Receptor Editing: An Approach by Autoreactive B Cells to Escape Tolerance

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Summary

To determine the fate of anti-DNA antibody-bearing B cells in normal mice, we generated transgenic mice bearing the heavy (H) and light (L) chain genes of a well-characterized anti–double-stranded DNA antibody. This antibody was originally isolated from a diseased MRL/*lpr* mouse and has characteristics common to spontaneously arising anti-DNA antibodies. Results show that the H/L transgene (tg) immunoglobulin receptor is not expressed by animals bearing both tgs, although single tg animals (H or L) express their transgenes. Young H/L tg animals express few B cells, whereas adult H/L tg animals maintain almost normal B cell numbers. Analysis of the immunoglobulin receptors used by adult B cells shows that all contain the tg H chain in association with endogenous L chains. These B cells transcribe the L tg as well as the rearranged endogenous L chain gene, and loss of endogenous L chain gene transcription results in resurrection of the 3H9 H/L tg product. Examination of the endogenous L chains used by these cells shows that they represent a highly restricted subset of V genes. Taken together, these data suggest that autoreactive transgenic B cells can rearrange endogenous L chain genes to alter surface receptors. Those L chains that compete successfully with the L tg for H chain binding, and that create a nonautoreactive receptor, allow the B cell to escape deletion. We suggest that this receptor editing is a mechanism used by immature autoreactive B cells to escape tolerance.

B cells expressing autoreactive Ig receptors are thought to be negatively regulated through inactivation or deletion. Inactivation was observed by Pike and Nossal (reviewed in reference 1), who demonstrated that low doses of antigen could render B cells specifically unresponsive to antigen and mitogen stimulation. This type of tolerance was shown in amplified form using mice with transgenes (tgs)[1] coding for an anti–hen egg lysozyme (HEL) antibody (reviewed in reference 2). These mice, when crossed with mice with a tg coding for HEL, yielded double-tg progeny whose B cells were refractory to antigenic or mitogenic stimulation. Deletion was dramatically demonstrated using a tg system in which genes of an anti-H-2^k antibody were introduced into mice of the H-2^k haplotype (reviewed in reference 3). These mice showed a great reduction in B cell number and absence of the tg idiotype on the remaining peripheral B cells.

These types of negative regulation operate at the cellular level through uncoupling signaling pathways or by initiating cell death. Another level at which regulation might occur is by changing the specificity of antiself receptors (4). Such "editing" might occur at the genetic level by replacing V genes coding for antiself receptors with V genes coding for "harmless" receptors. Mechanisms are available at both the H and L chain κ loci for V gene replacement. Here we describe another way of editing receptors. In this case, the L chain of an autoreactive antibody has been displaced by different L chains to yield nonautoreactive specificities. This form of editing occurs at the level of H/L pairing and depends upon the ability of a nonautoreactive L chain to displace an autoreactive L chain.

This phenomenon has been observed in a mouse with H and L tgs coding for an anti-DNA antibody. This antibody, termed 3H9, arose in a diseased MRL/*lpr* mouse and has the characteristics of autoantibodies associated with autoimmune disease: it binds single-stranded (ss)DNA, double-stranded (ds)DNA, and cardiolipin; it is a member of an expanded clone and is highly mutated to include at least one mutation that creates the specificity for dsDNA and cardiolipin (5, 6). We are unable to detect this antibody in tg animals. In addition, young tg mice exhibit greatly reduced numbers of B cells. Results indicate that the remaining B cells have altered their surface receptors through L chain competition. We pro-

[1] *Abbreviations used in this paper:* ANA, anti-nuclear antigen; ds, double stranded; tg, transgene.

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pose that this receptor editing has saved these B cells from deletion.

Materials and Methods

Cloning of the Vκ4 Gene. The hybridoma cell line 3H9 was used to clone the functionally rearranged Vκ4 gene (5). The 9.4-kb BamHI fragment was isolated and cloned following a procedure by Carmack et al. (7). The purified insert was subcloned into the BamHI site of the pBS plasmid.

Production and Identification of tg Mice. Tg mice were generated and identified as previously described (7). Presence of the 9.4-kb BamHI fragment identified tg-positive animals. Seven tg founder mice were identified and backcrossed onto BALB/c to establish lines. Tg copy number was established for each founder line by scanning Southern blots of BamHI-digested DNA probed with ^{32}P-labeled Jκ/Cκ-specific pECκ probe (8) using a two-dimensional proportional scanner (AMBIS, Inc., San Diego, CA). Total counts from transgene bands were compared with those of germline bands.

Hybridomas. Spleen cells (2×10^6 cells/well) were cultured for 2 d with *Escherichia coli* LPS (L2880; Sigma Chemical Co., St. Louis, MO) at a concentration of 20 μg/ml and then fused with either Sp2/0-Ag14 (9) or X63-Ag8 (10) cells as described (11). Cells were seeded into 96-well culture plates at limiting dilution. Hybridomas were selected for growth in azaserine-hypoxanthine (A9666; Sigma Chemical Co.) supplemented medium. Cells fused with x63-Ag8 fusion partners were grown in 5% ORIGEN (IGEN Inc., Rockville, MD). All cultures were maintained in RPMI 1640, 10% FCS, 5×10^{-5} M 2-ME. Supernatant of hybridoma-containing wells was tested for the presence of secreted Igs. Greater than 96% of the hybrids secreted an IgM; 10–20 Ig-producing hybrids were randomly selected from each fusion for further analysis. Membrane Ig was assayed by cytofluorography and secreted Ig by ELISA for binding to either anti-3H9 idiotypic antibody 1.209 or 1.3H9. Secreted Ig was also analyzed for cell nuclear binding using an anti-nuclear antigen (ANA) assay (12).

Antiidiotypic Antibodies. A/He mice were injected with protein A–purified 3H9 antibody using the immunization protocol for soluble protein antigens outlined by Harlow and Lane (11). Sera were monitored for anti-3H9 binding by radioimmunoassay (RIA) using ^{125}I-3H9 as described by Newby et al. (13). The best responder was boosted, rested for 4 d, and killed. Splenocytes were fused to SP2/0 cells and grown as described above. Hybridomas were screened for anti-3H9 antibody activity by RIA. Two hybridomas, 1.209 and 1.3H9, secreted antibodies that reacted specifically with 3H9 antibody. These hybrids were subcloned. 1.209 and 1.3H9 antibodies are of the IgG1 isotype.

ELISAs. ELISAs were used to detect hybridoma-secreted Ig products and to determine if these products were bound by either anti-3H9 idiotypic antibody 1.209 or 1.3H9. Goat anti–mouse κ or goat anti–mouse IgG1 antibodies (Fisher Biotech, Orangeburg, NJ) were coated on polystyrene plates (Immulon II; Dynatech Labs. Inc., Chantilly, VA) at concentrations of 5 μg/ml followed by blocking with 10% FCS. 1.209 or 1.3H9 antibodies were then bound onto goat anti–mouse IgG1 plates. Test culture supernatants were added to wells containing anti-κ antibody, 1.209, 1.3H9, or anti-IgG1 as a control. Bound antibody was detected using alkaline phosphatase–conjugated goat anti–mouse IgM antibodies and developed with phosphatase substrate (104; Sigma Chemical Co.). Binding was quantitated by measuring absorbance at 405 nm.

RNA Primer Extension Analysis and Sequencing. Poly(A)RNA was isolated from spleen cells and hybridoma cells as described by Badley et al. (14). Primer extension analysis was performed as described by Erikson et al. (15) using four Jκ primers, two λ primers, a Cκ primer (16), and a 3H9 Vκ4-specific CDR3 primer (5′-GAACGTGAATGGGTAACCACACCA-3′). Full-length cDNA products were sometimes analyzed for radioactivity by scanning with a two-dimensional proportional scanner (AMBIS, Inc.). Hybridoma mRNA was sequenced according to the protocol of Geliebter et al. (17).

Southern Analysis. Southern blot analysis was carried out as described (18). Blots were hybridized with ^{32}P-pECκ probe.

Cytofluorographic Analysis. Spleen cells and bone marrow cells (10^6/sample) from 4-d-old and from 2–4-mo-old animals or B hybridoma cells (3×10^5/sample) were incubated with 1.209 or 1.3H9 antibody, or no primary antibody, followed by incubation with a biotinylated goat anti–mouse IgG1 reagent (Fisher Biotech), then incubation with Texas red–conjugated avidin (Molecular Probes, Inc., Eugene, OR). Spleen and bone marrow samples were counterstained with FITC-coupled goat anti–mouse IgM (Fisher Biotech). 3H9 and 104.8 hybridomas (see Fig. 4) were incubated with FITC-coupled anti–mouse IgG2b (Fisher Biotech). Propidium iodide was used to exclude dead cells from analysis. Analyses were performed using a dual-laser FACStar Plus® (Becton Dickinson & Co., Mountain View, CA).

Results

Construction of the Vκ4 Fragment and Its Expression in tg Mice. The Vκ4Jκ4Cκ gene expressed by the 3H9 hybridoma was cloned as a 9.4-kb BamHI fragment (Fig. 1 *a*). The Vκ exon of this clone was sequenced and found to be identical to the Vκ cDNA sequence reported for the 3H9 κ chain (5). In addition, cotransfection of the Vκ4 construct with a 3H9 H chain construct into non-Ig-secreting cells led to secretion of an antibody with the specificity of 3H9 (data not shown).

Tg mice were generated using this Vκ4 construct. Fig. 1 *b* shows Southern analysis of tail DNA from the founder selected for all subsequent experiments. The predominant 9.4-kb band corresponded to the introduced Vκ4 fragment. Additional bands in the high molecular weight region and ~9.4 kb suggested that some tg copies had modified their BamHI ends. Scanning of blots with a two-dimensional proportional scanner and comparison of tg bands to germline bands showed this founder to carry 20–40 tg copies. Transcriptional analysis showed tg expression in bone marrow, spleen, and at very low levels in thymus. No tg transcripts were detected in heart, brain, lung, or liver (data not shown).

Endogenous L Chain Expression in L tg Mice. A key property of an Ig H or L tg is its ability to shut down endogenous Ig gene rearrangement. We analyzed the extent of endogenous L chain gene rearrangement and expression in hybridomas derived from LPS-activated spleen cells of Vκ4 L tg animals. Ig-secreting hybridomas were examined for unique κ rearrangements by Southern blot analysis. As shown in Fig. 1 *b*, DNA from these hybridomas exhibited the characteristic tg pattern as well as bands corresponding to κ genes from the fusion partner SP2/0. Unique bands representing endogenous rearrangements could be observed in certain hybrids (2748.9.1 and 2748.14.1; Fig. 1 *b*), whereas another (2748.10.1) exhibited no additional rearrangements. This latter case suggested that the L tg does exclude endogenous κ rearrangements in some B cells.

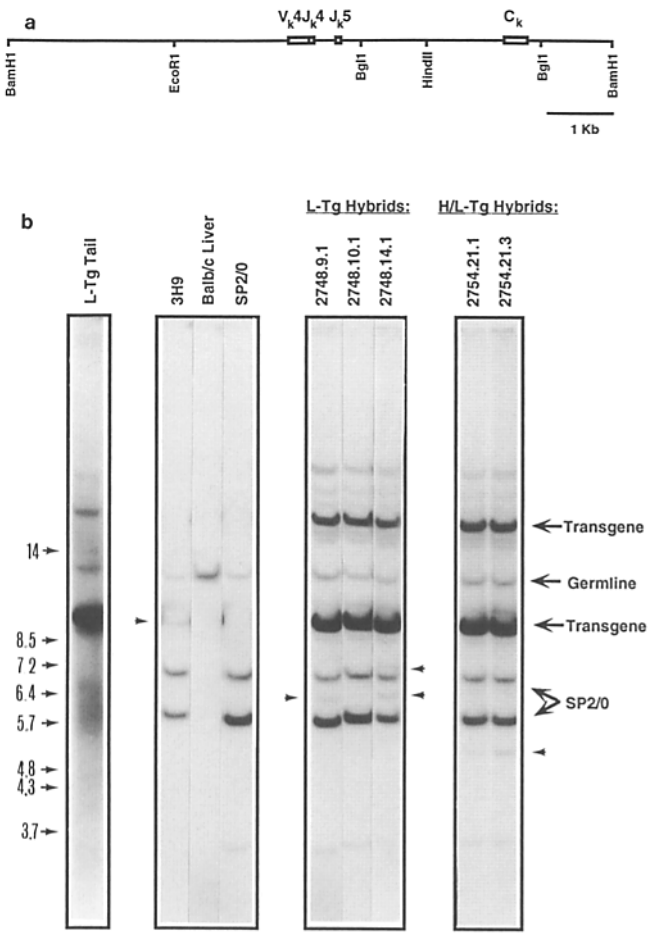

Figure 1. (*a*) The 3H9 L tg construct. (*b*) Southern blot analysis of L tg tail, L, and H/L tg splenic hybridomas. BamHI-digested DNAs from L tg tail, BALB/c liver, Sp2/0, 3H9, L tg splenic hybridomas 2748.9.1, 2748.10.1, 2748.14.1, and H/L tg splenic hybrids 2754.21.1 and 2754.21.3 were examined by Southern analysis as described in Materials and Methods. In addition to the hybridization pattern observed for all L tg–containing hybridomas, unique bands representing endogenous κ rearrangements were seen in 2748.9.1, 2748.14.1, 2754.21.1, and 2754.21.3; these bands are marked by adjacent arrows.

It was possible that additional endogenous rearrangements might be hidden in the complex Southern blot patterns of the tg hybridomas. As an alternative approach to determine the influence of the L tg on endogenous L chain expression, we tested these hybridomas for transcription of endogenous κ genes. Assays using primers specific for Jκ1, Jκ2, Jκ4, Jκ5, λ1, and λ2 showed that 19 of 25 hybrids derived from L tg animals transcribed an endogenous L chain transcript (Table 1). Fig. 2 *A* shows results using the Jκ1-specific primer. The lower band in each lane of Fig. 2 *A* corresponded to the cDNA extension product of the aberrant SP2/0 κ transcript found in all SP2/0-fused hybrids. In addition, hybridoma 2748.7.1 generated a second product presumably corresponding to an endogenously derived κ transcript. Hybridomas 2748.10.1 and 2748.5.3 did not show a product using the Jκ1 primer (Fig. 2 *A*), or other primers (data not shown), and are presumed to lack endogenous L chain transcripts.

Extension analysis using the L tg–specific primer showed that the tg was transcriptionally active in all 25 L tg hybrids (Fig. 2 *A*, and data not shown). Those hybrids transcribing only the L tg also secreted κ-containing Ig, indicating that the L tg can produce a functional protein.

Cytofluorographic Analysis of tg Animals. We followed expression of the 3H9 antibody in 3H9 H/L tg animals using two antiidiotypic mAbs. The specificities of these reagents are demonstrated in Fig. 3. 1.209 bound surface 3H9 Ig on 3H9 hybridoma cells and surface Ig composed of the 3H9 H chain in association with a Vκ8 L chain on 104.8 cells. Because Vκ4 and Vκ8 L chains are only distantly related, these data suggested that the idiotope bound by 1.209 is on the H chain. Further analysis showed that 1.209 bound other 3H9H chain–containing antibodies associated with a wide variety of L chains (J. Erikson, Wistar Institute, Philadelphia, PA, unpublished results). 1.3H9 bound 3H9 antibody, but this reagent failed to bind other antibodies using either 3H9 H or L chain in association with unrelated chains. We presume that 1.3H9 is specific for a 3H9 H/L combinatorial idiotope.

We analyzed day 4 and adult tg mice by cytofluorography using these antiidiotopes in conjunction with an anti-IgM reagent (Fig. 4). The anti-3H9 H chain reagent, 1.209, bound 50–60% of day 4 splenic and adult bone marrow IgM^+ cells and 90% of adult splenic IgM^+ cells from H tg animals and H/L tg animals (Fig. 4 *a*). The antiidiotypic reagent 1.3H9 failed to stain cells from any population tested (Fig. 4 *b*). Most importantly, this reagent bound neither cells from the adult nor cells from the day 4 H/L tg animals.

The above data suggested that 3H9-bearing B cells are deleted in these animals. Deleting tg mouse models have shown that loss of the autoreactive tg specificity is accompanied by profound B cell depletion (3, 19, 20). The number of IgM^+ B cells from day 4 H/L tg animals was greatly reduced compared with that of L tg or normal animals. The H tg animal showed less severe depletion. In contrast, bone marrow and spleen of adult H tg and H/L tg animals had near normal numbers of B cells.

Hybridomas from 3H9 H/L tg Mice. Hybridomas derived from LPS-activated splenic B cells of H/L tg animals and their littermates were examined for expression of the 3H9 idiotope using both antiidiotypic reagents 1.209 and 1.3H9. In addition, each Ig was assayed for ANA staining of fixed Hep-2 cells to look for the homogeneous nuclear staining pattern characteristic of 3H9. By these criteria, none of the 228 hybridomas produced an Ig with the properties of 3H9 (Table 2). Three H/L tg hybrids secreted antibody that produced a 3H9-like ANA pattern; however, none of these had the 3H9 idiotope, nor did they bind DNA. These three antibodies did bind to isolated histones (M. Monestier, Center for Molecular Medicine and Immunology, Newark, NJ, unpublished results), perhaps explaining their ANA pattern. 2 of 42 hybrids from L tg animals exhibited the 3H9 idiotope, but did not bind DNA or stain nuclei of HEP-2 cells. The L tg product in these hybrids may be associated with a nonautoreactive 3H9-like H chain.

Table 1. *Endogenous L Chains Used by L tg and H/L tg Hybridomas*

Hybrid	Vκ	Jκ
L tg:		
1878.3	None	None
2748.10.1	None	None
2748.6.1	None	None
2748.5.3	None	None
2748BM11	None	None
5176.11	None	None
1878.12	Vκ23	Jκ5
2748.7.1	Vκ1	Jκ1
2748.8.3	Vκ8	Jκ1
2748.9.1	Vκ9	Jκ1
2748.19	Vκ22	Jκ1
2748.23	Vκ19	Jκ1
2748.2.1	λ1	λ1
2748.14.1	ND	Jκ4
2748.15	ND	Jκ1
2748.3.1	ND	Jκ2
2748BM6	ND	Jκ2
5176.1	ND	Jκ5
5176.2	ND	Jκ1
5176.4	ND	Jκ1
5176.5	ND	Jκ5
5176.6	ND	Jκ5
5176.7	ND	Jκ1
5176.12	ND	Jκ5
5176.13	ND	Jκ1
H/L tg:		
2754.1.1	Vκ12–13*	Jκ2
2754.3.2	Vκ12–13	Jκ1
2754.5.5	Vκ12–13	Jκ1
2754.14	Vκ12–13	Jκ1
2754.17.8.4	Vκ12–13	Jκ1
2754.19	Vκ12–13	Jκ4
2664.1	Vκ12–13*	Jκ2
2664.5	Vκ12–13	Jκ4
2664bm4	Vκ12–13	Jκ5
2664bm2	Vκ12–13*	Jκ2
2754.21.3	Vκ21	Jκ1
2754.20	Vκ1	Jκ4
2754.17.6	Vκ19	Jκ4
2664bm5	Vκ19	Jκ4
2754.15.2	Vκ38C	Jκ4
2664.6	Vκ38C	Jκ1
2754.13.2	Vκ5	Jκ5
2754.2.2	Vκ5	Jκ5
2664.4	Vκ9	Jκ1

continued

Table 1. *(continued)*

Jκ use was determined by primer extension analysis of hybridoma mRNA (see Fig. 2). Endogenous L chains were presumed to be in germline configuration when only tg mRNA could be detected. L chain mRNA sequencing was carried out using the appropriate Jκ primer as described in Materials and Methods. Family identity was determined using the Vκ gene classification given by Strohal et al. (41).

* mRNA extension gel patterns proved highly characteristic for particular Vκ products. Pattern comparisons of such extensions provided assignment of Vκ genes to unsequenced mRNAs.

Although none of the antibodies from H/L tg hybridomas expressed the 3H9 idiotope, most had the 3H9 H chain as determined by binding to the 1.209 reagent. Sequencing of H tg mRNA from numerous H/L tg hybridomas revealed no V region mutations; therefore, failure to express the 3H9 idiotope seemed likely to result from lack of surface expression of the L tg product. This might result from impairment of the L tg or from 3H9 H chain association with endogenous L chains. To assay L tg expression as well as any endogenous L chain gene expression, primer extension analysis was undertaken on 19 H/L hybrids. As summarized in Table 1, all analyzed H/L tg hybrids transcribed an endogenous κ mRNA as well as the L tg. Sequencing of the L tg transcript from several of these hybrids revealed no V region mutations. Therefore, the presence of endogenous L chains rather than impairment of the L tg appeared to prevent expression of the H/L product in these hybrids.

The fact that 3H9 was undetectable indicated that either L tg expression was greatly reduced or that endogenous L chains successfully competed for 3H9 H chain binding in these hybrids. Relative levels of endogenous and tg L chain mRNA were compared by primer extension analysis using IgM H chain mRNA as an internal standard. Fig. 2 *B* shows results for two H/L tg hybrids, and Table 3 summarizes results for 20 hybrids. 10 of these hybrids expressed approximately equal or higher relative levels of tg mRNA than endogenous κ mRNA, indicating that in these hybrids at least, lack of 3H9 expression was not due to deficient L tg expression.

Restriction in the number of different endogenous L chains used was suggested by the fact that none of the antibodies from the H/L hybrids exhibited specificity for dsDNA despite previous work, which had shown that the 3H9H chain could associate with a wide variety of L chains to create this specificity (12). Sequence analysis of endogenous κ chains from

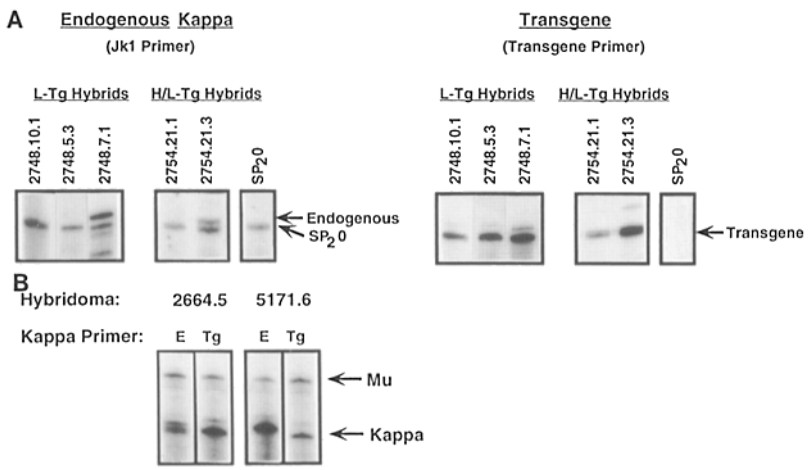

Figure 2. (*A*)3H9 L tg and endogenous κ transcription in L and H/L tg splenic hybridomas. Identical aliquots of mRNA from each hybridoma were annealed with either (*a*) a Jκ1-specific primer, or (*b*) a tg-specific primer, and converted to cDNA. Reaction products were analyzed by PAGE. Full-length products are shown here. In *a*, the Jκ1 primer crosshybridized with the Sp2/0 Jκ2-containing sterile κ transcript to create a full-length cDNA product. Endogenously derived products were revealed as additional bands. (*B*) Comparison of endogenous and tg L chain mRNA levels in H/L tg hybrids. Analysis was carried out as described in *A*, except that a Cμ-specific primer was included in each sample, and a Jκ4 primer was annealed to endogenous (*E*) mRNA from hybrid 2664.5, and a Jκ5 primer was annealed to 5171.6 E mRNA. Full-length cDNA products were analyzed for radioactivity by scanning with a two-dimensional proportional scanner (AMBIS, Inc.). Relative activity was quantitated by comparing counts from κ bands to those of the internal standard, the IgM H chain band. For each hybrid, approximately equal concentrations (less than a twofold difference) of H chain mRNA were found in samples containing either endogenous Jκ primer or Tg primer as determined by radioactivity of the full-length H chain cDNA product. H chain cDNA amounts were typically lower than κ cDNA amounts, possibly due to degradation of the longer H chain mRNA.

H/L tg hybridomas showed that, indeed, these antibodies expressed a very limited set of Vκ genes (Table 1). 10 of 19 antibodies from H/L tg hybrids used members of the Vκ12,13 group. Sequence analysis also indicated that Vκ groups 5, 19, and 38c were overrepresented.

In addition to overrepresentation of a few groups, a high degree of homology was noted between members of each group, suggesting use of one or several highly related members. Thus, Vκ12,13 sequences were 93–98% homologous (sequence ambiguities prevented more conclusive determination of homology). A high degree of homology (at least 96%) was also observed between the Vκ5 genes used by hybrids 2754.13.2 and 2754.2.2, suggesting that both originated from one member of this family. Vκ19 genes used by 2754.17.6 and 2664bm5 were ~93% homologous, again suggesting use of several highly related members. Clonal relatedness among hybrids using similar L chains was disproved by examination of endogenous H chain rearrangements (data not shown).

Generation of Endogenous L Chain Loss H/L Hybrids That Express the 3H9 Phenotype. In subcloning H/L hybrids, variants were isolated that had lost endogenous κ mRNA transcription. Fig. 1 shows Southern analysis of one subclone,

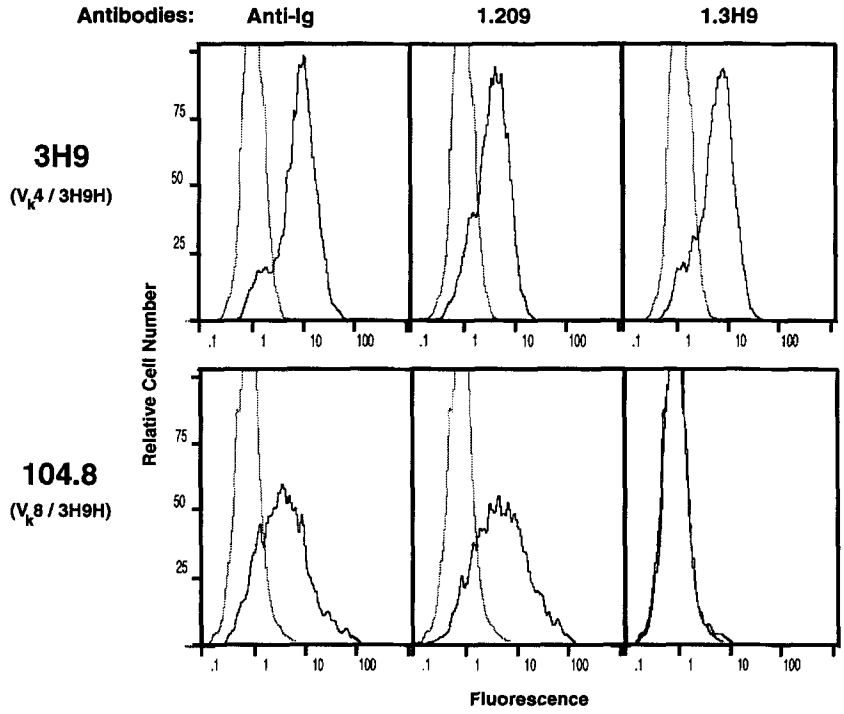

Figure 3. Binding of hybridoma cell surface 3H9 antibody and a related antibody by monoclonal anti-3H9 idiotypic reagents. Cytofluorographic analysis was carried out as described in Materials and Methods. 104.8 is a splenic hybridoma derived from a 3H9-H/Vκ8 tg animal.

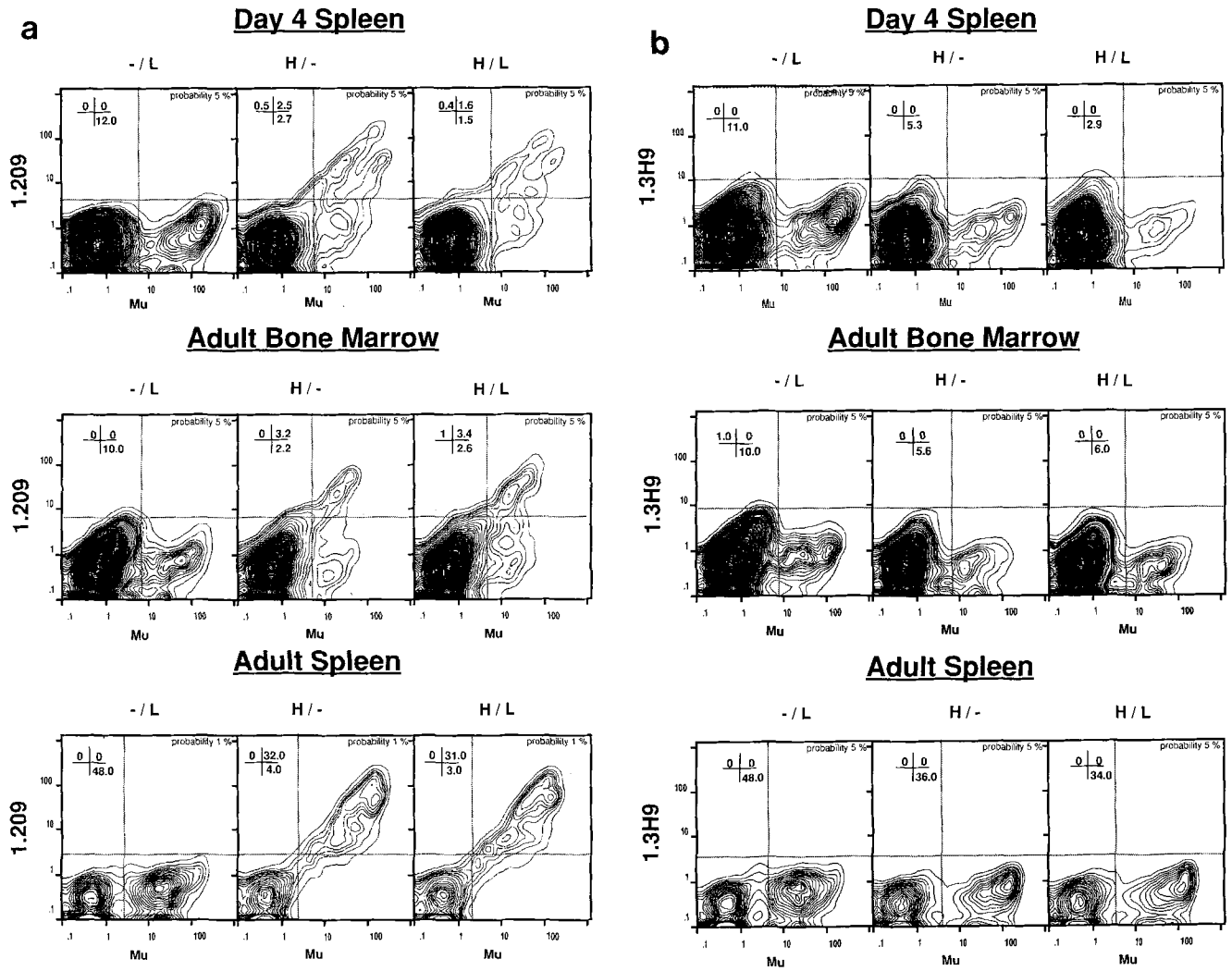

Figure 4 Cytofluorographic analysis of day 4 splenic and adult splenic and bone marrow B cells from tg animals using anti-3H9 monoclonal reagents. Spleen and bone marrow cells from animals carrying the L tg, the H tg, or both H and L tgs were costained with FITC-coupled anti-mouse IgM and (*a*) 1.209 or (*b*) 1.3H9 (revealed by Texas red-avidin binding) as described in Materials and Methods and analyzed by cytofluorography. Non-tg littermates had similar cytofluorographic patterns as L tg animals (data not shown). Results from four experiments analyzing day 4 mice and from eight experiments analyzing adult mice showed similar profiles.

Table 2. *Occurrence of the 3H9 Phenotype in Antibodies from Splenic Hybridomas*

Hybrids from:	Ig_s^+ Hybrids	Antibodies binding antiidiotypes: 1.3H9 H/L specific	Antibodies binding antiidiotypes: 1.209 H specific	Antibodies exhibiting 3H9 ANA pattern	Antibodies exhibiting 3H9 phenotype
−/− spleen*	58/58	0/58 (0)	0/58 (0)	0	0
L spleen	42/42	2/42 (5)	2/42 (5)	0	0
H spleen	56/56	0/56 (0)	56/56 (100)	0	0
H/L spleen	72/72	0/72 (0)	62/72 (86)	3	0

All splenic hybridomas secreted antibody of the IgM isotype. Antibody was assayed for binding to antiidiotypic reagents and to HEP-2 cells (ANA) as described in Materials and Methods.

* Number of animals represented from each group are: −/− spleen, two; L tg spleen, four; H tg spleen, two; H/L tg spleen, three.

Table 3. *Relative Levels of Endogenous and Tg L Chain mRNA in H/L Hybridomas*

Hybrid	Endogenous L chain mRNA*/ L Tg mRNA
2754.1.1	20.9
5171.2	14.2
5171.6	9.3
2754.15.2	7.2
2664BM5	5.7
2664BM2	3.4
2754.20	3.4
2664.1	3.4
2754.14	2.3
5171.4	1.7
2754.5.5	1.4
2754.21.3	1.3
2754.3.2	1.2
2754.19	1.1
2754.13.2	0.6
2754.17.8.4	0.6
2754.2.2	0.4
2664.5	0.4
5171.1	0.1

* Relative levels of L chain mRNA were quantitated by comparing radioactive counts from L chain cDNA bands to radioactive counts from an internal standard, IgM H chain cDNA. See Fig. 2 *B* for details. The ratio of counts of endogenous L chain or L tg-specific product to counts of IgM H chain-specific product, was taken as a relative concentration of L chain mRNA. The ratio of the relative amount of endogenous L chain mRNA to the relative amount of L tg mRNA is shown above.

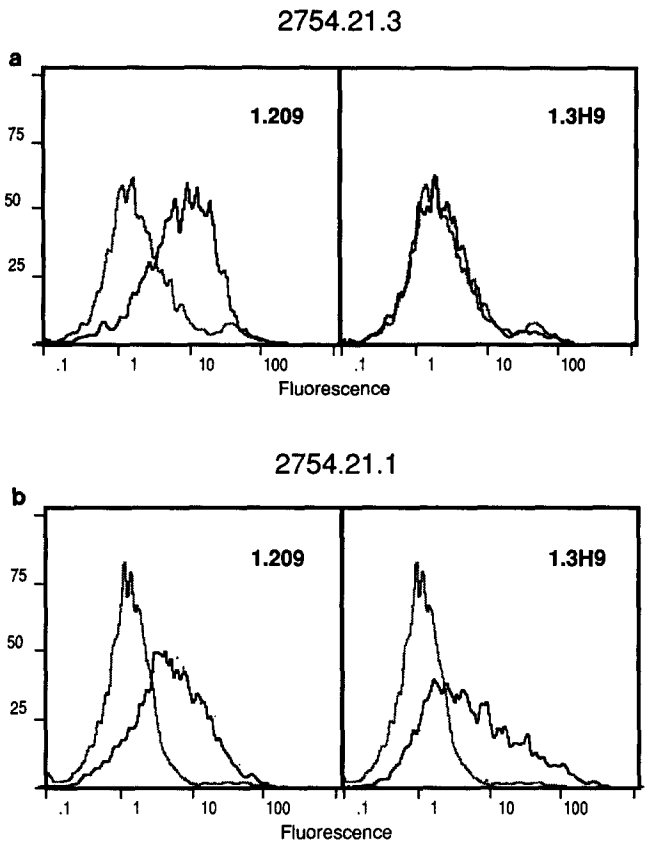

Figure 5. Cytofluorographic analysis of 2754.21 subclones using antiidiotypic reagents. Cytofluorographic procedures are as described in Materials and Methods.

2754.21.1, that had lost endogenous L chain transcription, and a sibling, 2754.21.3, that retained endogenous L chain transcription. They exhibited an identical banding pattern indicating that both arose from a single progenitor. Primer extension analysis showed that both transcribed the L tg, but that subclone 2754.21.3 transcribed an endogenous κ mRNA whereas 2754.21.1 did not (Fig. 2 *A*). Loss of endogenous κ transcription might be due to a mutation in the observed rearrangement (see Fig. 1) or by loss of a κ gene not discernible by this Southern analysis.

FACS® analysis of these hybrids showed that antibody on both bound the anti-3H9 H chain antibody 1.209 and thus expressed the 3H9 H chain (Fig. 5 *a*). However, 2754.21.1 also bound the antiidiotypic reagent 1.3H9 (Fig. 5 *b*), and produced a homogeneous nuclear pattern on fixed Hep-2 cells (data not shown). According to the above-described criteria, this cell expressed the 3H9 antibody.

Discussion

We are interested in understanding how anti-DNA antibodies, arising in diseased MRL/*lpr* mice, are negatively regulated in normal mice. To address this issue, we constructed tg mice carrying the H chain gene of an autoreactive anti-dsDNA antibody, 3H9 (15). A useful feature of this H chain is that it plays a dominant role in determining DNA specificity. This was evident from the wide range of L chains associated with the 3H9 H chain among spontaneous anti-DNA antibodies from autoimmune mice (5, 6, 21). Cotransfection studies showed that additional L chains could pair with this H chain to create an anti-dsDNA specificity (12). We reasoned that tg animals carrying the 3H9 H chain gene should, in the absence of negative regulation, express a large number of B cells with specificity for dsDNA. Examination of 3H9H tg animals showed complete absence of this predicted specificity, suggesting deletion of these B cells.

To directly study whether B cells expressing anti-dsDNA antibodies are deleted, we have produced an anti-dsDNA tg mouse by crossing the 3H9H chain tg with a tg animal carrying the L chain gene derived from the original 3H9 antibody. Indeed, we could not detect 3H9 in H/L tg animals using an antiidiotypic reagent specific for this antibody. This is not due to a defect in either the H or L chain tg since expression of these tgs can be demonstrated in the single H or L chain tg animals. Instead, we believe that the 3H9-expressing B cells are negatively regulated. Consistent with this idea are the low numbers of splenic B cells in young tg mice. B cell numbers of 4-d-old H/L mice were reduced

by as much as 75% as compared with L tg littermates. H tg mice also frequently bore fewer B cells presumably reflecting the dominant role of the 3H9 H chain in creating autoreactive specificities. Although these data suggest that anti-dsDNA antibody-bearing B cells are deleted in young animals, we cannot exclude the possibility that these lower numbers reflect abnormal development of B cells in tg animals.

To determine the site of regulation, we tested the bone marrow B cells of H/L tg mice for expression of the 3H9 idiotope. In contrast to other autoantibody tg models (3, 4, 19, 20), we could not detect 3H9 antibody. This is not due to competition for antiidiotype binding by self-antigen, as this antiidiotype is not ligand (DNA) inhibited (data not shown). Instead, this result suggests that regulation of these B cells must occur at the initial stages of surface expression.

Even though the majority of B cells in H/L neonates are deleted, significant numbers remain, and adult tg mice have near normal B cell numbers. These B cells express altered receptors that retain the H chain idiotope but not the H/L idiotope. Since the 3H9 idiotope requires both 3H9 H and L chain, this shows that the L tg is not expressed on the surface of these cells. Hybridomas from H/L animals exhibit the same phenotype as splenic B cells. Transcriptional analysis shows that all hybridomas examined transcribe the L tg as well as an endogenous κ gene. We think it unlikely that the L tg transcript is untranslated. Subclones of several hybridomas yield variants that have lost the endogenous κ transcript but have retained the tg transcript. Such variants now express the tg L chain–dependent 3H9 idiotope. We believe that the loss of expression of the 3H9 idiotope must be due to displacement of the 3H9 L chain by an endogenous L chain.

For the 3H9 receptor to change to a nonautoreactive receptor, three requirements must be met.

(*a*) *The tg-encoded Antibody Must Permit Rearrangement of Endogenous L Chain Genes.* Considerable evidence indicates that TCR-α and Ig L chain gene rearrangement can continue after functional rearrangement and even receptor expression on T and B cells. Sequential rearrangement on the same allele to replace existing functional rearrangements and rearrangement on both alleles to generate two functional products have been demonstrated (22–27). Also, expression of recombinase-associated genes (RAG-1 and RAG-2) has been observed in immature thymocytes and B cells bearing surface receptors (4, 28). In apparent contradiction to these findings, numerous groups have shown that expression of a tg antibody in Ig tg mice or expression of a tg TCR in α/β tg mice can lead to shutdown of endogenous rearrangement (29, 30). To reconcile these contrary findings, several groups have suggested that expression of selected receptors does shut down rearrangement, whereas expression of inappropriate or unselected receptors may not (4, 31). In the 3H9 tg model, we cannot distinguish between the possibilities that 3H9, as an autoreactive receptor, may actively promote rearrangement to encourage receptor editing, or that tg expression may not shut down rearrangement.

(*b*) *The Endogenous L Chain Repertoire Must Include Members That Change 3H9 Specificity.* Since the result of receptor alteration is to shift specificity from self to nonself, the L chain repertoire must include L chains that modify or change 3H9 specificity. This is bound to be a restricted set in view of the variety of L chains that sustain an anti-dsDNA specificity in combination with 3H9H (12). Indeed, sequence analysis of endogenous Vκ genes by these H/L hybrids shows considerable restriction in the genes selected (Table 1). Most strikingly, single members of the Vκ5, 12, and 19 families were repeatedly selected by B cells from two different H/L mice. In addition, comparison of Vκ genes used by H/L hybrids and those used by H hybrids shows considerable overlap (32). Highly homologous genes from Vκ1, 5, 9, 12, and 19 families are used by both H and H/L B cells, reflecting a common requirement to create nonautoreactive phenotypes with a fixed H chain (3H9H). In H/L cells, these L chains fulfill the added requirement of successfully competing with the L tg product for H chain binding. Other Vκ genes, observed in H hybrids have not been found in H/L B cells. These include members of the Vκ8, 2, 9A, and 21 families. The restricted use of these genes by H but not H/L B cells may simply result from variability in L chain use by different animals. Alternatively, it may reflect the inability of these gene products to compete with the 3H9 L chain in H/L B cells.

(*c*) *Certain Endogenous L Chains Must Be Favored over the 3H9 L Chain.* As shown above, we were unable to detect the 3H9 idiotope on either tg B cells or among secreted antibodies. We explain this by L chain replacement. Since we assume that the antiidiotope is reasonably sensitive, this result implies that the 3H9 H chain usually associates with endogenous L chain protein. This finding cannot be explained by an overabundance of endogenous L chain mRNA. As explained in Table 3, the ratio of endogenous L chain mRNA to tg mRNA varies, and sometimes tg mRNA levels far exceed those of endogenous L chain levels. Although the reason for this variation is not understood, the lack of tg L chains on H/L hybrids and presumably B cells is not due to a shortage of mRNA. One explanation for the preferential use of endogenous L chains is that they outcompete tg L chains for association with the 3H9 H chain. Preferences in H/L pairing have been seen in competition studies carried out either in the test tube or through hybridoma-hybridoma fusions (33–39). Often, the homologous (original) H/L combination prevails, but numerous examples have been reported in which an H chain prefers a heterologous (foreign) L chain (33–39). The latter case could explain our findings. It is, however, surprising that heterologous L chains are so strongly preferred by the 3H9 H chain in H/L hybrids, as evidenced by the fact that we do not observe even low concentrations of 3H9 on these cells. Potentially, the deletion process may be sensitive enough to get rid of such low expressors. Additionally, the 3H9 H/L association may not be particularly strong. The examples in the literature that have shown preferential homologous reassociation have undergone affinity maturation and may also have evolved better H/L association. Since our example is an autoantibody, the selection forces may be quite different. It may be of interest to carry out H/L reconstitution experiments with antibodies that have not been under positive selection.

Tiegs et al. (4) have reported similar findings in "centrally

deleting" anti-H-2^k antibody tg-bearing, H-2^k-positive mice. Residual B cells found in the periphery of these animals are idiotype negative. In agreement with our results, these cells express the H tg product in association with some endogenous L chain (frequently λ) that apparently competes with the L tg for H chain association. However, the anti-H-2^k tg system promotes more severe, long-term depletion of peripheral B cells, whereas the 3H9 tg system appears to provide a better environment for endogenous L chain gene rearrangement and subsequent escape from the deleting process. This difference most likely reflects an increased efficiency by the anti-H-2^k tgs to shut down endogenous rearrangement. Lack of the downstream enhancer in the 3H9 L tg construct may decrease its ability to exclude endogenous rearrangement (40). Conversely, in the anti-H-2^k tg system, cointegration of H and L tgs into one location might increase L tg expression through use of H gene enhancer and/or facilitate coordinated expression of H and L tgs. Either of these events might provide this system with a better means of suppressing endogenous gene expression.

The concept of receptor editing implies that recombinase activity is reactivated in immature autoreactive B lymphocytes. Given recombinase reactivation, an edited cell must often pass through a stage of dual L chain expression (an exception to this is editing by V gene replacement, yet even here the cell can also rearrange on the "other" κ allele or at the λ locus). Dual L chain expression may commonly lead to death. Perhaps the autoreactive receptor is not sufficiently diluted to escape deletion, or the density of any given receptor is too low to elicit positive selection. For those cases where the H chain favors one L chain and this H/L pair is not autoreactive, preferential pairing may be an important editing mechanism. On the other hand, V gene replacement that lacks the potential problems of dual L chain expression may also be observed in mature cells. In either case, the data presented in this paper suggest that receptor alteration through continued L chain gene rearrangement may be a valuable means of salvaging autoreactive B cells.

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