Contributions of Conventional and Heavy-Chain IgG to Immunity in Fetal, Neonatal, and Adult Alpacas

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In addition to conventional immunoglobulins, camelids produce antibodies that do not incorporate light chains into their structures. These so-called heavy-chain (HC) antibodies have incited great interest in the biomedical community, as they have considerable potential for biotechnological and therapeutic application. Recently, we have begun to elucidate the immunological functions of HC antibodies, yet little is known about their significance in maternal immunity or about the B lymphocytes that produce them. This study describes the application of isotype-specific reagents toward physiological assessments of camelid IgGs and the B cells that produce them. We document the specificities of monoclonal antibodies that distinguish two conventional IgG1 isotypes and two HC IgG3 variants produced by alpacas. Next, we report that the relative concentrations of five isotypes are similar in serum, milk, andcolostrum; however, following passive transfer, the concentrations of HC IgG2 and IgG3 declined more rapidly than the concentration of conventional IgG1 in the sera of neonates. Finally, we assessed the distribution of B cells of distinct isotypes within lymphoid tissues during fetal and adult life. We detected IgG1, IgG2, and IgG3 in lymphocytes located in lymph node follicles, suggesting that HC B cells affinity mature and/or class switch. One IgG3 isotype was present in B cells located in ileal Peyer’s patches, and one conventional IgG1 isotype was detected in splenic marginal zone B cells. Our findings contribute to the growing body of knowledge pertaining to HC antibodies and are compatible with functional specialization among conventional and HC IgGs in the alpaca.

Camelids produce functional IgG isotypes that do not incorporate light chains (19, 39). In addition to these heavy-chain (HC) isotypes (classified as IgG2 and IgG3), camelids produce conventional IgG1. First described in the dromedary, camelid isotypes were named according to the decreasing apparent molecular masses of their H chains in SDS-PAGE and, subsequently, by their differential binding to protein A and protein G (19, 27, 40). These binding properties have been exploited in purification schemes, and the fractions recovered have been used to estimate serum concentrations of antibodies (Abs).

Assessment of llama and camel genomic and cDNA sequences revealed the existence of at least six and nine γ chain genes, respectively (40; reviewed in reference 8). In the dromedary, four genes are likely to be pseudogenes and the remaining five encode two conventional γ chains, γ1a and γ1b, and three HC isotypes, γ2a, γ2c, and γ3. In the llama, a gene encoding an additional HC isotype, γ2b, has been reported (8, 44). The genes encoding HC isotypes have a mutation within the splice consensus sequence of the CH1 domain that results in the exclusion of this domain from the protein structure (29). In the dromedary, genomic and cDNA sequences have been obtained for a conventional μ chain, and cross-reactive anti-serum indicates the presence of IgA. Sequence analysis of the alpaca heavy-chain locus has revealed only two HC isotypes, together with conventional γ1a, γ1b, μ, δ, α, and ε-coding sequences (1). The immunoglobulins encoded by these genes have not been thoroughly characterized in the alpaca.

The V genes that encode HC V domains (VH) are distinct from those encoding conventional V domains (VH). VH genes are distinguished by the presence of codons corresponding to extended CDR3 loops and specific amino acid substitutions at five distinct positions within the framework 2 region (30, 40). Interestingly, the VH and VH genes rearrange with the same set of J and D genes, which is consistent with an interspersed arrangement (1, 8).

The biophysical attributes of HC Abs are comparable with those of conventional antibodies, with some important exceptions. The absence of a CH1 domain affords HC γ chains lower apparent molecular masses than conventional γ chains. This difference, taken together with the absence of light chains, makes HC Abs considerably smaller than conventional antibodies, which may allow them greater access to antigens (Aggs). HC Abs are bivalent, and the single VH comprises the Ag-binding platform. Extended CDR3 loops provide an increased Ag-binding surface, compensating for the loss of the VL and contributing to the high affinity of the binding site (12, 28). These structural features enable VH to bind epitopes within the catalytic sites of enzymes (13, 14, 24), suggesting potential as enzyme inhibitors. Evidence points to the presence of somatic hypermutation within the VH gene; however, it has not been ascertained whether this occurs in response to antigen or during lymphocyte development, or both (1, 19, 24). The ag-
aggregate physical features of HC Abs and the ease with which their V_{H}H domains can be expressed in bacterial and yeast (Pichia) systems make them attractive biotechnological and therapeutic tools (4, 34, 38).

HC Abs constitute approximately 50% of serum IgG, compatible with a significant role in camelid immunity. We have shown previously that alpaca IgG3 is highly efficient in neutralization of West Nile virus (WNV), and the effect is enhanced in the presence of complement (10). Furthermore, IgG2 and IgG3 can bind to surfaces of mononuclear cells, suggesting that specific Fc receptors are present. Both HC and conventional IgGs are capable of enhancing WNV infection of cultured mononuclear cells in vitro, providing further evidence of effector function (10). Nevertheless, the concentrations of IgG2 and IgG3 in body fluids have not been determined. Furthermore, the contribution of HC Abs to maternal immunity, which is critical to newborn health and well-being, has not been investigated. Information on the development and distribution of the B lymphocytes that produce these unusual antibodies is lacking.

This report describes the application of isotype-specific reagents toward physiological assessments of camelid IgGs and the B cells that produce them. First, we document the specificities of monoclonal antibodies (MAbs) that distinguish the two conventional IgG1 isotypes as well as the two HC IgG3 species produced by alpacas. Next, we report the concentrations of each IgG in serum, milk, and colostrum and assess the distribution of the B cells of distinct isotypes within lymphoid tissues during fetal and adult life.

MATERIALS AND METHODS

Reagents. MAbs specific for llama/alpaca IgG1 (clones 24F1, 26G11, 27E10, and 28G4), IgG2 (clones 10E6, 13A9, 16A4, 16H10, and 19D8), and IgG3 (clones 1C11, 1D1, 2B11, 3E6, and 8E1) have been described elsewhere (9). Horseradish peroxidase (HRP)-conjugated goat antibodies to llama IgG (H+L; Bethyl) were used in enzyme-linked immunosorbent assays (ELISAs) and Western blots. Mouse antibodies were detected using HRP-goat anti-mouse IgG (MP Biomedicals). LH41A and LT97A bind undetermined cell surface epitopes expressed by llama/alpaca B and T lymphocytes, respectively (Washington State University [WSU] Monoclonal Antibody Center) (11). TH14B binds major histocompatibility complex (MHC) class II expressed by llama/alpaca cells (WSU Monoclonal Antibody Center).

SDS-PAGE and Western blots. IgGs were resolved in discontinuous, 15-cm polyacrylamide gels under reducing and nonreducing conditions as described elsewhere (9). Briefly, samples and molecular weight markers (Bio-Rad Laboratories) were boiled for 7 min in sample preparation buffer, with or without 2-mercaptoethanol, before they were loaded. Gels were stained for 1 h with Coomassie blue or were transferred to nitrocellulose membranes.

Western blots were developed at room temperature. Each incubation step was conducted for 1 h unless otherwise specified. Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) and washed with TBS containing 0.05% Tween 20. The wells were blocked with 50 μl DPBS containing 2% skim milk–0.05% Tween 20 and then incubated with hybridoma supernatant diluted 1:10 in blocking solution. Bound MAbs were detected with 5 μg/ml HRP-goat anti-mouse IgG diluted in blocking buffer containing 10% normal goat serum. The assay was developed with 3,3′,5,5′-tetramethylbenzidine (TMB; KPL), and the reactions were terminated with 1 M H₃PO₄. Optical densities were measured at 450 nm.

Collection of alpaca blood and lacteal fluids. Samples collected from 14 healthy alpaca dam-cria pairs (Cas-Cas-Nac Farm, Perkinsville, Vermont) were evaluated. Lacteal secretions were collected at parturition (colostrum) and 24 h later (transitional milk). Blood samples were collected from dams and crias by jugular venipuncture in Becton-Dickinson Vacutainers and centrifuged to separate the serum, and sera were stored at −20°C until use.

Calculation of IgG concentrations in body fluids. Fluids were assayed directly for all isotypes except IgG3_8E1. In this case, samples (n = 3) were first depleted of IgG3_8E1 using 8E1-Sepharose affinity columns and reconstituted to their original volumes prior to assay.

The ELISA described above was modified to estimate IgG concentrations in lacteal fluids and sera. Conditions were as described above, except that microtiter plates were coated with 5 μg/ml MAbs, affinity-purified IgGs were used as standards, wells were incubated with sera or lacteal fluids, and bound alpaca antibodies were detected with 0.1 μg/ml HRP-goat anti-llama IgG.

Immunohistochemistry. Lymphoid tissues were collected from animal carcasses submitted to the necropsy service at the College of Veterinary Medicine at Cornell University. Tissues were collected from two fetuses (5 and 6 months of gestation) and seven adult male and female animals (2 to 14 years of age). Tissues were embedded in tissue-freezing medium (Electron Microscopy Sciences) and snap-frozen on dry ice. Sections were prepared (7 μm; Cytocut 1800; Reichert-Jung), mounted on glass slides, and stored at −80°C. Prior to immunohistochemical procedures, slides were washed to room temperature and then fixed in 100% acetone for 10 min. Endogenous peroxidase activity was quenched with phosphate-buffered saline (PBS) containing 0.3% H₂O₂ and 1% NaN₃. Nonpecific binding was blocked by incubating sections in PBS containing 5% skim milk and 10% normal goat serum. Subsequent procedures were conducted at room temperature, and the slides were washed in three changes of PBS following each incubation step. Sections were incubated with MAbs diluted in blocking solution. Bound antibodies were detected with HRP-conjugated goat anti-mouse IgG, and color development was carried out using 3-amino-9-ethyl-carbazole (AEC; Sigma). Sections were counterstained with Gill’s no. 2 hematoxylin (Vector Labs), rinsed in tap water, and mounted with Glycergel (Dako); and images were obtained using a BX51 microscope fitted with a MagnaFire SP digital camera system (Olympus).

RESULTS

Isolation of a novel HC Ab and a second conventional IgG1 from alpaca serum. During routine IgG purification procedures, we observed that repeated passage of alpaca sera over a series of Sepharose affinity columns prepared with MAbs 27E10, 19D8, and 8E1 failed to deplete samples of IgG-like antibody. Affinity chromatography was performed using fast-performance liquid chromatography (AKTA-FPLC; GE Healthcare).

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proteins, despite completely adsorbing all MAb-reactive proteins (confirmed by ELISA). To further investigate the nature of the unbound fraction, preparations were loaded onto a protein G affinity column and the eluted protein was resolved by SDS-PAGE under reducing conditions. This preparation contained four distinct protein bands, three of which were detected in a Western blot by polyclonal antibodies to llama IgG (H+L) (Fig. 1A). A fourth, weak band having the slowest electrophoretic mobility was not detected by the anti-IgG antibodies and was not characterized further. In order to identify the immunoglobulins, an ELISA was conducted using the entire panel of 14 isotype-specific MAbs that we have described previously (9). Four MAb clones were found to detect epitopes within the depleted preparation that bound to and that was eluted from protein G: three that were generated against llama IgG1 (24F1, 26G11, and 28G4) and one that was generated against llama IgG3 (1D1) (Fig. 1B). Clone 26G11 bound exclusively to the eluted protein, suggesting that an IgG isotype that was distinct from the IgG1 recognized by 27E10 was present. In contrast, clone 1D1 bound epitopes present within the protein G eluted fraction as well as on the 8E1-specific IgG3. Subsequent experiments documented additional distinctions between alpaca IgG1 antibodies recognized by 27E10 and 26G11 and also IgG3 antibodies recognized by 1D1 and 8E1 (see below).

To further investigate these novel IgGs, affinity columns were prepared with clones 26G11 and 1D1 and, together with 27E10, 19D8, and 8E1 columns, were used to purify IgGs from alpaca sera. Eluates from all five affinity columns were characterized by SDS-PAGE (Fig. 1C, quadrants i and ii) and detected with polyclonal anti-llama IgG antibodies in Western blots (Fig. 1C, quadrants iii and iv). IgGs eluted from the 27E10, 19D8, and 8E1 columns resolved in a manner similar to previously published results (9). A conventional IgG species was eluted from the 26G11 column that was comprised of H chains (49.9 kDa) and L chains (23.1 to 25.5 kDa) that resolved separately under reducing conditions (Fig. 1C, quadrants i and iii). Although visible in Coomassie blue-stained gels, the L

FIG. 1. Identification of novel IgGs in alpaca serum. (A) Affinity columns were prepared by coupling anti-IgG1 (27E10), anti-IgG2 (19D8), or anti-IgG3 (8E1) MAb to Sepharose. Serum was passed over all three columns before it was loaded onto a protein G column. Protein G eluate was resolved by SDS-PAGE under reducing conditions. Gels were stained with Coomassie blue (Cb) or blotted onto nitrocellulose membranes for development with HRP–goat anti-llama IgG (H+L) (Western blotting [WB]). LC, light chain. (B) Protein G eluate was analyzed in an ELISA using a panel of MAbs specific for llama IgG isotypes (10). Plates were coated with 1 μg/ml of the unknown protein or affinity-purified IgG1, IgG2, or IgG3. Supernatants from 14 hybridoma clones were applied to the wells at a 1/10 dilution. Bound MAbs were detected using HRP–goat anti-mouse antibodies. Arrows indicate MAbs that were selected for use in subsequent assays. Asterisks denote binding of llama IgG isotypes by the anti-IgG1 (27E10), anti-IgG2 (19D8), or anti-IgG3 (8E1) MAb that was used to prepare affinity columns. (C) Affinity columns were prepared by coupling anti-IgG isotype MAbs to Sepharose. Serum was loaded onto these columns, and bound proteins were eluted and characterized by SDS-PAGE under reducing (i and iii) and nonreducing (ii and iv) conditions. Gels were stained with Coomassie blue (i and ii) or blotted onto nitrocellulose membranes for Western blot development (iii and iv). Arrows highlight lanes containing novel IgG proteins isolated from alpaca serum.
chains of 26G11-specific IgG were not detected by polyclonal goat anti-llama IgG (H+L), a result that remains unexplained. In contrast, the L chains of 27E10-specific IgG reacted with the polyclonal antibodies (Fig. 1C, quadrants i and iii). Further, the electrophoretic migration of intact 26G11-specific IgG was retarded under nonreducing conditions compared to that of 27E10-specific IgG1 (Fig. 1C, quadrants ii and iv). The data are consistent with the conclusion that the 26G11 MAAb binds an IgG1 isotype that is distinct from 27E10-specific IgG1. We refer to these two proteins as IgG127E10 and IgG126G11, until amino acid sequences that relate them to published IgG1a and IgG1b gene sequences are obtained (1, 40).

The 1D1 eluate was evaluated and found to contain a single IgG protein that resolved in SDS-PAGE under reducing conditions as a small (44.3 kDa) HC species similar to 8E1-specific IgG3 (IgG38E1) (Fig. 1C, quadrants i and iii). Given that clone 1D1 was initially generated against protein G-enriched llama IgG antibodies and that it cross-reacts only with proteins bound by 8E1, we deduce that this MAAb detects a distinct IgG3, hereafter referred to as IgG31D1.

Concentrations of IgG isotypes in lacteal secretions and serum. In order to evaluate the concentrations of the different IgG isotypes in lacteal secretions and sera from alpacas, we performed capture ELISAs with colostrum, transitional milk, and serum collected from 14 female alpacas that had recently given birth. For the purpose of calculating IgG31D1 concentrations, a small subset of samples (n = 3) were depleted of IgG38E1, prior to assaying in the ELISA. Results are summarized in Fig. 2. As expected, alpaca colostrum was highly enriched in all isotypes of IgG compared with the amount in transitional milk (Fig. 2A). HC Abs represented 38.5% of the total colostral IgG (Fig. 2B). The predominant HC in colostrum was IgG127E10, with IgG126G11, IgG31D1, and IgG38E1 being equally represented and IgG2 being the least well represented. Within 24 h of parturition, IgG concentrations in transitional milk had fallen approximately 10-fold (Fig. 2C). All IgG isotypes were represented in similar relative proportions in colostrum, milk, and serum (Fig. 2D). The presence of HC Abs in lacteal secretions is consistent with a role for the antibodies in passive transfer of immunity to neonates.

Concentrations of IgG isotypes in sera of neonatal crias. To further evaluate transfer of passive immunity, blood samples were collected from crias born to the dams described above. We measured IgG concentrations in sera collected from crias between 1 day and 3 months of age (Fig. 3A). High concentrations of IgG in sera of 1-day-old crias reflected successful passive transfer of maternal antibodies (17, 22, 41, 43); however, there were no statistically significant correlations between the IgG concentrations in the colostrum of the dam and those in the serum of her 1-day-old cria (data not shown). In cria sera, the levels of IgG2 and IgG38E1 declined much more rapidly than those of conventional IgG1 over time. Concentrations of HC Abs fell by 50% within 14 days, as opposed to 25 days and 20 days for IgG127E10 and IgG126G11, respectively (Fig. 3B). Due to the limited sample size, IgG31D1 was not evaluated. In the majority of animals, the decline in serum antibodies ceased after 42 days of age (Fig. 3A and B), indicating that cria B cells had begun to secrete measurable quantities of conventional and HC antibodies. The data confirm that colostral HC Abs were efficiently transferred from dam to cria.

Distribution of B lymphocytes within adult lymphoid tissues. In order to describe the distribution of HC Ab-producing cells, we examined secondary lymphoid tissues of adult alpacas using immunohistochemical methods. The data shown are representative of results obtained from seven animals. Cells bearing IgG of all five isotypes were found dispersed throughout the lamina propria of the ileum, along with T cells and MHC class II-positive (MHC II +) cells (Fig. 4A and data not shown). Only antibody 1D1 stained cells in ileal Peyer’s patches. In spleen, B cells present in red pulp were predominantly IgM positive (IgM +) (data not shown), together with occasional IgG1 + and IgG38E1 + cells. Only IgG31D1 was detected among splenic follicular B cells. Of particular interest was the observation that B cells producing IgG126G11 were detected in the marginal zone. T cells were located within periarteriolar lymphoid sheaths (PALS; data not shown). Within the mesenteric lymph nodes, IgM + and IgG38E1 + cells were present mainly in extrafollicular sites and appeared to be excluded from follicles (Fig. 4C and data not shown), while IgG1 +, IgG2 +, and IgG31D1 + cells were present within follicles of lymph nodes (Fig. 4C and data not shown). In summary, HC Ab-positive (HC Ab +) cells were distributed in conventional B-cell compartments juxtaposed to T cells, suggesting that these cells are poised to execute functions characteristic of B cells, e.g., class switching and affinity maturation. The unique representation of IgG126G11 in the marginal zone and of IgG31D1 in Peyer’s patches supports an association with antibacterial and mucosal immunity, respectively.

Distribution of B lymphocytes in fetal tissues. To begin to describe the life history of IgG-producing B cells, we used immunohistochemical methods to examine lymphoid tissues from alpaca fetuses at 5 and 6 months of gestation. Images from the 6-month-old fetus are shown; however, results were similar in the animals at the two times of gestation. LH41-positive (LH41 +) cells (a population that includes B lymphocytes) were detected in fetal liver; however, these cells were rare and found mainly within endothelia or in close proximity to hepatic blood vessels (Fig. 5A). LH41 + cells were more prevalent in the fetal bone marrow and in follicles within the ileum. In contrast, neither IgM- nor IgG-producing cells were detected in fetal liver, bone marrow, or ileum at 6 months gestation. Of note, T cells were detected in the thymus in both fetuses at this time (data not shown). The architecture of the spleen was well developed at midgestation, and follicles in the white pulp were readily observed. This organ was the most active site for lymphocyte colonization of all fetal tissues examined. The LT97 MAAb identified T cells within PALS contiguous with cellular aggregates that stained with LH41, while MHC II + cells formed aggregates between the PALS and adjacent blood vessels (Fig. 5B). IgM (data not shown), as well as conventional and HC isotype-containing B cells, was detected in follicles (Fig. 5B) as well as the extrafollicular regions of the spleen. Our observations are compatible with the conclusion that at midgestation, conventional and HC Ab + B cells develop in the spleen.

DISCUSSION

Previously, we reported on the production and characterization of MAAbs generated against llama IgGs. The antib-
ies could be discerned among three isotypes of IgG and cross-reacted with alpaca isotypes (9). Additionally, we have validated the usefulness of these reagents in evaluating immunity induced by vaccination or by natural parasitic or viral infections (9, 10). Here we report the results of additional analyses showing that these reagents allow us to distinguish two isotypes of IgG1 and two variants of IgG3. The finding of three HC isotypes in the alpaca is inconsistent with the genetic analysis that revealed only two HC γ genes in the H-chain locus (1). The two IgG3 variants that we identified are unlikely to be allotypic variants, as the antibodies that distinguish them were raised against llama IgG and the B cells producing them are distributed in distinct locations. The apparent discrepancy between these findings and the genetic data merits further investigation.

Similar to other ruminants, camelids are born severely
hypogammaglobulinemic. The epitheliochorial placenta prevents passive transfer of antibodies in utero, and crias obtain maternal IgGs from colostrum (18, 26). Failure of passive transfer of immunity by this route correlates with morbidity and mortality in camelids (18, 41). Our estimates of total IgG concentrations in dam colostrum (196.2 ± 25.9 mg/ml) and day 1 cria sera (22.2 mg/ml ± 4.8 mg/ml) corroborate those of previous studies (17). Analysis of individual IgG isotypes indicated that conventional and HC isotypes are represented in serum, colostrum, and milk in similar relative concentrations, demonstrating that transfer is not selective. The neonatal Fc receptor (FcRn) has been detected in dromedary mammary gland tissue (21). On the basis of conserved contact residues in the Fc receptor and Fc regions of antibodies, this receptor is predicted to bind both conventional and HC dromedary IgGs (21). Thus, via FcRn transport, the repertoire of all maternal IgG isotypes would be delivered into colostrum. Following ingestion of colostrum, the relative concentrations of each isotype in cria sera parallel those in dam sera, demonstrating that absorption by the cria is not selective.

IgGs in alpaca cria sera declined during the first 4 to 6 weeks of life, results that correlate with those from studies conducted in the dromedary (39, 43). We found that HC Abs declined more quickly than conventional IgGs in cria sera (50% reduction in 14 days versus 20 to 25 days, respectively), suggesting that HC Abs may have a shorter half-life that may impact their function. Newborn crias are routinely vaccinated against *Clostridium*, rabies, and tetanus at 2 and 4 months of age. The increase or maintenance of serum IgGs after 6 weeks of age likely reflects the activation of the cria’s own immune response by vaccination and other antigenic stimuli.

B-cell ontogeny is described by the lymphoid compartment

FIG. 3. IgG concentrations in neonatal cria sera. (A) Concentrations of IgG1, IgG2, and IgG3 were obtained by ELISA, as described in the legend to Fig. 2. In all groups, n = 14, except for 1D1 values, where n = 3. Zero-concentration values are plotted as the lowest value on the y axes, 0.008 mg/ml, in order to represent these values on the graphs. (B) Mean concentrations and estimated times for IgG concentrations in neonatal sera to decline 50% (\(t_{1/2}\), half-life). Values were calculated on the basis of the IgG concentration in cria sera reported in panel A at between 0 and 14 days of life. In all groups, n = 14, except for 1D1 values, where n = 3.

| Isotype | Specific mAb | Mean conc. (mg/ml) | \(t_{1/2}\) |
|---------|--------------|--------------------|------|
| IgG1    | 27E10        | 6.34               | 25   |
|         | 26G11        | 3.18               | 20   |
| IgG2    | 19D8         | 1.12               | 14   |
| IgG3    | 8E1          | 1.46               | 14   |
|         | 1D1          | 5.88               | ND   |
within which the developmental process occurs, in addition to the mechanism and age at which gene diversification of the antibody repertoire occurs (6, 42). In contrast to humans and mice, B-cell development in cattle, sheep, pigs, and rabbits is restricted to the prenatal and neonatal stages of life and re- ceptor diversification occurs within gut-associated lymphoid tissues (GALTs) (42). In these species, commonly referred to as GALT species, gene conversion is the prevailing mechanism of antibody repertoire diversification (16). During gestation, lymphopoiesis occurs first in the fetal liver and is subsequently transferred to the bone marrow (3). In the sheep, B-cell development and expansion occur within the spleen (33), prior to the colonization of the GALT by mid- to late gestation (23, 36, 45). That is, IgM⁺ B cells appear first in the spleen (33), subsequent to populating the jejunum and ileum by days 65 and 68, respectively (2, 31). It is the ileal B cells that eventually disseminate to secondary lymphoid organs (35, 37). Recent studies of fetal bovine tissues collected throughout gestation implicate the bone marrow and lymph node in early B-cell lymphopoiesis, with subsequent development occurring in the GALT (15). Additionally, B cells were observed in the spleen throughout gestation.

Our results provide evidence that camelid B-cell development is likely analogous to that of the ruminants. Assessment of fetal alpaca tissues collected midgestation revealed that, similar to cattle, the bone marrow serves as a site for HC and conventional B-cell lymphopoiesis; however, the spleen appeared to be the most active and likely functions to support early development, expansion, and possibly, class switching of B lymphocytes. Structural incompatibilities in H and L chains would appear to preclude isotype switching from conventional IgM to an HC isotype (i.e., HC γ chains would not stably associate with L chains or surrogate L chains). To date, there is no evidence to support the existence of an HC IgM, suggesting that HC B cells may develop via a distinct pathway that substitutes a γ HC for the μ chain in B-cell precursors. In support of this, Zou et al. (46) have shown that HC IgG expression can instigate B-cell development independently of IgM expression. In contrast, Achour et al. (1) reported that 5% of IgM transcripts in alpaca blood incorporate unmutated VHH, supporting the conclusion that HC-producing B cells develop via an IgM-bearing precursor cell.

In adult alpacas, the distribution of HC Ab-producing B cells among and within secondary lymphoid tissues was similar to that of conventional B cells. HC Ab⁺ cells were found in all the lymphoid tissues examined, including those at mucosal sites. Collectively, mucosal surfaces encounter a vast array of antigens to which most of the resident lymphocytes become tolerant. The functions of the B cells found within Peyer’s patches and isolated lymphoid aggregates are distinct, and in the presence of antigens, cells in these locations become predominantly IgA-secreting plasma cells (7, 20). In intestinal tissues, the prevalence of IgG31D1⁺ cells was low; however, the presence of IgG31D1⁺ B cells within marginal zones of alpaca spleens implicates a role for this isotype in defense against...
blood-borne and T-cell-independent antigens in these animals. The observation merits further investigation.

In lymph nodes, the presence of HCAb⁺ cells within follicles provides evidence that these cells use this site to undergo affinity maturation and perhaps isotype switching. In other species, activated B cells migrate from this site to the bone marrow, where they terminally differentiate into plasma cells. Our data indicate that HCAb-producing plasma cells reside within the supportive environment of the bone marrow of the alpaca.

Our results indicate that the humoral branch of the camelid immune system is comparable to that of ruminants and other GALT species. HC IgGs are well represented in colostrum and milk and are likely to be important in passive transfer of immunity to neonates. Furthermore, HC Ab-producing B cells develop and are distributed in lymphoid tissue in ways that are similar to those for B cells producing conventional IgGs. Selective distribution of B lymphocytes of two isotypes suggests that these antibodies may have specialized functions. Our findings contribute to the growing body of knowledge pertaining to HC isotypes and are compatible with functional specialization among IgGs in the alpaca.

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FIG. 5. Distribution of B lymphocytes within fetal alpaca lymphoid tissues. Positive cells are red and are indicated by arrowheads. (A) B cells were detected in fetal liver, bone marrow, and ileal tissues at midgestation (6 months). Tissue sections were incubated with clone LH41, which identifies B cells. Scale bars, 50 μm. (B) Organization of follicles and distribution of IgG⁺ B cells within white pulp of the fetal spleen. Stippled lines delineate white pulp, including follicle. RP, red pulp; PALS, periarteriolar lymphoid sheath; B, B cell zone; A, arteriole. Scale bars, 50 μm.
