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Expression of a Dromedary Heavy Chain-Only Antibody and B Cell Development in the Mouse[1]

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In mature B cells of mice and most mammals, cellular release of single H chain Abs without L chains is prevented by H chain association with Ig-specific chaperons in the endoplasmic reticulum. In precursor B cells, however, surface expression of μ-H chain in the absence of surrogate and conventional L chain has been identified. Despite this, Ag-specific single H chain Ig repertoires, using μ-, γ-, ϵ-, or α-H chains found in conventional Abs, are not produced. Moreover, removal of *H* chain or, separately, *L* chain (κ/λ) locus core sequences by gene targeting has prevented B cell development. In contrast, H chain-only Abs are produced abundantly in Camelidae as H2 IgG without the C_H1 domain. To test whether H chain Abs can be produced in mice, and to investigate how their expression affects B cell development, we introduced a rearranged dromedary γ2a H chain into the mouse germline. The dromedary transgene was expressed as a naturally occurring Ag-specific disulphide-linked homodimer, which showed that B cell development can be instigated by expression of single H chains without L chains. Lymphocyte development and B cell proliferation was accomplished despite the absence of L chain from the BCR complex. Endogenous Ig could not be detected, although V(D)J recombination and IgH/L transcription was unaltered. Furthermore, crossing the dromedary H chain mice with mice devoid of all *C* genes demonstrated without a doubt that a H chain-only Ab can facilitate B cell development independent of endogenous Ig expression, such as μ- or δ-H chain, at early developmental stages. ***The Journal of Immunology,*** **2005, 175: 3769–3779.**

In the conventional mouse and human immune system, B cell development is initiated by VDJ recombination and surface IgM expression (Ref. 1, and refs. therein). At the pre-B cell stage, the associated surrogate L chain is replaced with a κ- or λ-L chain, and this initiates the process of Ab maturation, which is accompanied by cellular migration and class switching. At this stage, mature B cells undergo further selection and affinity maturation and can differentiate into Ab-secreting plasma cells or memory cells bearing other isotypes (IgG, IgA, or IgE). Developmental progression is blocked at the pre-B-I cell stage when H chain expression is prevented, although H and/or L chain transcripts may be found (2, 3). Likewise, silencing of both κ- and λ-*L* chain loci blocks B cell development, but at the somewhat later pre-B-II stage, which allows normal development up to pre-BCR expression (4). With the lack of L chain, μ-H chain is retained in the cytoplasm of immature bone marrow B cells, and their further development, with subsequent migration and colonization of the spleen, is prevented (4). Targeted modification of the *IgH* locus has permitted expression of truncated Ig polypeptides (5), and introduction of Ig transgenes consisting of shorter chains or removed domains has allowed single chain expression (6–8). Recently, it has also been shown that entire μ-H chains in association with the Igα coreceptor, but lacking surrogate or conventional L chain, can be expressed on the cell surface of pre-B cells. Single μ-H chain expression may induce differentiation signals and allow developmental progression possibly up to the immature B cell stage (9–11). Nevertheless, there are no examples where individual H chain polypeptides, on the surface or released from the cell without associated L chain, facilitate B cell differentiation to the mature and specialized stage leading to Ab repertoire formation in the mouse.

Abs, consisting of multiple units of paired H and L chains (12), emerged early in vertebrate evolution, and their presence is demonstrated in all of the jawed vertebrates studied to date (13). In addition to these conventional heteromeric Abs, sera of camelids (suborder Tylopoda, which includes camels, dromedaries, and llamas) contain a major type of Ig composed solely of paired H chains (14). Homodimeric H chain Abs in camelids lack the first C domain (C_H1) but harbor an intact variable domain (V_HH) encoded by different, clearly distinguishable, *V* genes (15). Using structural analysis, it has been concluded that it is impossible for a V_HH to pair with a normal V_L because the V_L-interacting side of the domain is reshaped by the hydrophilic V_HH hallmark amino acids and the long CDR3, which folds over this region (16). H chain Abs are absent in other mammals except in pathological cases, known as heavy chain disease, where parts of the V_H domain and/or C_H1 exon have been removed (17). Interestingly, H chain Abs are also present in some primitive fish; e.g., the new Ag receptor in the nurse shark and the specialized H chain (COS5) in ratfish (18, 19). Evolutionary analysis showed that their genes emerged and evolved independently, whereas *H* chain genes in camelids evolved from pre-existing genes used for conventional heteromeric Abs (20).

In camelids, the problem of developmental progression when single H chains are expressed may be circumvented because of

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structural differences in particular *V* and γ-*C* genes. As in conventional Ab production, the *H* chain gene of a H chain Ab is obtained after DNA rearrangements when specific V_HH germline genes, located within the V_H gene cluster, are assembled with commonly used *D* and J_H segments to code for the V_HH-domain (21). Genomic and cDNA analyses have revealed five functional dromedary γ genes, three of which (*γ2a*, *γ2c*, and *γ3*) are always used to form H chain-only isotypes, whereas two separate genes, *γ1a* and *γ1b*, are used for the production of heterodimeric IgG isotypes (22). Only a very low yield of H chain-only Ab transcripts (identified by their particular *V* genes) spliced to Cμ (V_HHDJ_H-Cμ) have been identified from dromedary spleen (23). Serum IgM devoid of L chains has not been found, and staining of camelid B cells for IgG H chain-only Ab is not yet possible due to a lack of specific Abs. These observations indicate that the IgM stage of H chain Abs may be transient and that the conventional differentiation events initiated by IgM expression may be circumvented.

The lack of C_H1 in H chain Abs is most likely to be the crucial factor in allowing their release from cells in the absence of L chains. Although H chain *C* region genes encode the first exon, it is spliced out during mRNA maturation, probably due to a point mutation at the canonical splicing donor site (24, 25). It has been established that the C_H1 domain participates actively in the regulation of assembly and secretion of conventional H2L2 Abs. The nascent-translated H chain polypeptide associates noncovalently with the H chain binding protein (BiP[5] or grp78) via BiP association sites in C_H1 (26). The BiP/H chain complex is retained in the endoplasmic reticulum by virtue of the KDEL sequence at the carboxy terminus of BiP (27), and an H chain with a C_H1 domain is not secreted unless BiP is displaced by the L chain (26, 28, 29).

It is possible that H chain-only Abs have been selected and maintained in Tylopoda for their complementary function in recognizing unusual epitopes, such as clefts on the Ag surface that are normally less antigenic for conventional Abs (30). So it could be argued that H chain Abs are maintained because they fulfill a complementary function in their humoral immune response. In the peripheral blood of all camelids, H chain Abs of different isotypes contribute to the immune response; they undergo Ag-mediated selection and affinity maturation, and their V_HHDJ_H domains are subjected to extensive somatic hypermutation (21, 22).

Our experiments focus on two essential questions. Can a dromedary H chain gene be processed and correctly expressed in the mouse? A feasibility study to evaluate the prospect of generating therapeutic camel H chain Abs in appropriately engineered mice is crucial to allow the future creation of H chain Ab repertoires. And, are conventional developmental processes capable of permitting B cell maturation without L chain association? This is important because L chain-dependent development or coexpression may impede the desired production of high-affinity dimeric H chains with their prospective use as enzyme inhibitors.

In this article, we show that H chain-only Ab expression of a rearranged dromedary *γ2a* gene in transgenic mice initiates B cell development without involvement of L chain or production of endogenous IgM. Allelic feedback signals are operative and seem to secure isotype exclusion by preventing expression but not V(D)J rearrangement and transcription of endogenous H and L chain genes. Multimeric IgG2a H chains are efficiently secreted, and their assembly on the cell surface allows Ag recognition. Expression of H chain Abs, with their particular configuration of the V_HH gene, their C_H1 exon removed by splicing, and lack of L chain, does not appear to be regulated by the presence of a classical BCR complex.

Materials and Methods

Derivation of mice

The dromedary V_HH-*Cγ2aTM* H chain Ab gene on an 11.7-kb *Not*I-*Sal*I fragment (31) was purified using a DNA purification kit (no. 28304; Qiagen). For the derivation of transgenic mice, DNA was microinjected into the male pronucleus of F_1 embryos (CBA × C57BL/6) according to standard methods (32), and several founders were produced. Transgenic animals were identified by PCR using dromedary Cγ2a-specific oligonucleotides: C_HH1 forward, 5′-GCACCTCGACCGGAAAGACCTTCATCTCC-3′; and hinge reverse, 5′-GGGACACGTGCATTCTGGTTCAGG-3′, which produced a 485-bp fragment (data not shown). Two animals, judged by PCR and Southern hybridization to be high (cam1) and low (cam2) copy number founders, were bred to homozygosity with μMT mice (33) and CΔ mice, in which all C genes had been removed by Cre-*lox*P-mediated deletion (3). Southern blot analysis (34) was conducted on *Kpn*I digests of tail DNA hybridized with a 1.4-kb *Not*I-*Bst*EII V_HH-gene probe (31).

Nucleic acid preparations and PCR

For transcriptional analysis of transgene expression, mRNA was prepared from bone marrow and spleen cells using the RNeasy Mini Kit (no. 74104; Qiagen), and cDNA was prepared with the Omniscript RT Kit (no. 205111; Qiagen) according to manufacturer's instructions. RT-PCR primers for identifying transgenic expression (see Fig. 2) were as follows: V3FR1B (V_HH), 5′-GAGGTGCAGCTGGTGGCGTCTGGAGGAGG-3; G2AH1F (H, hinge), 5′-GGGACACGTGCATTCTGGTTCA-3′; H (hinge) forward, 5′-CAACCAAAACCTGAACCAGAATGC-3′; C_HH2 reverse, 5′-G CACCTCAACGCCATCAATG-3′; C_HH3 forward, 5′-CAAGGACACCG TGAGCATAACCT-3′; 3′C_HH3 reverse (downstream of the stop codon), 5′-TGCCGGGGTGAGGCTCATTTA-3′; and M2 reverse, 5′-GCC CGATCATGTTTCTGTAGTCTG-3′. Lamin B1 served as a control to normalize the DNA concentration and also allowed discrimination of cDNA and genomic PCR products (3). PCR conditions were 93°C for 2 min followed by 32 cycles of 30 s at 93°C, 45 s at 58°C, and 90 s at 72°C, followed by 10 min at 72°C to complete the reaction.

For the analysis of D-J_H, V_H-D-J_H, and $V_κ$-$J_κ$-$C_κ$ rearrangement and transcription, as well as surrogate L chain transcription, RNA was prepared from bone marrow and spleen cells using Tri Reagent (Sigma-Aldrich), and cDNA was prepared using the Omniscript RT kit as above. Genomic DNA was prepared by lysis with proteinase K at 55°C, phenol/chloroform/isoamyl alcohol (25/24/1; Sigma-Aldrich) extraction and ethanol precipitation. Combinations of the following oligonucleotides were used: for the H chain, a 1/1 mixture of DF (5′- GCATGTCTCAAAGCACAATG-3′) and DQ52 (5′-ACCCTGGA CACAGGAAACAC-3′); VJ558L (5′-ATGGGATGGAGCTGGATCTT-3′) for DNA; and a 1/1 mixture of VJ558L and VJ558CL (5′-ATGGAATG GAGCTGGGTCTT-3′) for cDNA and V7183 (5′-ATGAACTTCGGGCT CAGCTT-3′) forward primers in combination with J_H1-4 reverse primer (5′-GAGACDGTGASHRDRGTBCCTKSRCC-3′) (3); for the L chain $V_κ$ forward (5′-GGCTGCAGSTTCAGTGGCAGTGGRTCWGGRAC-3′) (35); universal Jκ (5′-GTTTKATTTCCARYYTKGTSCC-3′) and Cκ reverse (5′-GCTCATGCTGTAGGTGCTGTCTTTGCTGTC-3′); and for surrogate L chain λ5 forward (5′-AGTTCTCCTCCTGCTGCTGCTGT-3′); λ5 reverse (5′-TACCTTCCAGTCCACCACCAAAG-3′); VpreB forward (5′-TGGT CAGGKCCCAGGAGCAGTGG −3′); and VpreB reverse (5′-CCGGAGC CCCACRGCRCAGTAAT-3′). PCR conditions were 30–35 cycles of 93°C for 20 s, 62–64°C for 30–35 s, and 72°C for 40–50 s, followed by 10 min at 72°C to complete the reaction, and RT-PCR conditions were 2 min at 94°C followed by 32 or 33 cycles of 93°C for 20 s, 62–64°C (70°C for λ5 and Vκ-Cκ, 50°C for J558-J_H1-4 RT-PCR) for 30–45 s, and 72°C for 50–60 s, followed by 10 min at 72°C to complete the reaction. The hybridization probe for the V558-J_H PCR was a 450-bp *Eco*R1 fragment from a cloned J558 family member, kindly supplied by A. Wood (Babraham Institute).

ELISA and Western blot analyses

Serum Abs were identified by ELISA (36) on Falcon plates (no. 353911; BD Biosciences) coated with 10 μg/ml goat anti-llama IgG (no. A160-100A; Bethyl Laboratories). Bound Abs were detected either with HRP-conjugated goat anti-llama IgG (no. A160-100P; Bethyl Laboratories) or biotin (BIO)-conjugated rat anti-mouse κ L chain (no. 04-6640; Zymed Laboratories) or BIO-conjugated anti-mouse $λ_{1,2,3}$ L chain (BD Pharmingen) developed with streptavidin-biotinylated HRP (no. RPN1051; Amersham Biosciences).

[5] Abbreviations used in this paper: BiP, H chain binding protein; BIO, biotin; HEL, hen egg lysozyme; KO, knockout.

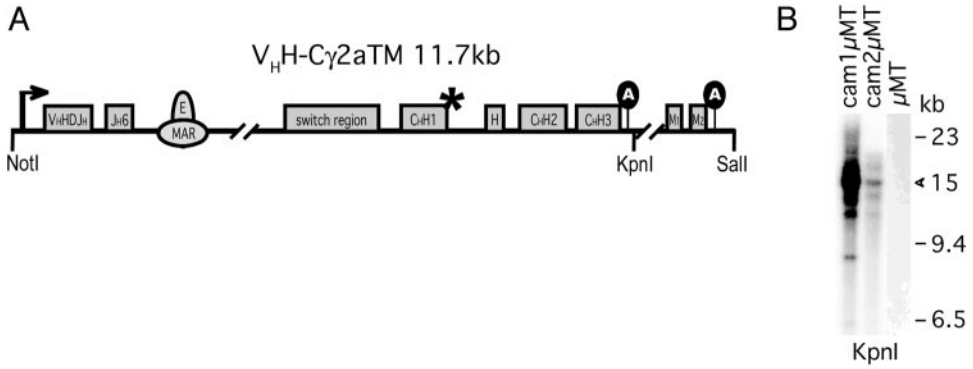

FIGURE 1. Integration of the dromedary *IgG2a* gene in the mouse germline. *A*, The dromedary H chain gene on an 11.7-kb *Not*I-*Sal*I fragment comprises a rearranged V_HHDJ_H gene, intervening sequences, including matrix attachment region (MAR), and Eμ enhancer (E), followed by the switch region and the genomic region of the dromedary *Cγ2a* gene (exons C_HH1, hinge (H), C_HH2, and C_HH3), including the membrane exons (M1 and M2) (31). The mutation in the donor splice site (*), the poly(A) sites (*A*), and the *Kpn*I site, for mapping analysis, are indicated. *B*, Southern blot analysis of tail DNA from cam1μMT and cam2μMT mice. DNA was digested with *Kpn*I, blotted, and hybridized with a 1.4-kb V_HH-specific probe (see *Materials and Methods*) to identify transgene integration and copy number. μMT DNA served as a control. λ*Hind*III was used as a size marker.

Goat anti-llama IgG (1 mg/ml) was coupled to CNBr-activated Sepharose 4B (no. 17-0430-01; Amersham Biosciences) in 0.1 M sodium carbonate buffer (pH 8.5) and stirred gently overnight at 4°C. Coupled Sepharose was left in 1 M glycine for 2 h and then washed and stored in PBS 0.5% sodium azide. Serum (20 μl) was incubated with ~20 μl of anti-llama IgG Sepharose overnight, and unbound proteins were removed by washing with PBS. For Western blot analysis, proteins were separated on precast 4–15% Tris-HCl Ready-Gels (no. 161-1104; Bio-Rad) and transferred to nitrocellulose membranes as described previously (31). Filters were incubated with HRP-conjugated goat anti-llama IgG, or BIO-conjugated rat anti-mouse κ L chain or anti-mouse $\lambda_{1,2,3}$ L chain, followed by incubation with streptavidin-biotinylated HRP as described above, and chemiluminescent substrate (SuperSignal West Pico, no. 34080; Pierce) was used for detection according to the manufacturer's protocol. Restore Western blot stripping buffer was used in some experiments (no. 21059; Pierce). The m.w. marker was All Blue Standards (no. 161-0373; Bio-Rad).

Flow cytometry analyses

Bone marrow and spleen cell suspensions were prepared from cam1μMT, cam2μMT, μMT, cam1, cam2, cam2CΔ, RAG2$^{-/-}$, and normal F_1 mice. Cells were stained in combination with allophycocyanin-conjugated anti-mouse CD45R (B220) (no. 01129A; BD Pharmingen), FITC-conjugated anti-mouse IgM (no. 04-6811; Zymed Laboratories), PE-conjugated anti-mouse c-*kit* (CD117) (no. 09995B; BD Pharmingen) and/or BIO-conjugated anti-mouse CD43 (no. 01602D; BD Pharmingen), FITC-conjugated anti-mouse IgM, PE-conjugated anti-mouse Igκ (no. 559940; BD Pharmingen), FITC-conjugated anti-mouse Igλ (no. 021174D; BD Pharmingen), FITC-conjugated anti-mouse CD21/35 (no. 553818; BD Pharmingen), and BIO-conjugated hen egg lysozyme (HEL) (31). Reactions with BIO-conjugated Abs were subsequently incubated with Tri-color-conjugated streptavidin (no. SA1006; Caltag Laboratories). Cytoplasmic staining was conducted using a fix and perm cell permeabilization kit with reduced background formulation (GAS-004; Caltag Laboratories) according to the manufacturer's instructions. Cells were analyzed on a FACSCalibur (BD Biosciences), and CellQuest (BD Biosciences) was used for data analysis. A FACSAria (BD Biosciences) was used for sorting B220$^+$ and B220$^-$ lymphocyte populations at up to 12,000 cells per second, which resulted in a purity >95%.

Results

Integration of a dromedary H chain gene construct in the mouse germline

The H chain Ab gene, *V_HH-Cγ2aTM*, has been constructed using a rearranged dromedary V_HHDJ_H gene with specificity for HEL and a dromedary *Cγ2a* gene in germline configuration, including the transmembrane exons (31). Figure 1*A* shows the 11.7-kb *Not*I-*Sal*I fragment, which was microinjected into fertilized mouse oocytes. From the animals born, two were selected (cam1 and cam2, identified by PCR) for further breeding. These represented high copy number and low copy number transgenic founder mice. The animals were crossed with μMT mice in the C57BL/6 background (33), which resulted in cam$^{+/-}\mu$MT$^{-/-}$ and μMT$^{-/-}$ animals, which were used for detailed analyses. In Southern blotting, *Kpn*I digests showed several bands containing the transgene (Fig. 1*B*) with a predominant ~15-kb fragment, suggesting multiple and tandem integration as the construct harbors a ~7.4-kb *Not*I-*Kpn*I fragment. Comparison of signal intensities estimated that the cam1μMT mice have the transgene integrated at a high copy number (>40 tandem copies), whereas cam2μMT is a low copy number (≥2 copies) line.

H chain transcripts are correctly spliced

An important question was whether the introduced H chain gene would be transcribed and whether the resulting product would match the transcripts found in Camelids. To investigate possible mRNA splice products, we used RT-PCR and sets of oligonucleotides that would reveal the exon usage (Fig. 2). Employment of V forward and hinge reverse oligonucleotides revealed a product of 500 bp from cam1 and cam2 mice (Fig. 2*A*), which corresponded to V_HHDJ_H-hinge splice products lacking the C_H1. Inclusion of the C_H1 exon would have increased the size to ~800 bp (31). The hinge region is followed by C_HH2 (Fig. 2*B*). Transcripts that allow Ig secretion (Fig. 2*C*) and surface expression (Fig. 2*E*, *top bands*) were found, both in bone marrow and spleen cells, as has been described for μ mRNA products in mouse B cells (37). Figure 2, *D* and *F*, illustrate the exon usage of the PCR products, which have been confirmed by cloning and sequencing (supplemental Table I).[6] Simultaneous amplification of lamin B1 (Fig. 2*E*, *bottom bands*) served as a semiquantitative reference and suggested that secretory and membrane transcript levels are higher in spleen and bone marrow cells from cam2 mice. In cam1 mice, we found little transmembrane product and a diminished intensity of the amplification bands. This implies low transcription levels of the dromedary transgene in cam1 mice, which may be due to the integration site, e.g., in a transcriptionally silent region, and is reaffirmed by ELISA and flow cytometry analysis shown below. Despite differences in expression levels, the results show correctly spliced H chain products, without C_H1, in bone marrow and spleen, which

[6] The online version of this article contains supplemental material.

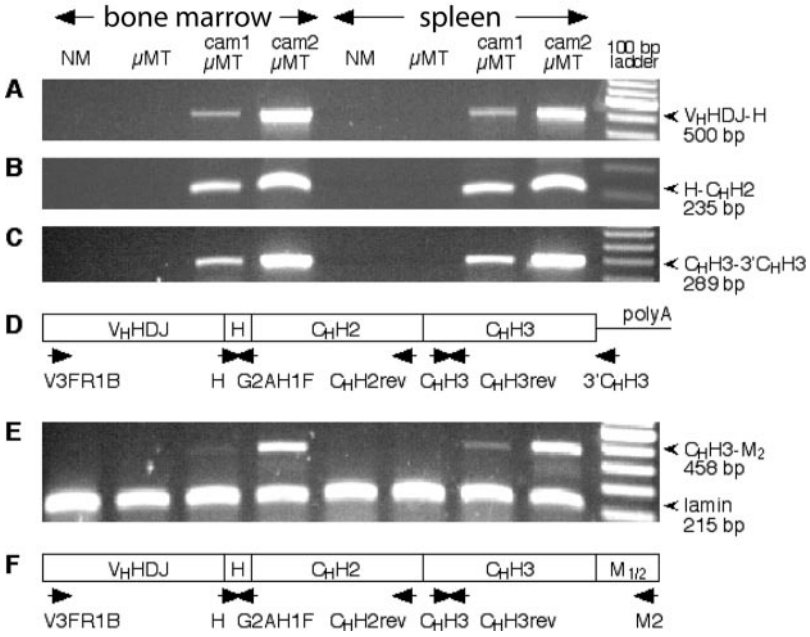

FIGURE 2. Transcription of dromedary H chain in bone marrow and spleen. RT-PCR analysis was conducted using oligonucleotides priming in the following regions: *A*, V_HH and hinge (H); *B*, H and C_HH2; *C*, C_HH3 and 3′ of C_HH3 stop codon; *E*, C_HH3 and membrane exon 2 (M2) and lamin, as control conducted in parallel, to verify matching cDNA concentrations. *D* and *F,* Maps established from the product sizes. These show that the C_HH1 exon is omitted and that the correctly transcribed dromedary H chain consists of V_H-HDJ-H-C_HH2-C_HH3-M1/2. The secreted (*D*) and membrane (*F*) forms are found in both bone marrow and spleen cell populations. As a size marker, a 100-bp ladder was used. In addition, the exact size of each band was established by DNA sequencing (supplemental Table I).

implies that the introduced dromedary *H* chain gene is faithfully expressed in both secreted and transmembrane form.

Multimeric Ig is secreted in serum

To analyze secretion of dromedary H chain Ig, we captured serum Abs from the camμMT mice in a sandwich ELISA using goat anti-llama IgG for detection. Figure 3 illustrates strong Ab binding of two representative cam2μMT mice (termed a and b), the low copy transgenic line, with good detection of up to 1/1000 dilution. The high copy line, cam1μMT, had a low Ab titer (detectable only in 1/3 (data not shown) and 1/10 serum dilutions), whereas background binding was obtained when using μMT and normal mouse serum. Because binding to anti-llama IgG did not reveal the assembly of the secreted dromedary IgG2a, we further tested serum Abs for the presence of L chain. None of the cam1μMT, cam2μMT, and μMT sera showed binding to anti-mouse Igκ or anti-mouse Igλ L chain; however, normal mouse serum revealed some cross-reactivity, in that weak binding to anti-llama Ig could be detected with anti-Igκ.

To assess the assembly and m.w. of the secreted H chain Ig, we conducted Western blot analyses. To overcome a high background,

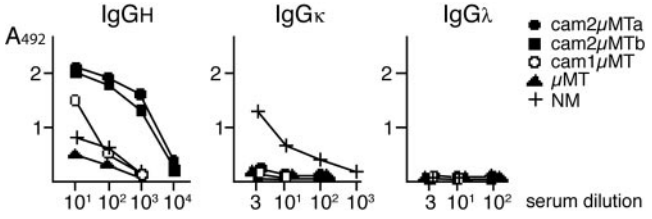

FIGURE 3. Expression of H chain-only Abs in serum of cam mice. H chain Abs (IgG_H) were identified in ELISA by coating and detection with anti-llama IgG. A lack of L chain Abs was affirmed by coating with anti-llama IgG and detection with anti-κ or anti-λ Ig. Coating with anti-κ and, separately, anti-λ Ig and detection with anti-κ and anti-λ Ig, respectively, gave similarly negative results for the camμMT mice (data not shown). The low copy number line cam2μMT showed good levels of H chain IgG with no L chain. This line is represented with serum titrations of two mice (a and b, ~3-mo-old) chosen from four separate experiments with similar results, using serum from at least eight mice, each between 6 wk and 10 mo of age. Normal mouse serum (NM) showed some cross-reactivity.

pronounced by the presence of serum albumin and separation under reducing conditions, we coupled anti-llama IgG to Sepharose for the purification of H chain Abs. Bound Abs from serum were separated on 4–15% polyacrylamide gels and visualized with HRP-coupled anti-llama IgG. The results (Fig. 4*A*) showed under reducing conditions a major band of ~46 kDa in cam1μMT and cam2μMT mice. A second, fainter, band of ~64 kDa is only seen in cam2μMT mice. Cloning and sequencing of a 1.6-kb fragment, as compared with the normal 1.2-kb band, obtained from RT-PCR using V3FR1B (V_HH) and 3′C_HH3 oligos, revealed an aberrant splice product due to tandem integration of the construct, incorporating an extra V_HHDJ domain (supplemental Table I). This particular dromedary H chain is made up from V_HHDJ-V_HHDJ-H-C_HH2-C_HH3, which would add ~18 kDa to the normal size and explains the additional band obtained in Western analysis. However, this band is not a product using the C_H1 exon as verified by RT-PCR. The samples separated under reducing conditions showed faint H and L chain background bands from IgG coupled to Sepharose due to leakage (38). A reason could be that the different preparations of anti-llama Ig used for capture and visualization allowed some cross-detection, possibly enhanced by the sensitivity of the Western analysis. Separation of captured Ig under nonreducing conditions (Fig. 4*A*, *right*) revealed a major band of ~91 kDa, which represents H chain dimers. There are two larger bands, one of ~112 kDa and a much fainter band of ~135 kDa, which are likely to account for different multimers. Although the separation suggests that the secreted dromedary IgG2a H chain Ab produced in cam mice is largely associated as H2 homodimer, it may also associate as H3 multimer and, depending on resulting transcription products, in extended (2 × 64 kDa) or unequal (46 + 64 kDa) form. The longer exposure used to visualize H chain Ig products from cam1μMT mice is due to the lower levels produced (see Fig. 3).

Because no endogenous Ig could be identified in serum from camμMT mice, we conducted further Western separation on cam2 mice bred into the normal mouse background. Serum samples were applied to the gel in different amounts to allow a meaningful comparison. Figure 4*B* shows that no Igλ or Igκ could be detected in total cam2 serum, whereas significant amounts of dromedary H chain Ig remained as the only serum Ab. In summary, serum from

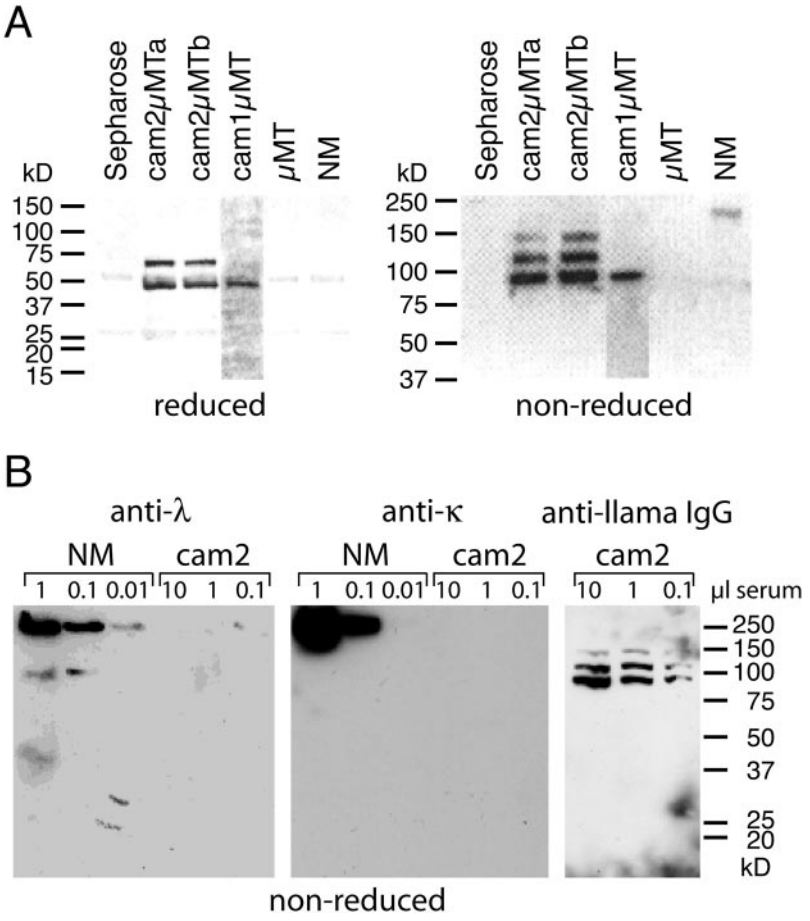

FIGURE 4. Western blot analysis of dromedary IgG2a. *A*, Serum Abs were captured by incubation with anti-llama IgG coupled to Sepharose, separated on Ready Gels, and stained with HRP-conjugated anti-llama IgG. Reducing conditions (*left*) revealed a ~46-kDa H chain band for cam2μMT (from mouse a and b) and cam1μMT (which was visible after longer exposure), and an additional band of ~64 kDa for cam2μMT. The fainter bands of ~26 kDa and ~53 kDa in all of the samples are the result of leakage of the Ab-coupled Sepharose (38) and cross-reactivity of different batches of anti-IgG, which contain IgG themselves. Samples separated under nonreducing conditions (*right*) revealed a major band of ~91 kDa (H2 configuration) for cam1μMT and cam2μMT. Additional bands found for cam2μMT of ~112 kDa and ~135 kDa may represent other multimers. Negative controls to affirm the specificity of the detection were anti-llama IgG-coupled Sepharose incubated with PBS, normal mouse (NM), and μMT serum. *B*, Total serum in 10-fold dilutions from cam2 in the normal mouse background and NM was separated under nonreducing conditions. Detection with anti-λ and anti-κ did not identify associated or residual L chain in cam2 mice, whereas development with anti-llama IgG showed the expected dromedary H chains. The filter was stripped between reactions. Detection with anti-κ necessitated a short exposure because of the strong NM signal. However, even longer exposures did not reveal any L chains in cam serum. The sizes of the marker bands are indicated.

camμMT or cam2 mice did not reveal any free or differently associated L chain by identification with anti-L chain reagents in ELISA and Western blotting, which showed that camelid H chain Ig can be exclusively produced in a mouse.

Progression of B cell development in bone marrow and spleen without L chain

A central question was how B cell development could progress in mice that express H chain Ig without L chain. Flow cytometry analysis of bone marrow cells (Fig. 5 left) from normal, μMT, and cam1μMT mice showed quite similar levels of pro- and pre-B cells, c-*kit*$^+$B220$^+$, and CD43$^+$B220$^+$, which are increased in cam2μMT mice (10% compared with 5% in normal mice or 4% in μMT mice). The reduced level of immature B220$^+$CD43$^-$ B cells in cam1μMT mice, 2% compared with 46% in a normal mouse, is barely detectable. However, cam2μMT mice, the low copy number but high expresser line, showed good recovery of the B lymphocyte population (14% compared with 46% B220$^+$ cells), which suggests induced B cell recovery by expression of the dromedary H chain gene. The effect is more dramatic in the spleen of cam2μMT mice (Fig. 5, *right*), where recovery of mature Ab-producing B cells reached about half the normal levels as shown in stainings with anti-CD21/35. Only poor recovery, 1.1%, is found in cam1μMT mice in concordance with the low level of expression found in this line. There is no expression of mouse IgM or Igκ or Igλ (data not shown) L chains. In the experiments, age-matched 3-mo-old mice were used. But very similar results were obtained, in flow cytometry, ELISA, Western, and PCR detection, when separate and parallel comparisons of 3-mo-, 6-wk-, and 6–10-mo-old animals were conducted. These results show that introduction of the dromedary H chain gene reconstitutes B cell development in the μMT or IgM-negative background without a requirement for L chain.

The H chain BCR signals exclusion of endogenous Ig

The purpose of crossing the dromedary H chain transgenic mice into the μMT background was to visualize H chain production without interference of mouse Ig. Initially, this was important, because the Abs that recognized the dromedary γ2a H chain cross-reacted with mouse Ig (see Figs. 3, and 4*A*). However, from the detailed analysis of the camμMT mice and serum Ig comparisons with cam2 mice bred into the normal mouse background, it became clear that dromedary H chain was well expressed and that B cell development progressed without L chain production. These results prompted further investigations to determine whether Ag-specific H chain Ig could be expressed on the cell surface and in the cytoplasm solely on its own or whether expression was accompanied by endogenous H or L chain polypeptides. Staining of bone marrow and spleen cells from cam2 transgenic mice with labeled HEL showed the presence of the Ag receptor on the cell surface of B220$^+$ cells (Fig. 6*A*). Binding of HEL was conducted in parallel using cells from MD-4 transgenic mice, which express HEL-specific Abs of high affinity (39). Although prominent surface staining with HEL was achieved in cam mice, the intensity was reduced compared with that of the MD-4 mice, perhaps due to low H chain density or reduced affinity. Receptor expression was not accompanied, even in the normal mouse background, by surface expression of endogenous IgM or IgL. Because this did not rule out the presence of endogenous Ig intracellularly, for example by chaperone retention, we used cytoplasmic staining. The results in Fig. 6*B*

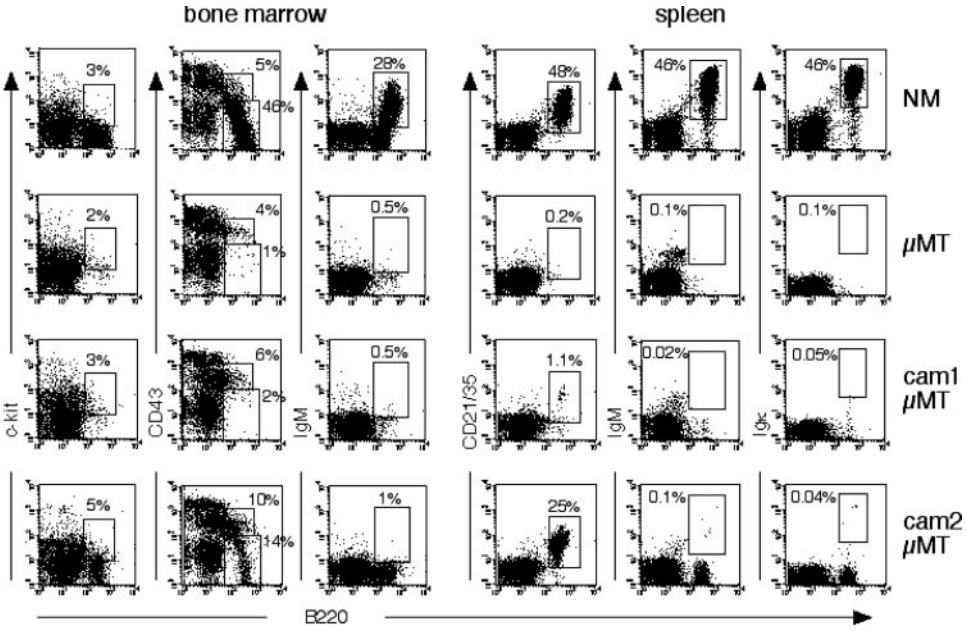

FIGURE 5. B cell development in mice expressing H chain-only Abs. Bone marrow (*left 3 rows*) and spleen cells (*right 3 rows*) from normal mice (NM), μMT, cam1μMT, and cam2μMT mice were stained with Abs against B cell differentiation markers. B220 served as a universal B cell marker and in combination with c-*kit*, and CD43 identified pro- and pre-B cells, which were well maintained in cam1μMT and cam2μMT mice. IgM is only expressed in normal mice, but cam2μMT mice showed good recovery of immature B220$^+$ cells, which are lacking in μMT and cam1μMT mice. Near normal levels of CD21/35$^+$ mature B cells were present in the spleen of cam2μMT mice (25%), but no L chain was found. The histograms were chosen from one of six independent experiments with very similar results, using ~3-mo-old mice.

showed HEL-specific Abs without the presence of mouse Ig. Unfortunately, some nonspecific background remains with this method (see *Materials and Methods*), which could point to residual mouse Ig expression at low levels. However, a similar background staining is found when using mice without endogenous C genes (3), reemphasizing that endogenous Ig levels are negligible.

H chain-only Abs are equally well expressed in C_H locus deletion mice

The findings that cam transgenic mice express dromedary H chain-only Ig in the absence of any mouse Ig chains and yet maintain appropriate levels of B220$^+$ cells, raised the question of whether endogenous H chain genes may be important for the early developmental stages. For example, pre-B cell development could be facilitated by VDJ recombination and endogenous H chain expression (μ, or δ in the μMT mice) initially accompanied but later replaced by transgene expression. It has been shown that the introduction of a rearranged murine γ transgene does not promote B cell development because joint expression with endogenous μ is required (40, 41). This finding suggested that IgG could not replace IgM, and it was speculated that their feedback signals to control B cell maturation must be different. To determine whether the dromedary γ2a H chain could be expressed without the help of other Ig genes, we crossed the cam2 mice with a recently derived line (C$\Delta^{-/-}$) where all constant region genes had been deleted (3). These animals cannot express any H chain isotypes. As can be seen in Fig. 6, *A* and *B*, cam2C$\Delta^{-/-}$ mice show the same level of B cells as cam2 mice and do not express any L chain. Indeed, it is worth noting that lymphocyte development, B cell levels, and Ab expression were very similar in camμMT$^{-/-}$, cam, and

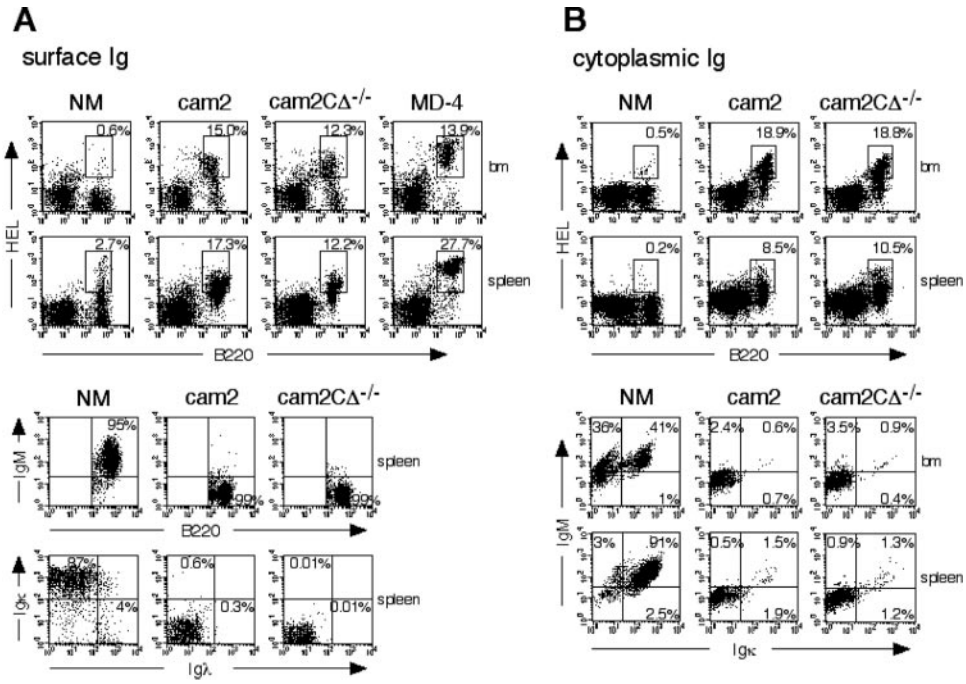

FIGURE 6. Make-up of the surface receptor and cytoplasmic Ig in bone marrow and spleen of cam2 mice in the normal mouse background and cam2C$\Delta^{-/-}$ mice with removed endogenous C_H locus. *A*, Identification of HEL-specific surface Ig plotted against B220 expression in normal mice (NM), cam2 mice, cam2C$\Delta^{-/-}$ and MD-4 (39) control mice (*top*), and stainings of μ-H and κ- and λ-L chain shown for the gated B220$^+$ lymphocytes (*below*). *B*, Cytoplasmic stainings confirmed the presence of HEL-specific Ig (*top*) and the lack of endogenous IgM and L chains in B220$^+$ cells from cam2 and cam2C$\Delta^{-/-}$ mice (*below*). The stainings show that bone marrow and splenic B220$^+$ cells from mice carrying the dromedary H chain transgene express HEL-specific Ig not accompanied by endogenous IgM or L chain.

camC$\Delta^{-/-}$ mice, which established that the dromedary H chain transgene promotes B cell development independent of μ or expression of other endogenous H and L chains. Expression of HEL-specific H chain Abs on the cell surface suggests H chain association without L chain in dimeric or multimeric form. Such association presents a new type of BCR, with the capacity to initiate B cell development independent of conventional Ig expression.

Endogenous H and L chain loci are rearranged and transcribed

The lack of endogenous Ig expression suggested that the H chain BCR elicits the appropriate signals to prevent endogenous rearrangements. However, when analyzing bone marrow and spleen cell DNA from cam2 mice by semiquantitative PCR, we found, apart from the expected D-J_H bands, similar levels of V_HDJ_H and VκJκ rearrangement in cam2 and normal mice (Fig. 7). Using forward oligos representing different V_H gene families, *J558* and *7183*, gave a clear indication of diverse rearrangements in the cam2 mice, but we cannot completely rule out that endogenous V(D)J rearrangements are detected in B cells that have lost transgenic expression of the dromedary H chain. Hybridization with a full-length *J558* V_H gene further confirmed the similar levels of VDJ rearrangement in cam and normal mice. The obtained PCR fragments were of the expected sizes (3, 35). In the experiments, DNA from RAG2$^{-/-}$ tissues, used as a negative control, showed some background amplification. Such background was not seen when, for example, mouse embryonic stem cell DNA was used for V_HDJ_H amplification (data not shown). Different RAG2$^{-/-}$ mice sources and DNA preparations did not prevent this but always showed a much reduced signal compared with cam2 and normal mouse DNA. However, because we did not see any background in RT-PCR using RAG2$^{-/-}$ RNA, this could indicate a low level of nonproductive recombination products. Using lamin B1 as reference (3) for the semiquantitative comparison using serial dilutions, we found no indication of reduced levels in the cam mice. Control reactions conducted in parallel using normal mouse DNA for dromedary H chain amplification and dromedary *V_HH-Cγ2aTM* plasmid DNA for mouse V(D)J PCR did not result in nonspecific bands (data not shown).

To our surprise, RT-PCR signals, reflecting RNA levels, were also very similar in cam2 and normal mice. To assess whether the V558-J_H RT-PCR bands from cam2 mice accounted for nonfunctional VDJ rearrangements or represented potentially productive transcripts, we cloned and sequenced the ~400-bp fragments (supplemental Table II). Sequence comparison established that fully functional and diverse murine V_HDJ_H transcripts were produced in cam2 mice. To investigate whether endogenous transcripts were only expressed in cells that did not produce dromedary H chain Ig or whether endogenous and exogenous transcription was jointly operative in the same cell, we separated B220$^-$ and B220$^+$HEL$^+$ lymphocytes by flow cytometry (Fig. 8). Semiquantitative RT-PCR analysis of B220$^+$HEL$^+$ bone marrow and spleen cell RNA from cam2 mice showed extensive V558-J_H and Vκ-Cκ amplification similar to those from normal mice. In B220$^-$ cells, V(D)J transcripts were also well maintained, and certainly in the cam mice, there were no amplification differences. As this raised the possibility that the calculated purity, >95%, of the sorted cell populations may not have been reached, we used further RT-PCR to identify surrogate L chain transcripts. With VpreB and λ5, surrogate L chain polypeptides are well expressed in B220$^+$ bone marrow cells, but no expression is found in the spleen (Refs. 9, 11, 42, and refs. therein). This was exactly what we found and provided reassurance of the purity of the analyzed cell populations. A comparison of sorted B220$^+$ and B220$^-$ bone marrow and spleen cells from cam2 and normal mice showed no difference in transcription levels of the surrogate L chain (Fig. 8). Expression of surrogate L chain in bone marrow but not spleen B220$^+$ lymphocytes from cam mice was independently confirmed by cytoplasmic staining with anti-λ5 (data not shown). Control reactions (Fig. 8*C*) using cDNA prepared from bone marrow and spleen cells of RAG2$^{-/-}$ mice, bone marrow cells from SL (surrogate L chain triple knockout (KO))$^{-/-}$ mice (42), and DNA from normal mouse spleen cells confirmed the validity of the RT-PCR. The lack of VpreB and λ5 transcription in mature cam2 B cells rules out that dromedary H chain expression relies on the presence of surrogate L chain.

Our comprehensive analysis of intra- and extracellular expression of endogenous murine Ig revealed that very small amounts, if any, were retained in the cell. This may mean that either no translational products were being produced or that there was rapid degradation. Staining with anti-L chain confirmed a lack of endogenous Ig. In addition, we did not identify dromedary H chain transcripts in other nonlymphocyte tissues (data not shown). In

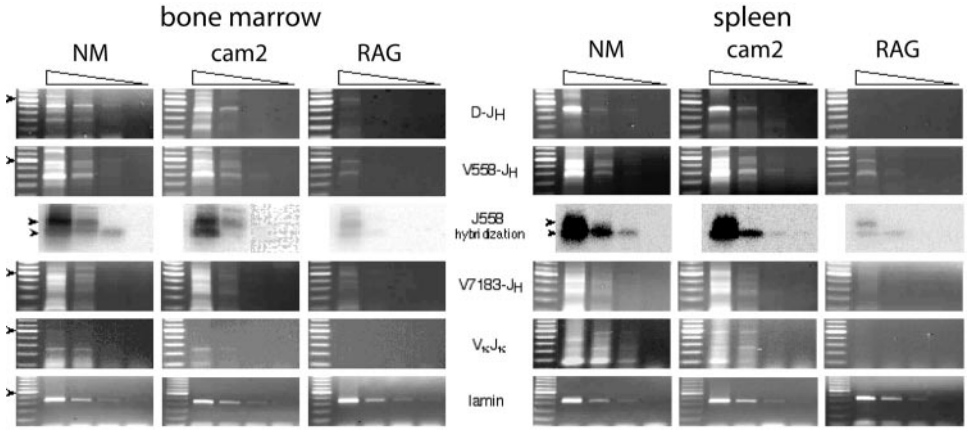

FIGURE 7. DNA rearrangement of endogenous *H* and *L* chain genes is maintained in cam mice. V(D)J recombination in bone marrow and spleen cells from normal mice (NM) and cam2 mice was examined by semiquantitative PCR using combinations of D-J_H, V558-J_H, V7183-J_H, and Vκ-Jκ primers. The DNA starter concentration was ~10 ng followed by three further dilutions, 10^{-1}, 10^{-2}, and 10^{-3}, each. RAG2$^{-/-}$ mice served as a negative control, and amplification of lamin B1 permitted normalization. Hybridization of blotted V558-J_H amplifications with a *J558* V_H gene probe confirmed the specificity of the reactions. Bands of the expected approximate sizes, 400–800 bp for V_HDJ_H and 200–400 for $V_\kappa J_\kappa$, depending on J segment usage (3, 35), were maintained in the cam mice. As a size marker, 100-bp ladders were used with the 600-bp band of increased intensity marked by an arrow, followed by a 100-bp size increase above and decrease below. For the J558 hybridization, 600 bp is marked by a normal arrow, and 400 bp is marked by a small arrow.

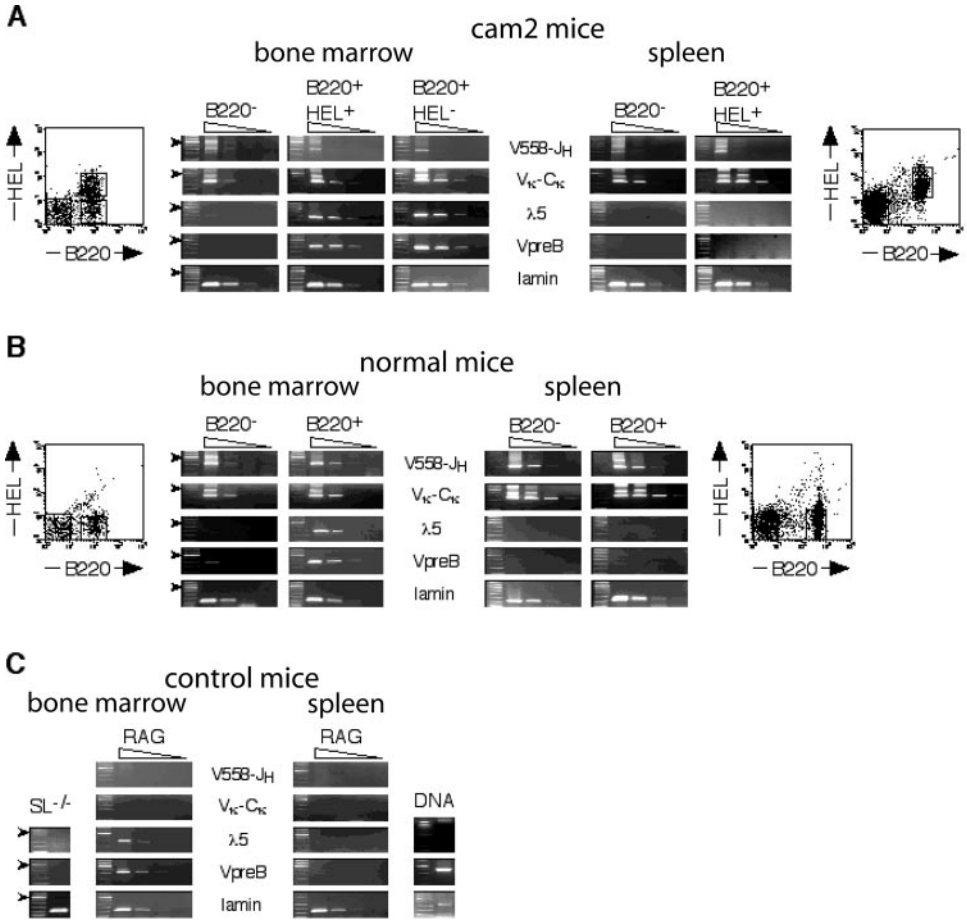

FIGURE 8. V(D)J transcription is operative in cam mice. Bone marrow and splenic lymphocytes from cam2 and normal mice, stained for B220 and HEL binding, were sorted by flow cytometry to >95% purity. RNA was produced from 10^5–10^6 sorted cells, and equal amounts of cDNA from $\sim 5 \times 10^3$ cells were analyzed in parallel by PCR in successive 10-fold dilutions. Oligo combinations of V558-J_H and V_κ-C_κ determined the level of Ig transcription, λ5 and VpreB examined the developmental stage of the cell populations, and lamin provided an independent reference of the cDNA concentrations. *A*, RT-PCR analysis of gated bone marrow and spleen cell populations, $B220^-$, $B220^+$, and $B220^+HEL^+$, from cam2 mice. *B*, $B220^-$, $B220^+$ cells from normal mice. *C*, Control reactions using RT-PCR analysis using tissue from surrogate L chain KO mice (42) and $RAG2^{-/-}$ mice, and PCR using normal DNA, which confirmed the size difference of the genomic products.

conclusion, this suggests that cam transgenic mice express the dromedary H chain-only Ab without association or attendance of significant amounts of endogenous Ig. Nevertheless, it may be possible that the transgene can only be expressed after endogenous V(D)J rearrangement has been completed. However, dromedary H chain expression in $C\Delta^{-/-}$ mice, which do not express rearranged H chains without C region (3), rules out that B cell differentiation is driven by endogenous IgH expression. The results show that endogenous H and L chain genes, despite being fully rearranged and transcribed, are excluded from expression at the translational stage by a feedback signal originating from a H chain BCR without L chain.

Discussion

Introduction of a rearranged dromedary *H* chain gene into the mouse germline showed that Ag-specific H chain-only Abs could be correctly expressed, without the C_H1 domain, and assembled as multimer. H chain Ig was secreted and also presented on the cell surface, which led to progression in B cell development. Expression of H chain IgG might exclude translation of endogenous H and L chain polypeptides, which established a BCR without L chain association.

The rearranged H chain expressed in transgenic mice was constructed with no alteration that would favorably bias expression in mouse B cells. Thus, secretion and surface expression of HEL-specific H chain Abs in a heterologous system established that RNA processing, H chain assembly, and cellular transport use commonly recognized signals provided by the dromedary V_HH-γ2aTM construct. The likely reason why dromedary H chain Ig can be expressed in the mouse seems to be due to two gene adaptations in camelids, not found in other jawed vertebrates. Their V_HH genes are distinct from conventional V_H genes; they accommodate changes in key residues normally in contact with the V_L domain in the Ag binding site of conventional Abs (16). Apparently, neither the V_HH hallmark amino acids, nor the presence of a long CDR3 loop of 24 aa, caused folding problems (43). Nevertheless, the genomic organization of the V_HH genes (i.e., promoter, leader signal, intron, V-exon, and recombination signal sequence) is otherwise remarkably similar to that of the conventional V_H counterparts (44). It has been reasoned that V_HH genes have recently evolved from conventional V_H genes after the emergence of the Tylopoda (>50 million years ago), which makes it likely that both types are accommodated in the *V* gene cluster of the *H* chain locus (44). This is supported by the observation that both the V_H and V_HH gene segments appear to rearrange to the same *D* and J_H gene segments to form either a conventional Ab or a H chain Ab (21). The other adaptation concerns a subset of their *Cγ* genes (24, 25). It was proposed that in these genes, a point mutation at the canonical splice signal sequence might cause the excision of the first C region domain (24). Although the precise mechanism is not known, this removal seems to permit assembly and secretion of homodimeric H chains (20). Interestingly, accurate and highly efficient removal of the C_H1-containing sequence from the RNA transcript of *H* chain genes appears to be performed with equal efficiency in camelids and transgenic mice. Neither in the dromedary nor in our transgenic mice could *γ2a H* chain genes with retained C_H1 exon be identified by RT-PCR and sequencing. Thus, the removal of the C_H1 exon appears to be essential to permit expression of H chain Ig. However, exclusive H chain-only Ab production in camelids was predicted to involve interaction with

species-specific cellular factors important for the expression of H chain Ab genes, processing of their transcripts, and the assembly of the translation products into functional Ag binding entities (45). For this reason, it was unexpected to see that a heterologous system produced functional H chain Abs at quite respectable protein levels. This suggested that intrinsic alterations of the dromedary H chain Ab are well recognized and dealt with by the mouse B cells and that dromedary-specific factors are either not essential or can be bypassed by the mouse transcription, translation, and secretion machinery.

The C_H1 domain participates actively in the regulation of the assembly and secretion of conventional H2L2 Abs via association with BiP (26–29). A lack of C_H1 is likely to permit unhindered transit of the H chain polypeptide through the endoplasmic reticulum to allow secretion and appropriate surface deposition. Furthermore, the loss of BiP association may also prevent degradation of the H chain. H chains with the long hydrophobic transmembrane region anchor in the lipid bilayer, whereas the short hydrophilic C-terminal region of secretory form H chains ensures their release from the cell in the absence of associated BiP. The importance of the C_H1 domain is well recognized because hybridoma or myeloma cell lines harboring Ig genes with deleted C_H1 exon retain the ability to secrete homodimeric H chains without associated L chains (46, 47). In heavy chain disease, truncated H chains are readily secreted without L chain (17, 48). For the dromedary H chain, not being dependent on IgM expression may allow the expansion of a different lymphocyte subset, which may be able to restore normal B cell development. Extensive levels of $B220^+$ cells, some with dendritic cell characteristics, have been found in bone marrow and spleen (49) and may be maintained in the dromedary H chain mice. Alternatively, expression of the rearranged dromedary H chain gene could facilitate progression in B cell development to a mature stage without the differentiation stages from pro- to pre-B cells ($B220^+CD43^-$ cells in Fig. 5). In this context, it is notable that staining of camel lymphocytes for Ig H and L chain on the cell surface has been attempted but did not unambiguously demonstrate surface IgG H chain-only expression. A reason for this may be that the staining reagents raised against ruminant Ig fail if there is broad epitope diversity (50). Despite this setback, camelids readily produce Ag-specific Abs in H2 and H2L2 configuration, and there is no indication that mixed molecules are expressed (16, 51). Unfortunately, there is no information about pre-B cell development in camelids or whether an H chain without C_H1 can associate with a surrogate L chain to form the pre-BCR necessary to progress B cell development. However, from gene targeting studies in the mouse, it is clear that B cell development without surrogate L chain can progress (42), whereas B cell development without L chain is blocked after H chain expression and maturation up to the immature B cell stage (4).

The various Ig classes seem to form distinct oligomeric BCR complexes, which may differ in their threshold levels for BCR signaling (Ref. 52, and refs. therein). For example, the IgG BCR complex, in contrast to the IgM or IgD BCR complex, cannot give an efficient positive selection signal. Perhaps, contradictory to expectation, the H chain BCR may be able to provide an adequate differentiation and proliferation signal to secure survival. In transgenic mice carrying rearranged conventional *H* chain genes (μ, δ, γ, or α), feedback inhibition can prevent DNA rearrangement of the endogenous *IgH* locus (References 53–55, and references therein). However, the expression of the transmembrane form of introduced Ig transgenes does not necessarily prevent DNA rearrangement of the endogenous loci to secure allelic exclusion (56–58). It has also been shown that *γ2b* transgenes are coexpressed with endogenous μ, and that *γ2b* cannot by itself promote B cell development in the μ KO background (40, 55, 59). The few mature B cells that do develop in the transgenic mice express both endogenous μ and transgenic γ2b, and in addition, L chain is expressed. Although these experiments show that B cell development is critically dependent on signaling of a μ-H chain associated as BCR, there are exceptions. In a particular *γ2b* transgenic mouse line, it appears that transgene expression by itself can promote B cell maturation and allelic exclusion, possibly by expanding a particular B cell subset (55). In separate founders, most likely carrying a γ2b transgene integration at diverse chromosomal locations, it was discovered that alternative expression pathways were used, maybe dictated by different expression levels. Despite these contradictory results, which may largely depend on site of integration and copy number of particular *IgH* transgenes, the overall conclusion from transgenic IgH mouse studies is that B cell maturation can progress, but the developmental state of the lymphocytes appears to be critically dependent on the onset of (endogenous) μ expression. This was not seen in the cam mice, where even in a normal mouse background, IgM expression was prevented without causing developmental cessation.

The two cam transgenic lines we describe in this study, derived from independent microinjections, are most likely to carry the transgenes at different chromosomal sites. Despite this and the low H chain expression level in cam1 mice, developmental progression is very similar. The recent finding that, in the mouse, entire μ-H chains can be transported and expressed on the surface of pre-B cells without associated L chains (9–11) contradicts previous discussions that free H chain polypeptides are toxic and that they have to be neutralized to allow progression in B cell development (Ref. 60, and refs. therein). The observations may be compatible if lower H chain expression levels in early B cells are taken into account and if apoptosis, which may be induced when insoluble (accumulated) H chain complexes damage the cell, occurs at a later differentiation stage. The lack of L chain in the cam mice must be the result of the failure of the dromedary H chains to associate with L chains, which arises from the difference in important residues in V_HH genes compared with V_H genes (44). Prohibited L chain association may act as a feedback signal that stops L chain translation. This would be in agreement with the observation that in healthy individuals, H chain expression balances L chain synthesis to accomplish equimolar levels (61). In the dromedary H chain mice, which are perfectly healthy, the induced lack of L chain may prevent expression of endogenous H chains, which, if not removed, could be toxic for the cell. In addition, this emphasizes that the introduced dromedary H chain appears to be fully active in securing allelic feedback, albeit at the very late translational stage, which still allows productive DNA rearrangements and transcription of potentially functional endogenous H chains.

The presence of the BCR is essential to govern B cell survival and differentiation (62). Thus, H chain Ab deposition on the cell surface is of key importance for the formation of the H chain Ab repertoire (63, 64). The formation of H chain Abs in camelids is decided by rearrangement of a V_HH gene to commonly used D and J_H segments (44) and (switch?) recombination to a $C\gamma$ gene that permits the removal of C_H1 (22, 24). We speculate that transitory surface expression of μ-H chain without L chain association, as described in the mouse (9–11), may also occur in camelids, and perhaps, unlike in mice, may facilitate successful switching and expression of H chain IgG isotypes with their particular *V* genes, which do not tolerate association with L chain (15, 16, 23). Expression of the membrane form strongly suggests the presence of memory B cells for H chain Abs in camelids. Such cells would undergo an Ab maturation process, leading to H chain Abs with

improved affinities for the Ag. The finding of extensive diversification of H chain Abs (21), but the failure to detect an IgM isotype without L chains in camelids (22, 23), has unexpected implications for H chain Ab ontogeny because it questions the involvement of μ^+ B cells bearing conventional IgM as precursors of H chain Ab-producing cells. For this reason, it becomes important to reassess the developmental progression of B lymphocytes, which can express H chain Abs. The successful generation of transgenic H chain Abs paves the way for the creation of single-chain repertoires in the mouse by introduction of modified V_H genes and splice site mutation in a γ-H chain gene. Retention of V gene variability allows recognition of novel epitopes regarded as poorly accessible by conventional Abs and provides the advantage that single-chain binders are not dependent on successful V_H/V_L pairing (16).

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Disclosures

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