Fig. 1). In sum, the restricted production of B cells that recognize a single Ag and the bias toward anti-dsDNA point toward a stage of Ag activation during cGVH.

The IgG anti-DNAs have other properties of Ag-driven immune responses, in that they are members of expanded clones and are mutated. In our study of eleven randomly selected IgG2a anti-dsDNAs, two sets had highly homologous VH and VH CDR3 regions, suggesting that each set represents an expanded clone (Figs. 3 and 4). Additionally, some mutations among members are shared, a property that usually results from their accumulation during clonal expansion. Taken together, the homologous CDR3 sequences and the hierarchical pattern of shared mutations provide strong evidence that members of these sets have descended from a single B cell precursor. As discussed above, the V gene usage and pattern of mutations indicate that the IgG anti-DNA B cells were selected for and driven by DNA.

Even though the sd-tg is associated with relatively high affinity anti-DNAs, it appears to be excluded in the DNA-driven phase of the 56R model. Although every B cell at the initial stage of development inherits the 3H9/56R sd-tg, none of the GVH induced IgGs are encoded by the sd-tg. In the anti-dsDNA IgG subset, all cells used an upstream VH gene that replaced the sd-tg or a VH derived from the untargeted allele. How these arise could be explained in three different ways (see Fig. 6):

(a) A leaky H-STOP control. It is thought that a functional H chain, presumably in combination with surrogate L chain, stops further rearrangement at the H locus. This STOP signal explains why the majority of B cells are incompletely rearranged (DJ) on the silent allele. Accordingly, an H chain tg should prevent rearrangement on the untargeted allele. Nevertheless, H chain rearrangement is found in H chain transgenics, either at the untargeted allele or in the form of VH replacement of the tg VH. The frequency of such rearrangements varies; in the anti-DNA sd-tgs LPS-activated B cells 2% (3H9), 14% (3H9/56R), and 38% (3H9/56R/76R) have one or the other form. The site at which these secondary VH rearrangements occur could be in the pro-B cell, because the sd-tg does not completely stop rearrangement, or later because of editing. At least some VH replacement must occur early because N addition is found at the V-D-J junctions. Thus, the tg^- B cells may be selected from a subpopulation of B cells with a VH repertoire encoded by endogenous genes. Alternatively, TdT could be reengaged in mature B cells under cGVH.

(b) Mutation correction. Endogenous VH genes may be introduced after Ag selection of the 3H9/56R B cells. We and others have noted that V gene replacement occurs during clonal expansion. In the 3H9/Vκ8 MRL/*lpr*, L chain rearrangement followed (and rescued) a nonsense-mutated L chain (28). VH replacement is seen in expanded clones from RA synovium (40). As noted above, the clones observed in GVH-induced anti-DNAs have unusually high frequencies of somatic mutations. The R:S of these mutations in certain FRs is low, showing that many lethal mutations have occurred during clonal expansion. Thus, it is conceivable that inactivation of the sd-tg followed by replacement or rearrangement at the untargeted allele explains the shift to endogenous VH gene usage. This model implies that rearrangement can be reinitiated after Ag-selection. This may be special to those cells in a clone with a lethal H or L chain mutation.

(c) Autoimmune V gene replacement. VH and/or VL rearrangement may be induced in autoimmunity. In fact, it has been reported that recombination activating gene levels are unusually high in SLE (41). This would lead to "reediting." The key distinction between editing and reediting is the setting in which the secondary rearrangement occurs. Editing refers to rearrangement that takes place in imma-

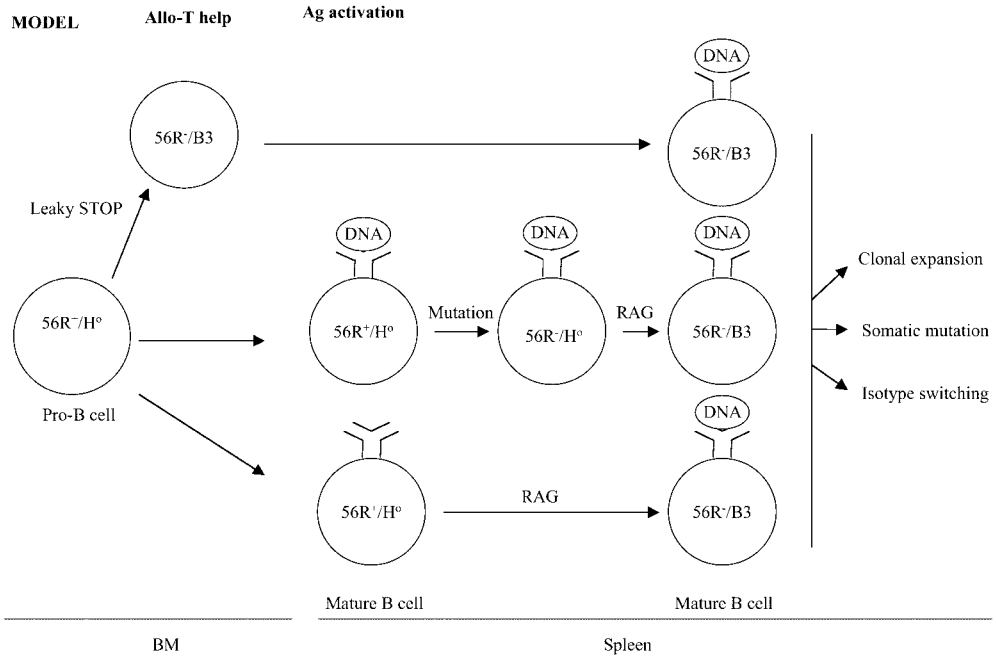

Figure 6. Models.

ture cells in the bone marrow. By contrast, reediting takes place in mature peripheral cells, and is associated with Ag-driven immune responses. In our bm12→56R fusion, a clear distinction between peripheral subsets provided evidence of reediting under cGVH. In the IgG cells, secondary H chain rearrangement occurred frequently (see above), whereas among IgM cells, most of which used an unmodified 3H9/56R H chain, it must have been rare. In addition, the IgG clones displayed characteristics typical of B cells that have undergone a secondary immune response, as indicated by clonally related sequences (discussed above) and a high frequency of somatic point mutations. In this subset, oligoclonality and hypermutation, along with isotype switching, clearly associates secondary VH rearrangment with Ag activation under cGVH, and indicates reediting. The increased frequency of such H chain rearrangement in anti-dsDNA IgG cells, and the fact that the anti-dsDNA IgG subset has been implicated in SLE pathology, implies that reediting during clonal expansion could play a key role in disease development. The mechanism of reediting is also more clearly established in light of these data. Within the anti-dsDNA IgG subset, very few cells derived from VH replacement in the sd-tg allele (see clone 2). This can be explained by the high percentage of such VH rearrangements that might result in nonfunctional joints (42). Most interestingly, a high percentage of these cells (80%) used the second allele. Along with the fact that none of these cells expressed both alleles simultaneously, this strongly suggests that most VH reeditings result in deactivation of the first (here, the transgenic) allele. This in turn enables the more efficacious classical rearrangement process to occur at the untargeted allele.

Our data do not allow us to distinguish among these models. The reediting model or the lethal mutation model are appealing because they explain the shift from tg to endogenous gene usage. If we assume that DNA selects and drives anti-DNA B cells, then we would expect the response to include, indeed to prefer, the tg^+ B cells. The alternative explanation for the shift would mean that the subpopulation of cells arising because of a leaky STOP signal has a selective advantage. Although this possibility may appear initially unlikely, further consideration of our sequence data could be supportive. The endogenous VH usage we found was highly restricted. This implies that certain endogenous VH genes may have DNA binding properties. In fact, most members of the anti-dsDNA IgG clones sequenced used the J558.B3, J558.B4 genes. These genes are unique to the B6 VH locus (30) and have CDR1/CDR2 regions that are rich in asparagine residues. These residues form a pocket of positive and negative charges that should attract DNA/protein complexes. These features suggest that B cells carrying H chains with increased affinity for dsDNA may be preferentially selected and expanded. Additionally, in the nearby FWR2 area of these hybridomas, residues located between positions 24 and 30 sustained somatic mutations to arginine codons. These arginines occupy the space adjacent to the previously mentioned pocket of asparagine residues, suggesting that these mutations actively increase the tendency of this region to bind dsDNA. The VH characteristics of these clones strongly suggest a process of positive selection, with DNA as the Ag.

This analysis prompts us to consider the role of strain-specific loci in the development of disease (SLE susceptibility loci). These and other studies have shown that lupus autoantibodies may be determined by allotype-linked gene(s). For example, [MRL/lpr (Igh^j) × B6/lpr (Igh^b)]F1 mice express SLE-Abs mainly of the b allotype (43). This bias is borne out in our study. We found that most IgG2a anti-dsDNA mAbs rescued in the bm12→56(+) were of the Igh^b allotype. These findings may provide clues to the cause of this allotype dependence. In our mouse model, which possesses a diploid number of ~150 VHJ558 (a and b), the VH germline repertoire used by the anti-dsDNA IgG Abs does not represent a random sample. Rather most (73%) of the monoclonal anti-dsDNA Abs sequenced were encoded by VH genes that shared the positively charged motif in the CDR2 region. This region, not found in most other J558 VH, represents a highly restricted population. Therefore, in this model, it is likely that the higher predisposition to form anti-dsDNA B cells on a B6 background would be due to availability of these VH germlines, and, specifically, that differences such as charge between the b and a allotype V regions would make the b-allotype more likely to bind charged Ag, such as DNA.

In summary, then, we have identified two stages of activation during cGVH: a stage of nonspecific activation represented by the IgM subset, and a stage of Ag activation represented by the IgG subset. In the former, allo-T cells provided signals that stimulated most B cells nonspecifically. In the latter, Ag selected and expanded specific B cell clones. The Ag is most likely dsDNA, as evidenced by the selection of cells carrying a dense, positively charged region in the CDR, and by arginine mutations proximate to this area. The fact that all members of clone 1 bound only DNA also makes it the likely driving molecule.

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