The Rearranged V<sub>H</sub> Domain of a Physiologically Selected Anti-Single-Stranded DNA Antibody as a Precursor for Formation of IgM and IgG Antibodies to Diverse Antigens

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The Rearranged V_H Domain of a Physiologically Selected Anti-Single-Stranded DNA Antibody as a Precursor for Formation of IgM and IgG Antibodies to Diverse Antigens

Jing Li,* Luis Fernandez,† Kevin C. O’Connor,‡* Thereza Imanishi-Kari,† and B. David Stollar*‡*

It has been proposed that autoreactivity of modest affinity contributes to positive selection of a preimmunization B cell repertoire, whereas high-affinity autoreactivity leads to negative selection. This hypothesis predicts that a B cell producing a physiologically selected unmutated ssDNA-binding Ab should be a precursor of cells that respond to diverse exogenous Ags. To test this prediction, we prepared transgenic mice bearing the rearranged V_H domain of an IgM Ab from a nonautoimmune mouse immunized with a DNA-protein complex, poly(dC)-methylated BSA. The Ab, dC1, binds both poly(dC) and ssDNA. It is encoded by V_H and V_L gene segments with no mutations, suggesting that the producing cell may have been selected before and activated during immunization. The dC1V_H transgene was targeted to the IgH locus. In heterozygous mice, on a nonautoimmune C57BL/6 background, the transgene allotype was expressed on B cell surfaces and in serum Ig, but about one-third of B cells expressed the endogenous allele instead. Total serum Ig concentrations were normal and included both transgene- and endogenous gene-coded IgM and IgG. The transgene V_H D_LJ_H was expressed in splenic IgM cDNA with few or no mutations, and in IgG cDNA with multiple mutations. The transgene allotype was also expressed in Abs formed on immunization with thyroglobulin, pneumococcal polysaccharide, and ssDNA-methylated BSA. Consistent with the hypothesis, cells with a rearranged autoreactive V_H domain selected for reactivity with a form of ssDNA did serve as precursors for cells producing IgM and IgG Abs to diverse Ags. The Journal of Immunology, 2001, 167: 3746–3755.

The balance between potential for autoreactivity and the ability to recognize foreign or dangerous substances is a fundamental feature of the immune system. Processes that occur during B cell development shape that balance and give rise to a very nonrandom repertoire of new B cells even before exposure to foreign Ag. The best defined of the early processes are those that test for effective association of a μ-chain with pseudo L chain (1, 2) and those that abrogate potentially damaging autoreactivity by cell deletion, anergy, or receptor editing (3–8).

Positive as well as negative selection shapes the repertoires of both B cells (9–12) and T cells (13). The effect of B cell selection can be seen, for example, in the different frequencies of usage of particular V_H genes in productive as compared with nonproductive rearrangements (14). It has been proposed that autoreactivity plays a role in positive as well as negative selection (15). Indeed, B cells expressing transgenic anti-Thy-1 Ab actually express deletion of the Thy-1 autoantigen for normal development and maturation (16).

Several lines of investigation underlie a hypothesis that ssDNA and/or substances that it mimics are among ligands that play a role in positive selection of a preimmunization B cell repertoire. A significant fraction of hybridomas made from LPS-stimulated lymphocytes of normal newborn mice (17) or nonimmunized adult mice (18, 19) makes polyreactive IgM autoantibodies that bind ssDNA and have unmutated V domains (20). Anti-DNA Abs are also produced by normal B lymphocytes activated by EBV in tissue culture (21). Immune responses to immunogenic exogenous nucleic acids (i.e., those for which tolerance has not developed) also include a substantial component of IgM Abs (22, 23). Even some selective IgG Abs to Z-DNA (Ab Z22) (24) or poly(dC) (25) can be formed with few or no complementarity-determining region (CDR) mutations. These findings probably reflect the nucleic acid-binding potential of numerous B cells that were selected even before immunization with exogenous Ag. For many anti-DNA Abs, the V_H domain alone can also bind ssDNA and polypyrimidines, with selectivity that is similar to that of the natural autoantibodies from LPS-stimulated cells (26, 27). Furthermore, a significant fraction of V_H domains encoded by unmutated cDNA in libraries prepared from normal B cells of healthy adults (28) or newborns (Y. Chen and B. D. Stollar, unpublished data) can bind ssDNA and polypyrimidines. This DNA reactivity of the V_H domain may be a factor in positive selection of developing B cells in bone marrow.

The hypothesis that anti-ssDNA autoreactivity of modest affinity can play a physiological role in positive selection predicts that cells producing natural ssDNA-binding autoantibodies before immunization can respond to diverse exogenous Ags and that.

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Abbreviations used in this paper: CDR, complementarity-determining region; ES, embryonic stem; MBSA, methylated BSA; PI, propidium iodide; scFv, single-chain variable fragment; wt, wild type.
through Ig locus mutations, their Ab products lose autoreactivity and gain affinity for the new immunogen (15). Such a progression was observed in use of the unmutated 36–65 VH gene segment in IgM ssDNA-binding natural autoantibodies before immunization and in IgM and IgG Abs to the Ars hapten after immunization (20). In that case, however, it was not possible to follow the fate of a particular rearranged VH DNA rearrangement to be followed, we have prepared mice bearing a targeted transgene for the rearranged H chain of a physiologically selected ssDNA-binding Ab. The mAb, named dC1, was obtained by PCR primers were designed according to the germline sequence of VH10 and amplified with T4 DNA ligase (Life Technologies, Bethesda, MD). The upstream primer, containing a SalI site, was 5’-dGAGGTGTCATATCGTTAGAATACGAA, a sequence ~400 bp upstream of the TATA box. The downstream primer, containing a ClaI site, was 5’-dCCATCGAATGGCATGTTAAGATCGAATCGA, a sequence ~100 bp downstream of the end of the JH4 segment. The genomic DNA was isolated from 10^7 dC1 hybridoma cells with the QIAamp Blood kit (Qiagen) and used in PCR and Southern blot analysis to identify heterozygous knockin mice. The targeted ES clones were microinjected into blastocysts of C57BL/6 or C57BL/6J females, and chimeras were obtained in the unmutated JH domain of dC1, like natural autoantibody, binds ssDNA, poly(dT), and poly(dC). We have tested the prediction that the targeted rearranged dC1VH domain can participate in diverse IgM and IgG Abs.

Materials and Methods

Construction of dC1VH gene-targeting vector

The dC1 H chain rearranged DNA, with adjacent upstream and downstream regions, was cloned from genomic DNA of the dC1 hybridoma. PCR primers were designed according to the germline sequence of VH10 family member B4, to which the dC1VH segment is identical; genomic sequence data upstream of the VH10 B4 segment were provided by P. Brodeur (Tufts University, Boston, MA). The upstream primer, containing a SalI site, was 5’-dGAGGTGTCATATCGTTAGAATACGAA, a sequence ~400 bp upstream of the TATA box. The downstream primer, containing a ClaI site, was 5’-dCCATCGAATGGCATGTTAAGATCGAATCGA, a sequence ~100 bp downstream of the end of the JH4 segment. The genomic DNA was isolated from 10^7 dC1 hybridoma cells with the QIAamp Blood kit (Qiagen, Valencia, CA) and amplified by PCR. The 1-kb PCR product was purified and ligated with T4 DNA ligase (Life Technologies, Bethesda, MD) to the targeting vector pHIVL2neo, provided by K. Rajewsky (Harvard Medical School, Boston, MA) (29). The ligated vector was transformed into XL1-blue Escherichia coli cells. Plasmids were isolated, and the presence of a correctly constructed insert was confirmed by restriction digestion with SalI and ClaI. Automated sequencing was performed using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, PerkinElmer, San Francisco, CA). Gel electrophoresis and sequence determination were performed by the Protein Chemistry Facility at Tufts University School of Medicine.

Transfection and selection of embryonic stem (ES) cells

The homologous recombination construct was linearized with NotI (New England Biolabs, Beverly, MA) and purified with the QIAEXII gel purification kit (Qiagen) before electroporation. A total of 10^7 J1 ES cells of 129 mouse origin, prepared in the laboratory of R. Jaenisch (Whitehead Institute, Cambridge, MA) and obtained from S. Tonegawa (Massachusetts Institute of Technology) was transfected with 20–50 µg linearized vector, with the Gene Pulser II (Bio-Rad, Richmond, CA). The cells were allowed to recover for 24 h after transfection and then plated in selective medium containing 150 µg/ml G418 (Sigma, St. Louis, MO) and 2 µM ganciclovir (Merck, Whitehouse Station, NJ) on a feeder layer of mitotically inactivated embryonic fibroblast cells (30). The embryonic fibroblast cells, which are G418 resistant, were extracted from the embryos of a pregnant CD43 knockout female mouse maintained from B. Ardman (Tufts University).

Clones were picked and each divided into two parts: one was maintained in undifferentiated state on feeder layer, and the other was grown without feeder cells. The latter cells were used for PCR screening and Southern blot.

PCR screening and Southern blot

Cells for screening were centrifuged at 300 x g for 5 min and resuspended in 10 µl lysate buffer (1x PCR buffer (Life Technologies), 0.45% Nonidet P-40, 0.45% Tween 20, and 60 µg/ml proteinase K (Sigma)) at 55°C for at least 2 h, and the lysates were used directly as PCR templates. Identification of a potentially targeted clone was performed by diagnostic PCR using an upstream primer within the CDR3 of the dC1VH gene 5’-dGAGATC CGATGCTATGGACTACTGGGG and a downstream primer outside the targeting vector, flanking the EcoRI site of the H chain J intronic enhancer (5’-TTTAAATCATTTTGAAGTCTGAGCAT-3’). As an internal control to ensure that the PCR conditions would amplify a segment present in all cells (Fig. 1), a portion of the lysate was amplified with a JH4 upstream primer (5’-dAGATTTCTTGTACCCCATGTC CATAG) and the same downstream primer used in the diagnostic PCR.

Once candidate clones were identified by PCR diagnostic for homologous recombination, targeted gene construction was verified by Southern blotting. The genomic DNA, purified from the cell pellet, was digested with EcoRI (Boehringer Mannheim, Mannheim, Germany), electroelosed in 1% agarose gel, blotted to Nytran membrane, and hybridized to 30 ng of a 1.2-kb HindIII-EcoRI JH4 fragment probe (Fig. 1) that was 32P-labeled by random priming using a Random Primers DNA Labeling System (Life Technologies). The resulting autoradiographs were developed for 2–7 days.

Generation of knockin mice and deletion of the neo’ gene

The targeted ES clones were microinjected into blastocysts of C57BL/6 or BALB/c mice. Injected blastocysts were implanted in SWR pseudopregnant females, and chimeras were obtained in <3 wk. The male chimeras were bred with C57BL/6 females to generate heterozygous knockin mice. The tail genomic DNA of pups was extracted with a DNeasy tissue kit (Qiagen) before electroporation. A total of 10^7 J1 ES cells of C57BL/6 or C57BL/6J mice were bred with C57BL/6 females to generate heterozygous knockin mice. The targeted ES clones were microinjected into blastocysts of C57BL/6 or C57BL/6J mice. The targeted ES clones were microinjected into blastocysts of C57BL/6 or C57BL/6J mice. The targeted ES clones were microinjected into blastocysts of C57BL/6 or C57BL/6J mice. The targeted ES clones were microinjected into blastocysts of C57BL/6 or C57BL/6J mice. The targeted ES clones were microinjected into blastocysts of C57BL/6 or C57BL/6J mice.
Selsing (Tufts University). Pups were screened for the Cré transgene by PCR with Cré-specific primers (5’-dCTGATGACCTTGTTCCAGGATGC and 5’-dGCTAATGCTTCTCCTACACTCCG). Heterozygous male knockin mice (dC1VH-/+) were bred with Cre-positive C57BL/6 female mice to delete the neo gene. PCR and Southern blot analyses were used to identify progeny in whom the neo gene was removed by Cre-loxP-mediated recombination. Further breeding of Cré-negative knockin males with normal female C57BL/6 mice yielded succeeding generations of heterozygotes on the C57BL/6 background. Mice of the third or fourth generation were used for immunization.

Flow cytometry analysis

The spleens of heterozygous knockin and control littermates were crushed with a syringe bar and filtered with a cell strainer (BD Labware, Franklin Lakes, NJ). Bone marrow cells were washed out of a femur with DMEM and filtered through the cell strainer. Cells were washed with PBS buffer by centrifugation for 5 min at 300 × g and resuspended in staining buffer (PBS buffer, pHe 7.2; 1% normal rabbit serum and 0.2% NaN3). A total of 100 cells was stained with 50 μl containing 1 μg/ml monoclonal PE-conjugated anti-mouse IgM and 1 μg/ml FITC-conjugated anti-mouse IgM Abs (BD Pharmingen, San Diego, CA), or combinations of FITC anti-IgM (Southern Biotechnology Associates, Birmingham, AL), PE-conjugated anti-CD43 (BD PharMingen), and biotin-labeled anti-B220 (BD PharMingen) followed byallophycocyanin-conjugated streptavidin (Molecular Probes, Eugene, OR). After 15 min, the cells were washed with staining buffer, centrifuged for 5 min at 300 × g, and resuspended in 0.5 ml of staining buffer. The cells were analyzed by FACScan (BD Biosciences, San Jose, CA) using the Lysis II program. Ten thousand events were counted.

Immunization

Heterozygous knockin mice and wild-type (wt) littermates (2 mo old) were immunized i.p. with 25 μg of the mixtures were added to wells coated with 2 μg/ml ssDNA. Wells of UV-irradiated polystyrene Immulon 1 plates were coated with 2 μg/ml nucleic acid. OD 405 nm was used to detect bound Ig. The biotin-conjugated reagents were further competed at similar concentrations, reflecting similar affinities; however, poly(dU) and poly(dT) were not effective inhibitors (Fig. 2b). The VH and VL domains of dC1 were cloned from hybridoma cDNA and inserted into a plasmid vector for expression of a single-chain variable fragment (scFv) domain fused to a single B domain of staphylococcal protein A (33). The monoclonal scFv bound to (dC)2αβ in a filter-binding assay (Fig. 3a); by Scatchard analysis, the estimated Kd was 5 × 107 M−1 (L/mol of (dC)2αβ). The VH domain alone bound only weakly in the filter-binding assay (data not shown) but, at submicromolar concentrations, was able to bind to immobilized poly(dC), ssDNA, or poly(dT) (Fig. 3b). The VL domain alone did not bind to these nucleic acid.

ELISA

ELISA was used for measurement of serum IgG and IgM and specific Ab titers on wells coated with anti-IgM, anti-IgG, or Ag. Wells of microtiter plates (Immulon II; Dynatech, Alexandria, VA) were incubated at room temperature for 1 h with 100 μl/well goat anti-mouse IgM Ab (2 μg/ml; Boehringer Mannheim), goat anti-mouse IgG (γ-specific) Ab (2 μg/ml; Boehringer Mannheim), human thyroglobulin (2 μg/ml), or Pneumovax (2.3 μg/ml; NEMC Pharmacy, Boston, MA). For coating the wells, these reagents were diluted in 125 mM borate buffer (pH 9) with 50 mM NaCl and 0.1% Tween 20. For the anti-ssDNA assay, wells of UV-treated (32) Immulon I microtiter plates (Dynatech) were incubated at room temperature for 1 h with 100 μl/well heat-denatured salmon DNA (Sigma) at a concentration of 2 μg/ml in PBS. A total of 100 μl of biotin-conjugated allotype-specific anti-mouse IgMAb, IgMβ, IgG1A, or IgG1B (1 μg/ml) or 100 μl of biotin-conjugated anti-mouse Ig (1 μg/ml; all from BD Pharmingen) was used to detect bound Ig. The biotin-conjugated reagents were further reacted with 100 μl of streptavidin-conjugated alkaline phosphatase (0.2 U/ml; Southern Biotechnology Associates), followed by p-nitrophenyl phosphate as developing substrate. OD405 or OD50 was read after 1 h of color development with a Molecular Dynamics (Sunnyvale, CA) Microplate Spectrophotometer System, with SOFTmaxPro software or a Dynatech MR600 ELISA reader.

cDNA library construction and sequence analysis

Total RNA was prepared from 1 × 107 spleen cells from a heterozygous knockin mouse with the RNAeasy kit (QIAGEN). Reverse transcription was performed with a 1 μl primer (5’-GGA AAT GGT GCT GGG CAG) or Cy5 primer (5’-GGA CAG TGG ATA GAC), complementary to a sequence near the 5’ end of the mouse Cμ1 or Cly C region. PCR was used to amplify the knockin gene segments in the cDNA, with nested primers Cμ2 (5’-GGA CAC GAG GCC GGC ATG TGG GAA GGA) or Cy2 (5’-GAT CTA GAT GGG GGG CTT GTG TTG GCT G) complementary to the 5’ end of the mouse Cμ1 or Cly C region, but upstream of primer Cμ1 or Cly1, and the dC1VH leader sequence (5’-ATG GTG TGG GGG CTG TGA AGG TGG TTG TTC TTT G). The PCR products were directly ligated into TA cloning vector PCR2.1 (Invitrogen, Carlsbad, CA). Automated sequencing was performed with the Big Dye Terminator cycle sequencing kit (Applied Biosystems-PerkinElmer), according to the manufacturer’s instructions. Gel electrophoresis and sequence determination were performed by the Protein Chemistry Facility at Tufts University School of Medicine.

Results

Properties of mAb dC1

mAb dC1 is an IgM product of a hybridoma from a mouse immunized with poly(dC)-MBSA complexes. Assayed in ELISA, it bound to immobilized poly(dC) and ssDNA (Fig. 2a). In competitive ELISA, with ssDNA-coated wells, both poly(dC) and ssDNA competed at similar concentrations, reflecting similar affinities; however, poly(dU) and poly(dT) were not effective inhibitors (Fig. 2b). The VH and VL domains of dC1 were cloned from hybridoma cDNA and inserted into a plasmid vector for expression of a single-chain variable fragment (scFv) domain fused to a single B domain of staphylococcal protein A (33). The monoclonal scFv bound to (dC)2αβ in a filter-binding assay (Fig. 3a); by Scatchard analysis, the estimated Kd was 5 × 107 M−1 (L/mol of (dC)2αβ). The VH domain alone bound only weakly in the filter-binding assay (data not shown) but, at submicromolar concentrations, was able to bind to immobilized poly(dC), ssDNA, or poly(dT) (Fig. 3b). The VL domain alone did not bind to these nucleic acid.

FIGURE 2. Nucleic acid binding by dC1 IgM from tissue culture fluid. a. Binding of varying concentrations of IgM to immobilized poly(dC) (●), ssDNA (○), or wells with no nucleic acid (Ag (□)). Wells of UV-irradiated polystyrene Immulon 1 plates were coated with 2 μg/ml nucleic acid. OD405 (A 405 nm) readings were recorded after 1-h incubation with substrate. b. Competitive ELISA for binding to soluble nucleic acids. The mAb dC1 was preincubated for 30 min in PBS with no inhibitor (○) or with varying concentrations of poly(dC) (●), ssDNA (○), poly(dT) (△), or poly(dU) (Δ), and the mixtures were added to wells coated with 2 μg/ml ssDNA.
Sequencing of the cDNA (GenBank accession number AF045483) revealed that the V_H domain consisted of V_H10-B4 and J_H4 segments and the V_L domain of V_L/H92601A5 and J_L/H92601 segments, all with no mutations from germline sequences; the short D_H segment was not assigned to a germline sequence. The IgM isotype, lack of V_H or V_L mutations, and binding of ssDNA are characteristic of natural autoantibodies present before immunization with exogenous Ag. The preferential reactivity with poly(dC) appears to reflect activation of a preexisting B cell receptor by the immunogen.

Generation of dC1V_H knockin mice

The targeting vector pIVhL2neo-dC1V_H was constructed by insertion of the rearranged genomic V_H DNA, along with upstream and downstream sequences, into the SalI/ClaI site of pIVhL2neo vector (Fig. 1). The dC1V_H cassettes, including promoter and rearranged VDJ genes, were verified by sequencing. NotI-linearized targeting constructs were transfected by electroporation into J1 ES cells. Transfected cells were selected with G418 (150 μg/ml) and ganciclovir (2 μM). The 368 doubly resistant clones carrying the vector were screened with PCR using a dC1V_H CDR3 5′ primer (a sequence specific to the insert in the homologous recombination construct) and a 3′ primer located in 3′ end of the IgH J-C intronic enhancer (a sequence inherent in the endogenous Ig H chain locus) (Fig. 1). Three of the 368 clones yielded the predicted 1.3-kb PCR fragment. These candidate targeted clones were further analyzed by Southern blotting with a Hinfl-EcoRI J_H4 probe that hybridizes to a sequence intrinsic to the homologous recombination construct. Targeted insertion in these three clones was confirmed by the presence of a 2.3-kb band, predicted for the inserted dC1V_H targeted allele, in addition to the 6.5-kb band representing the wt allele.

ES clones bearing the rearranged dC1V_H gene were microinjected into blastocysts of C57BL/6 and BALB/c mice and implanted in SWR pseudopregnant females. We obtained 27 male chimeric mice, of which 10 were able to generate knockin F1 mice when mated with C57BL/6 females. PCR analysis (Fig. 4a) and Southern blotting (Fig. 4b) of DNA from the tail identified heterozygous knockin (dC1V_H*, a/b) and nontransgenic control (+/+, b/b) mice. To delete the neo gene, we bred heterozygous knockin mice (dC1V_H+, a/b) with Cre-positive C57BL/6 mice. PCR and Southern blot identified progeny that had successfully removed the neo gene by Cre/loxP-mediated recombination (data not shown).

Transgene expression by splenic and bone marrow cells in heterozygous dC1V_H knockin mice

Splenic and bone marrow B cells of heterozygous mice were analyzed with flow cytometry, with use of the antiallotypic reagents to distinguish between the targeted transgenic allele (IgM*) and the endogenous allele (IgM°). In both locations, the majority of IgM-
positive splenic cells (67%) in the heterozygous knockin mice expressed only the transgenic IgM<sup>a</sup> allotype (Fig. 5, c and d). However, a significant fraction (up to 31%) expressed the endogenous IgM<sup>b</sup> allele exclusively (Fig. 5, c and d); this result may reflect attempted H chain gene revision (34, 35) and transgene inactivation in those cells. Allelic exclusion at the individual B cell level was maintained in the transgenic mice, as few splenic or bone marrow B cells from knockin mice (~1%) scored as doubly stained. In specificity control experiments, B cells of BALB/c mice expressed only IgM<sup>b</sup> allotype (Fig. 5a), and C57BL/6 expressed only IgM<sup>b</sup> (Fig. 5b).

**Transgene expression in serum Ig of heterozygous dC1V<sub>H</sub> targeted mice**

As measured with a capture ELISA, heterozygous knockin mice expressed the same amount of total serum IgM and IgG as nontransgenic C57BL/6 control mice (Fig. 6, a and b). Consistent with that result, there was no difference in total spleen B cell numbers between knockin mice, with 1.9 ± 0.5 × 10<sup>7</sup> cells/spleen for four animals, and two C57BL/6 mice with 1.65 ± 0.4 × 10<sup>7</sup> cells/spleen. Washes of bone marrow from thigh bones yielded 2.65 ± 0.8 × 10<sup>7</sup> cells from two knockin mice and 2.95 ± 0.4 × 10<sup>7</sup> from two nontransgenic mice. Fractional distributions of B cell developmental subsets in bone marrow from transgenic and nontransgenic animals were also identical (Table I). Thus, no obvious developmental block or large scale deletion occurred in the transgenic animals. Again consistent with the FACS data, serum Ig<sub>a</sub> in the transgenic mice consisted of both transgene-coded IgM<sup>a</sup> (Fig. 6c) and endogenous gene-coded IgM<sup>b</sup> (Fig. 6d), and the serum IgM<sup>a</sup> concentration was lower than that of BALB/c control mice (Fig. 6c). The expression of cell surface and serum IgM<sup>a</sup> in heterozygous knockin mice indicated that the targeted allele, whether revised or not, was functional.

**cDNA library construction and sequence analysis**

As an additional test for expression of the transgene, and to determine whether the transgene can be diversified by mutation, we prepared cDNA libraries with RNA extracted from spleen cells of heterogeneous mice. The dC1V<sub>H</sub> leader sequence was used as the upstream primer for subsequent PCR, so that mRNAs coded by the transgene, or related VH10 family members, were captured. The Cμ2 or Cγ2 downstream primers were complementary to the 5′ end of the mouse Cμ or Cγ regions, but upstream of the Cμ1 or Cγ1 sequences used for initial cDNA synthesis. The dC1V<sub>H</sub> gene was expressed in both μ and γ cDNAs. Sequences for 16 of 18 clones analyzed from the μ library, including the three CDRs, were identical or nearly identical with that of the dC1V<sub>H</sub> gene, with only four mutations in all of the 16 sequences (Fig. 7). The other two μ clones, which appear to have arisen from the endogenous allele, had distinctly different CDR3 segments, and one used a J<sub>μ</sub> segment different from that of dC1. Eight of 17 γ libraries that we analyzed had the V<sub>H</sub>, D<sub>H</sub>, and J<sub>H</sub> segments used for initial cDNA synthesis. The dC1V<sub>H</sub> gene did undergo class switching and mutation, serving as a direct precursor for diversified V<sub>H</sub> domains. Nine other γ clones, apparently derived from an endogenous V<sub>γ</sub>10 family member, had distinctly different CDR3 and, in some cases, J<sub>H</sub> sequences (Fig. 8b).

**FIGURE 5.** Representative flow cytometric analysis of B cells from transgenic mice and control mice. a, BALB/c splenocytes; b, C57BL/6 splenocytes; c, heterozygous knockin mouse splenocytes; d, knockin mouse bone marrow cells. Spleen or bone marrow B cells were stained with monoclonal PE-conjugated anti-mouse IgM<sup>a</sup> and FITC-conjugated anti-mouse IgM<sup>b</sup> Abs. Ten thousand lymphocytes, as defined by forward/ sideward scatter, were recorded; data are representative of three knockin, three C57BL/6, and three BALB/c mice.

**FIGURE 6.** Analysis of IgM, IgG, and their allotypes in serum of heterozygous knockin (●) and nontransgenic C57BL/6 (○) and BALB/c (▲) mice. Ab concentration was determined with a capture ELISA. Wells of microtiter plates were coated with goat anti-mouse IgG (γ-chain specific) and IgM (μ-chain specific), respectively. For detection of total serum IgM and IgG (a and b), biotin-labeled goat anti-mouse Ig was added, followed by alkaline phosphatase-conjugated streptavidin. For detection of serum IgM<sup>a</sup> and IgM<sup>b</sup> (c and d), biotin-labeled goat anti-mouse IgM<sup>a</sup> and IgM<sup>b</sup> were used, respectively, followed by alkaline phosphatase-conjugated streptavidin. The color was developed with p-nitrophenyl phosphate as substrate. OD<sub>410</sub> (A410) was read after 1 h of color development. The curve and SD represent data from six mice of each group.
Active immunization of targeted mice

To test whether the transgenic B cells can be activated by exogenously administered Abs, nontransgenic control mice and heterozygous knockin mice were immunized with thyroglobulin, pneumococcal polysaccharide vaccine (Pneumovax), and ssDNA. Preimmunization sera of both knockin and nontransgenic control mice were negative at a 1/100 serum dilution in ELISA for antithyroglobulin or anti-pneumovax Abs, developed with an anti-mouse Ig reagent that could detect IgM, IgG, or IgA Abs. Low reactivities, with readings between 0.15 and 0.3 at a 1/100 serum dilution, were obtained with both knockin and control mice tested on immobilized ssDNA and developed with anti-total Ig reagents.

**Table I. Developmental subsets of B cell precursors and B cells in bone marrows of transgenic and non-transgenic mice a**

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<th>B Cell Population</th>
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<th>Pre-B</th>
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<td>% of B220&lt;sup&gt;-&lt;/sup&gt;CD43&lt;sup&gt;-&lt;/sup&gt; cells from BM</td>
<td>% of B220&lt;sup&gt;-&lt;/sup&gt;CD43&lt;sup&gt;-&lt;/sup&gt; cells from BM</td>
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* Bone marrow (BM) cells were flushed from femurs of mice, washed, and stained with PE-anti-CD43, FITC-anti-IgM, or biotin-anti-B220 followed by allophycocyanin-avidin, as described by Hardy et al. (48).

**FIGURE 7.** \( V_\delta 4 \) domain sequences of IgM-derived clones from the cDNA library of splenocyte B cells of heterozygous mice. A total of 18 clones yielded 6 distinct sequences.
FIGURE 8. V_{H} domain sequences of dC1-derived IgG clones (a) and non-dC1 IgG-derived clones (b) from cDNA library of splenocyte B cells of heterozygous mice. Derivation from the dC1 transgene was defined by retention of its CDR3 combination.
Titers were not raised by injection of RIBI adjuvant alone, but were raised by injection of Ag plus adjuvant (data not shown).

Immunization of heterozygous knockin mice with a protein Ag, thyroglobulin, resulted in immune responses of both IgM and IgG isotypes, each comprised of both transgenic a allotype (Fig. 9, a and c) and endogenous b allotype (Fig. 9, b and d). Cells expressing the targeted allele, therefore, were able to respond to Ag and undergo class switching to produce IgG1a. Other B cells in the knockin mice used the endogenous allele to produce IgMb and IgG1b Abs to thyroglobulin. Titers measured with anti-b allotype reagent were higher than those measured with anti-a allotype Ab, perhaps reflecting a larger choice of gene usage among endogenous Ig gene segments. In sera of nontransgenic control mice, as expected, only b allotype Abs were detected.

The response to Pneumovax immunization was typical for responses to a thymus-independent Ag, yielding IgM, but no IgG Abs (Fig. 10). As in the response to thyroglobulin, the presence of specific Abs of both IgM a and IgMb allotype in the knockin mice (Fig. 10, a and b) indicated that both the targeted and endogenous alleles were used in anti-polysaccharide Abs. The polysaccharide-specific IgMa level, however, was low compared with that of the endogenous IgMb Ab. The IgM a titer was similar in both knockin and nontransgenic mice (Fig. 10b).

Immunization with ssDNA yielded sera with modest titers (Fig. 11), again including both IgMa and IgMb Abs. The transgene-coded IgM a response to ssDNA (Fig. 11a) was stronger than the corresponding anti-polysaccharide response (Fig. 10a), but the endogenous IgM a titer (Fig. 10b) in the knockin mouse was still higher than the IgM a titer. In the anti-ssDNA response, the endogenous gene, but not the transgene, gave rise to IgG Abs (Fig. 11, c and d). Soluble nucleic acids were compared as competitors for ssDNA binding by the IgM a Ab in a 1/200 dilution of pooled serum from immunized transgenic mice. A concentration of 0.1 µg/ml soluble ssDNA caused 50% inhibition, whereas a much higher 5 µg/ml concentration of poly(dC) or poly(dT) caused only 25% inhibition. Thus, although the dC1V a was originally part of an anti-poly(dC) Ab, immunization with ssDNA yielded transgene-coded IgMa that reacted selectively with ssDNA in preference to poly(dC), perhaps as a result of association with a different V L domain.

Discussion
These experiments reveal that B cells expressing the rearranged V H domain of a physiologically selected autoreactive Ab, with ssDNA-binding activity, can be precursors of cells that participate
in responses to diverse exogenous Ags. The rearranged gene can undergo both class switching and mutation. This result is consistent with a role for autoreactivity in positive physiological selection of a preimmunization repertoire of B cells.

Expression of the targeted transgene allele in heterozygous mice was demonstrated both serologically, by analysis of spleen and bone marrow cells and serum Ig with anti-allotype reagents, and by analysis of cDNA libraries. In a library prepared with a γ-specific cDNA primer, clones with the D1 CDR3 had numerous mutations, as expected for cDNAs of Abs from experienced B cells that have responded to exogenous Ag.

Study of cell surfaces, serum Ig, and cDNA showed that a substantial fraction of B cells in heterozygous mice expressed the endogenous IgH allele rather than the rearranged targeted transgene, while allelic exclusion was maintained in individual B cells. It may be that the presence of a rearranged functional gene even at the earliest stages of B cell precursor development precludes operation of a mechanism that normally prevents rearrangement and expression of the endogenous allele at a particular developmental stage. It is more likely, however, that the transgene was inactivated in some cells by attempted H chain revision, either to eliminate strongly autoreactive cells or to enhance diversity (34–36). To test this question more explicitly, and to determine whether functionally revised VH domains contribute to specific immune responses, we have prepared mice homozygous for the targeted gene and are preparing Ag-specific hybridomas for study of expressed mRNA.

**DNA binding and selection**

A great deal has been learned about negative selection based on anti-DNA autoreactivity, i.e., about mechanisms of tolerance to DNA, from studies of mice bearing transgenes for chains of anti-DNA Abs derived from lupus mice. Those experiments have used both nontargeted and, more recently, knockin H and L chain transgenes targeted to the JH or Jκ region of the Ig H or Ig L chain locus (35, 37–39).

The fate of a B cell expressing an anti-DNA transgene depends on the properties of the H chain–L chain combination in that cell. In cells with just an H chain transgene, combination with endogenous L chains can form a variety of Ig molecules, including Abs with high affinity for dsDNA and ssDNA, others that bind only ssDNA, and still others with no DNA-binding activity (40). In cells with both H and L chain transgenes, secondary rearrangement can also yield a variety of DNA-binding or nonbinding Ig molecules. Most cells in which a transgenic H chain combines with an L chain to form a high-affinity anti-dsDNA Ab are deleted in the bone marrow of nonautoimmune strains of mice (8). They escape deletion if they undergo receptor editing, i.e., a secondary rearrangement of either L chain or H chain (35, 39), to yield an Ig without the autoreactivity. Receptor editing is not possible in the absence of RAG-2. As a result, in RAG-2-deficient mice with an H chain–L chain transgene combination for high-affinity anti-dsDNA Abs, virtually all B cells are eliminated by apoptosis in the bone marrow once a receptor is formed (41). In RAG-2-deficient mice with a transgene combination for anti-ssDNA, B cells mature and reach normal numbers in the periphery. Still, they do not secrete anti-DNA Ab in vivo, even though some of them have surface markers reflecting in vivo activation (41).

Nearly all of the experiments demonstrating negative selection have been done with transgenes for pathogenic IgG anti-dsDNA Abs, with mutated V gene segments, derived from autoimmune lupus mice (38, 42–44). In addition, one such gene (D42 V\_\text{H}) was back-mutated to a germline sequence, yielding an Ab that bound DNA with an affinity lower by an order of magnitude than that of the D42 V\_\text{H} (38). The back-mutated Ab retained the arginine-rich CDR3 of D42 and, with its residual DNA-binding activity, was subject to regulation by deletion and decreased density of surface IgM. Another set of experiments used the H chain of an unmutated dsDNA-binding IgG mAb, RA4 (containing an S107 family V\_\text{H} segment). It was obtained from a BALB/c mouse treated with an antidiotypic reagent and then immunized with phosphorylcholine; the transgene was expressed as an IgG2b H chain (45). With dsDNA-binding activity, it too was subject to negative selection in nonautoimmune mice. The unmutated H chain of a nophenotypic MRL/lpr IgM autoantibody, which bound laminin and ssDNA, has also been studied as a transgene (46). One of four lines of transgenic mice expressed the transgene, producing a pathogenic kidney-localizing Ab.

mAb dC1 differs from anti-DNA Abs used in most previous transgenic experiments. It was identified among mAbs made by a mouse of the nonautoimmune C57BL/6 strain, immobilized with a nucleic acid-protein complex. The immunizing nucleic acid was poly(dC), and the preference of the Ab for that polynucleotide suggests that the cell making mAb dC1 was in fact activated during immunization. However, the Ab and its V\_\text{H} domain alone also bound ssDNA, resembling natural autoantibodies and some of the anti-ssDNA Abs that occur in sera of systemic lupus erythematosus patients. The absence of mutations in any segment of the dC1 V\_\text{H} or V\_\text{\kappa} domains indicates that its nucleic acid-binding activity existed, and may have been selected, even before exposure to the poly(dC)-protein immunogen. By using the dC1-rearranged V\_\text{H} domain as a transgene targeted to the Ig locus, we have been able to follow its fate under physiological development and immunization.

Binding of ssDNA or a substance that it mimics could, in theory, play a role in preimmunization B cell selection at any time after expression of a corresponding receptor, either the pre-B cell receptor comprising μ- and surrogate L chains, or the B cell receptor comprising μ- and true L chains. Substantial apoptosis of the B-lineage cells occurs in bone marrow at a stage in which a μ-chain-containing receptor is expressed (47). Cells with strongly autoreactive receptors are deleted, as may be cells with receptors lacking any corresponding ligand. Low-affinity binding of available self-Ags may provide a survival signal that helps to determine which cells comprise the 10–15% of B cells that do emerge from the bone marrow.

If the autoreactive B cell is a physiological precursor for diversified humoral responses, it may, at the same time, be a potential precursor for producers of pathogenic autoantibodies, giving rise to the need for extensive peripheral tolerance mechanisms to restrain that potential. In a test of this potential for pathogenicity, the transgene is currently being bred onto the autoimmune background of the MRL/lpr lupus mouse strain.

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**References**


