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

Xiangang Zou,* Jennifer A. Smith,* Viet Khong Nguyen,2† Liming Ren,3* Kattie Luyten,* Serge Muyldermans,† and Marianne Brüggemann4*

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 Cγ1 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 disulfide-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 L chains found in conventional Abs, the associated surrogate L chain is replaced with a conventional heteromeric Abs (20). The Journal of Immunology, 2005, 175: 3769–3779.
structural differences in particular V and γ-C genes. As in con-ventional Ab production, the H chain gene of a H chain Ab is obtained after DNA rearrangements when specific V_{γ2}H germline genes, located within the V_{γ2}H gene cluster, are assembled with commonly used D and J_{H} segments to code for the V_{γ2}H-H-domain (21). Genomic and cDNA analyses have revealed five functional droma-edyr γ genes, three of which (γ_{2a}, γ_{2c}, and γ_{3}) are always used to form H chain-only isotypes, whereas two separate genes, γ_{1a} and γ_{1b}, are used for the production of heterodimeric IgG isotypes (22). Only a very low yield of H chain-only Ab transcripts (iden-tified by their particular V genes) spliced to C_{μ} (V_{H}HD_{1}H-C_{μ}) have been identified from dromedary spleen (23). Serum IgM de-void of L chains has not been found, and staining of cameld 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 differen-tiation 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 CH1 domain participates actively in the regu-

\[2a\] lineation and affinity maturation, and their VHHDJH domains are

\[2a\] rephrased to the peripheral blood of all camelids, H chain Abs of different isotypes

\[2a\] valid for their humoral immune response. In the pe-

\[2a\] recognized in particular V genes that are expressed by the mature B cell and are not secreted unless BiP is displaced by the L chain (26, 28, 29). The lack of CH1 in H chain Abs is most likely to be the crucial

\[2a\] gene in transgenic mice initiates B cell

\[2a\] L chain (no. 04-6640; Zymed Laboratories). Bound Abs were detected either with HRP-

\[2a\] g/ml goat anti-llama IgG (no. A160-

\[2a\] 3770 H CHAIN Ab EXPRESSION

\[2a\] 1 Abbreviations used in this paper: BiP, H chain binding protein; BIO, biotin; HEL, hen egg lysozyme; KO, knockout.

\[2a\] does not appear to be regulated by the presence of a classical BCR complex.

Materials and Methods

Derivation of mice

The dromedary V_{γ2}H-Cys\textsubscript{2}TM H chain Ab gene on an 11.7-kb Norf-\textsubscript{Sf} frag-ment (31) was purified using a DNA purification kit (no. 28304; Qagen). For the derivation of transgenic mice, DNA was microinjected into the male pro-

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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 μg of antillama 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 λ1,2,3 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, cam2CA, RAG2+/−, and normal F1 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 IgD (no. 01602D; BD Pharmingen), FITC-conjugated anti-mouse Igκ (no. 559940; BD Pharmingen), FITC-conjugated anti-mouse Igα (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 permeabilization kit with reduced streptavidin (no. SA1006; Caltag Laboratories). Cytoplasmic staining was conducted using a fix and permeabilization kit with reduced streptavidin (no. SA1006; Caltag Laboratories). Cytoplasmic staining was conducted using a fix and permeabilization kit with reduced streptavidin (no. SA1006; Caltag Laboratories). Cytoplasmic staining was conducted using a fix and permeabilization kit with reduced streptavidin (no. SA1006; Caltag Laboratories). Cytoplasmic staining was conducted using a fix and permeabilization kit with reduced streptavidin (no. SA1006; Caltag Laboratories).

Results

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

The H chain Ab gene, \( V_H^{\text{H}}-\text{Cy}2aTM \), has been constructed using a rearranged dromedary \( V_H^{\text{H}}-\text{HDI}_{\text{H}} \) gene with specificity for HEL and a dromedary \( \text{Cy}2a \) gene in germline configuration, including the transmembrane exons (31). Figure 1A shows the 11.7-kb NotI-SalI 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 cam1/−/μMT−/− and μMT−/− animals, which were used for detailed analyses. In Southern blotting, KpnI digests showed several bands containing the transgene (Fig. 1B) with a predominant ~15-kb fragment, suggesting multiple and tandem integration as the construct harbors a ~7.4-kb NotI-SalI 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 Camelds. 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. 2A), which corresponded to \( V_H^{\text{H}}\text{HDI}_{\text{H}}-\text{hinge} \) splice products lacking the C4l. Inclusion of the C4l exon would have increased the size to ~800 bp (31). The hinge region is followed by C4lH (Fig. 2B). Transcripts that allow Ig secretion (Fig. 2C) and surface expression (Fig. 2E, 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 1). Simultaneous amplification of lamin B1 (Fig. 2E, 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 C4l1, in bone marrow and spleen, which

\[ \text{HindIII} \]
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, V3FR1B and hinge (H); B, H and C2H2; C, Cγ3H3 and 3′ of Cγ3H3 stop codon; E, Cγ3H3 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 Cγ3H1 exon is omitted and that the correctly transcribed dromedary H chain consists of V1H-HDJ-H-Cγ2H2-Cγ3H3-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 cam2mt mice in a sandwich ELISA using goat anti-llama IgG for detection. Figure 3 illustrates strong Ab binding of two representative cam2mt 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 Igκ, we conducted Western blot analyses. To overcome a high background, 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. 4A) 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 (V1H) and 3′Cγ3H3 oligos, revealed an aberrant splice product due to tandem integration of the construct, incorporating an extra V1H-HDJ domain (supplemental Table I). This particular dromedary H chain is made up from V1H-HDJ-V1H-HDJ-H-Cγ2H2-Cγ3H3, 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 Cγ3H1 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. 4A, 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 Igκ products from cam1μMT mice is due to the lower levels produced (see Fig. 3).

Because no endogenous Ig could be identified in serum from cam2mt 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 4B shows that no Igλ or Igκ could be detected in total cam2 serum, whereas significant amounts of dromedary H chain Igκ remained as the only serum Ab. In summary, serum from

FIGURE 3. Expression of H chain-only Abs in serum of cam mice. H chain Abs (IgGκ) 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. A lack of L chain Abs was affirmed by coating with anti-llama IgG. A lack of L chain Abs was affirmed by coating with anti-llama IgG. A lack of L chain Abs was affirmed by coating with anti-llama IgG. A lack of L chain Abs was affirmed by coating with anti-llama IgG. A lack of L chain Abs was affirmed by coating with anti-llama IgG.
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 4A). However, from the detailed analysis of the cam1μMT 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. 6A). 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. 6B...
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 Cμ 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 γa H chain could be expressed without the help of other Ig genes, we crossed the cam2 mice with a recently derived line (Cμ/δ-H9004/H11002/δ-H11002) 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, cam2Cμ/δ-H9004/H11002/δ-H11002 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 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.

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 cam2Cμ/δ-H9004/H11002/δ-H11002 mice with removed endogenous Cμ locus. A. Identification of HEL-specific surface Ig plotted against B220 expression in normal mice (NM), cam2 mice, cam2CΔ⁻⁻⁻ mice 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 cam2CΔ⁻⁻⁻ 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.
camCΔ−/− 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-J₅⁄₄H bands, similar levels of V₅₅₈D-J₅⁄₄H and V₅₅₈J₅⁄₄K rearrangement in cam2 and normal mice (Fig. 7). Using forward oligos representing different VH 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 VH 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 V₅₅₈D-J₅⁄₄H 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 mouse. Control reactions conducted in parallel using normal mouse DNA for dromedary H chain amplification and dromedary V₅₅₈H-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-J₅⁄₄H RT-PCR bands from cam2 mice accounted for nonfunctional VDJ rearrangements or represented potentially productive transcripts, we cloned and sequenced the ~400-bp fragments (Table II). Sequence comparison established that fully functional and diverse murine V₅₅₈D-J₅⁄₄H 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-J₅⁄₄H and VκCκ 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 A5, 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-A5 (data not shown). Control reactions (Fig. 8C) 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 A5 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
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 \( \Delta^C \) 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\(_{\text{H}1}\) 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\(_{\text{H}}\)-\( \gamma_2a\)TM construct. The likely reason why dromedary H chain Ig can be expressed in the mouse seems to be due to two gene adaptations in camlids, not found in other jawed vertebrates. Their \( V_{\gamma}\)H genes are distinct from conventional \( V_{\gamma}\)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 \( V\gamma\)H hallmark amino acids, nor the presence of a long CDR3 loop of 24 aa, caused folding problems (43). Nevertheless, the genomic organization of the \( V_{\gamma}\)H 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 \( V_{\gamma}\)H 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 \( V_{\gamma}\)H and \( V_H\) 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 \( C\gamma\) 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 \( C_{\text{H}1}\)-containing sequence from the RNA transcript of \( H\) chain genes appears to be performed with equal efficiency in camlids and transgenic mice. Neither in the dromedary nor in our transgenic mice could \( \gamma_2a\) H chain genes with retained \( C_{\text{H}1}\) exon be identified by RT-PCR and sequencing. Thus, the removal of the \( C_{\text{H}1}\) exon appears to be essential to permit expression of H chain Ig. However, exclusive H chain-only Ab production in camlids was predicted to involve interaction with

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**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 \( 10^3 \)–\( 10^6 \) sorted cells, and equal amounts of cDNA from \( 5 \)× \( 10^5 \) cells were analyzed in parallel by PCR in successive 10-fold dilutions. Oligo combinations of V558-Jg and \( V_{\gamma}\)Cg determined the level of Ig transcription, A5 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.
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 CH1 domain participates actively in the regulation of the assembly and secretion of conventional H2L2 Abs via association with BiP (26–29). A lack of CH1 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 CH1 domain is well recognized because hybridoma or myeloma cell lines harboring Ig genes with deleted CH1 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 IgH 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, camels 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 camels or whether an H chain without CH1 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 μKO 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_{H2}H genes compared with V_{H2} 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 camels is decided by rearrangement of a V_{H2}H gene to commonly used D and J_{H1} segments (44) and (switch?) recombination to a Cγ gene that permits the removal of CH1 (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 camels, 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 camels. 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 camelds (22, 23), has unexpected implications for H chain Ab ontology because it questions the involvement of μ chain Abs 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 reper-}

**References**


**Supplementary Information**

**Table 1.** Alignment of transgene RT-PCR sequences from cam2 spleen RNA (A) and their translation products (B) with the dromedary V\_HHDJ\_H sequence. RT-PCR products of 1.2 and 1.6 kb using V3FR1B-3’C\_H3 primers were cloned and sequenced. This established 2 translational products: V\_HHDJ\_H-H-C\_H2-C\_H3 and V\_HHDJ\_H-V\_HHDJ\_H-H-C\_H2-C\_H3. Base differences are shown in bold which also highlights the reverse primer sequence resulting in amplification and cloning of the larger product. A novel region joining the two V\_HDJ\_Hs is indicated in blue. Sequencing of additional RT-PCR products (Fig. 2) confirmed the results and also established the sequence of the transmembrane part. The exon layout is summarized below.
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| camIgG2a normal | 412-810 | 811-915 | 916-1245 | 1246-1569 | 1570-1701 |
| camIgG2a 2x(VDJ) | 1-411, 412-810 | 811-915 | 916-1245 | 1246-1569 |

### VDJ  hinge  CHH2  CHH3  M1/M2

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| camIgG2a normal | GYTIGPCMGWFRQAPKEREQGVAAINMGGITYYADSVKGRTFTISQDNAKNTYVL | 81 |
| camIgG2a 2x(VDJ) | GYTIGPCMGWFRQAPKEREQGVAAINMGGITYYADSVKGRTFTISQDNAKNTYVL | 81 |


patent accession number: A68583
Table 2. A variety of cDNA sequences (A) and their translation products (B) were obtained from spleen RNA of cam2 mice by cloning ~400bp RT-PCR products using V558-J_H primers. The alignment with dromedary H-chain (camel VDJ) and the most closely matching mouse HV14 V_H-gene from the J558 family showed that the sequences were of mouse origin and fully functional. These rearranged and transcribed endogenous V_H DJ_H genes in the cam2 mice do not carry extensive CDR3s typical for dromedary H-chain antibody genes.
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