THE SIGNIFICANCE OF HEAVY-CHAIN ANTIBODIES IN CAMELID IMMUNITY

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Camelids produce IgG isotypes that do not conform to the rules governing conventional antibody structures. Typically, immunoglobulins combine homodimeric heavy and light chains to produce characteristic tetrameric structures. Unlike IgG1, the camelid IgG2 and IgG3 isotypes do not incorporate light chains into their structures. These unusual antibodies, generally referred to as heavy-chain antibodies (HCAbs), comprise approximately 50% of serum IgGs, compatible with a significant role in camelid immunity. Hitherto, the effector functions of camelid HCAbs remain largely undefined primarily due to the dearth of available isotype-specific reagents. As it was our objective to investigate camelid HCAbs, we produced and characterized monoclonal antibodies that discern among the IgG isotypes produced by both llamas and alpacas. These reagents were employed in affinity chromatography, serologic analyses, virus neutralization assays, flow cytometry, and immunohistochemistry to differentiate between the B cell sub-populations that synthesize conventional and heavy-chain IgGs, determine whether expression of the different IgG isotypes was regulated differently, and to investigate the clinical and physiological relevance of HCAbs.

Our investigations led to the discovery of a novel IgG3 protein, and the production of immunological reagents that discern among two conventional IgG1, IgG2 and the two IgG3 HCAbs. We have documented that the B cell sub-populations

developed within similar lymphoid compartments during gestation. At birth, HCAbs obtained through colostrum contributed to the passive transfer of immunity that is critical in protecting the newborn until maturity of its immune system. Throughout life, the B cell sub-populations continued to occupy similar compartments, although, B cells that locate within splenic marginal zones expressed one sub-isotype of IgG1 exclusively. Also, follicular B cells within adult, ileal Peyer's patches expressed only the novel IgG3. In response to pathogen infections, camelid B cells elicited IgG2 to helminths and IgG3 to viral infections: IgG1 expression was ubiquitous. Anti-viral IgG2 were induced only by hyperimmunization. While IgG3 proved to be as potent as IgG1 in neutralizing activities, anti-viral IgG2 was ineffective. The data presented here document for the first time a dichotomy in the effector functions of camelid IgG2 and IgG3 HCAbs.

BIOGRAPHICAL SKETCH

Lisa Patryce Daley was born January 9, 1975. At age six, she attended the Spanish Town Primary school where she sat the Common Entrance examinations in 1986. Her performance in this examination allowed her to be accepted into the Holy Childhood High School for girls. There she realized her love for the sciences, which she decided to pursue. Lisa attended the University of the West Indies, Mona Campus where she obtained a Bachelors of Science in Zoology with a minor in Chemistry. During her final year in college she was introduced to the world of Parasitology, which she found quite fascinating and decided to pursue as a Masters of Philosophy degree. The story of parasites and their relationships with their hosts seemed incomplete and, as a result, Lisa became curious about host responses to parasites. She then decided to pursue a Doctor of Philosophy degree in Immunology. She selected Cornell University because Dr. David Pimentel, who had conducted similar work as she had been doing for her M.Phil. research, was a professor there. His kind responses to her emails made her believe that Cornell would be a wonderful environment for such studies. She contacted Professor Judith Appleton after reading of her excellence in teaching and decided that this criterion would make her the ideal mentor. She entered the laboratory with the intention of studying *Trichinella spiralis*, but became intrigued by some 'rebel' antibodies produced by animals she had never seen in her life. Lisa decided to conduct Ph.D. research on these heavy-chain antibodies.

Dedicated to

my mother,

Sandra Rose Marrie Taylor

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CHAPTER 1

Introduction

The Camelids

Characteristics

Camelids are socio-economically important animals that are used as pack animals, sources of fiber and meat, as well as companion animals. These animals are well adapted for inhabiting extreme environments where nutrient-rich food sources are scarce. Accordingly, camelids are highly efficient at ruminating, even more so than ruminants (1). These animals are often classified as ruminants, which is a misnomer as camelids are a distinct group of Artiodactyls. Apart from the obvious differences in physical features, camelids are distinct from ruminants in that they have a three compartment stomach rather than four, their dentition is unique in that the upper jaw contains a single, isolated incisor, the upper lip is bifurcated and independently mobile, and they have padded two-toed feet as opposed to hooves.

Old World camels have characteristic dorsal humps and represent the largest species of camelids, weighing up to ~ 1500 pounds. The one-humped dromedary (Figure 1-1*A*) may grow to 6 ft 11 inches at the poll (part of the head between the ears) while the two-humped Bactrian camel (Figure 1-1*B*) is slightly taller and may attain 7 ft 6 inches. These animals have wide, padded feet and hairy coats. In contrast, New World camels, which include the guanaco, vicuña, llama and alpaca, are much smaller. The two wild species, the guanaco (Figure 1-1*C*) and the vicuña (Figure 1-1*D*) are similar in coloration but have several distinguishing characteristics. The guanaco has a grey face, and both the tail and ears are longer than those of the vicuña (1). Guanacos are slightly taller (~ 3 ft 6 inches at the withers) than the vicuña (~ 3 ft 6 inches) and heavier (≤ 265 lbs vs. ≤ 200 lbs) (1). The coats are also dissimilar in that the guanaco is double-coated with course guard hairs and a soft undercoat while the vicuña lacks guard hairs. Of the domesticated species, the llama (Figure 1-1*E*) is larger than the alpaca (Figure 1-1*F*). In fact the llama is the largest of these species

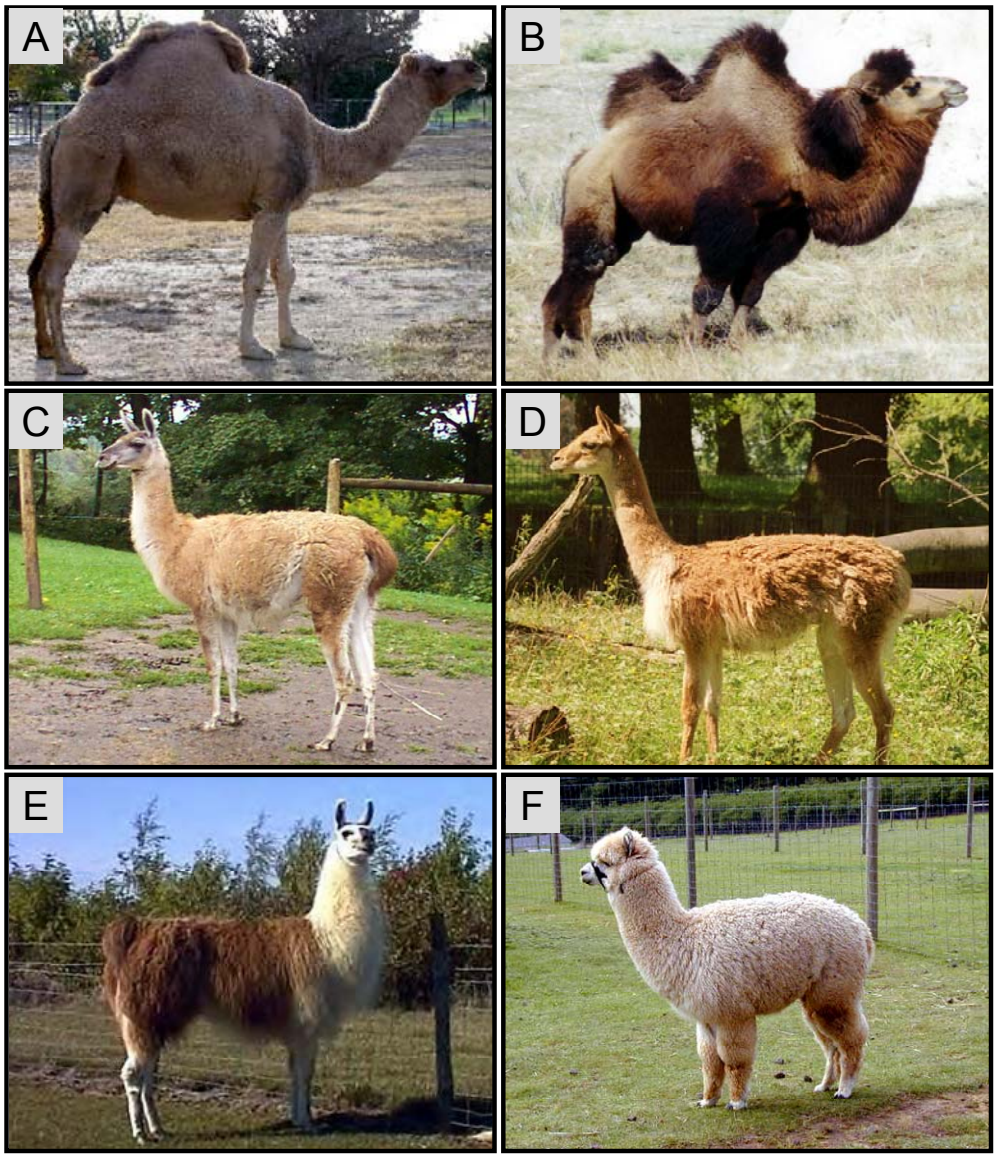

Figure 1-1. Characteristic features of the Old **(A and B)** and New **(C - F)** World camelids. **(A)** dromedary; **(B)** Bactrian; **(C)** guanaco; **(D)** vicuña; **(E)** llama, and **(F)** alpaca.

(~ 4 ft at the withers and ≤ 550 lb) while the alpacas are similar in size and weight to the vicuñas (1). The llama is double-coated while the alpaca is not. In general, amongst all the camelid species males are larger than females. In terms of fiber quality, the vicuña has the finest fiber followed by the guanaco, alpaca then llama.

All the camelids have identical karyotypes ($2n = 74$) and are capable of interbreeding to produce fertile hybrids (2-4). Anthropogenic and natural hybridization have been known to occur with members of the same tribe (see below). In addition, it has been proven that it is possible to obtain a fertile offspring from hybridization between a guanaco and a dromedary camel (5).

Classification

The three sub-ordinal clades of Artiodactyla include Ruminantia, Suiformes and Tylopoda (Figure 1-2). The Camelidae is the only extant family of Tylopods; Oromerycidae, Protoceratidae, and Xiphodontidae have become extinct. Originally, five sub-families of Camelidae existed; Alticamelinae, Camelinae, Poëbrotheriinae, Pseudolabidinae, and Stenomylinae. Fossil records document that Camelinae was the most diverse of the sub-families. All the species, except members of Camelinae, have become extinct. Camelinae is further divided into three tribes; Lamini, Camelini and Camelops. Species within the latter tribe have all become extinct; however, extant species of the Lamini tribe include the wild *Lama guanicoë* (guanaco) and *Vicugna vicugna* (vicuña), and their domesticated descendants, *L. glama* (llama) and *V. pacos* (alpaca), respectively. There is further sub-classification of New World lamoids into breeds. Of the Camelini tribe, it is believed that the *Camelus dromedarius* (dromedary) derived from the *C. bactrianus* (Bactrian). Today, a small population of wild Bactrian camels dwells within the Gobi Desert; however, wild dromedaries became extinct c. 3000 B.C.

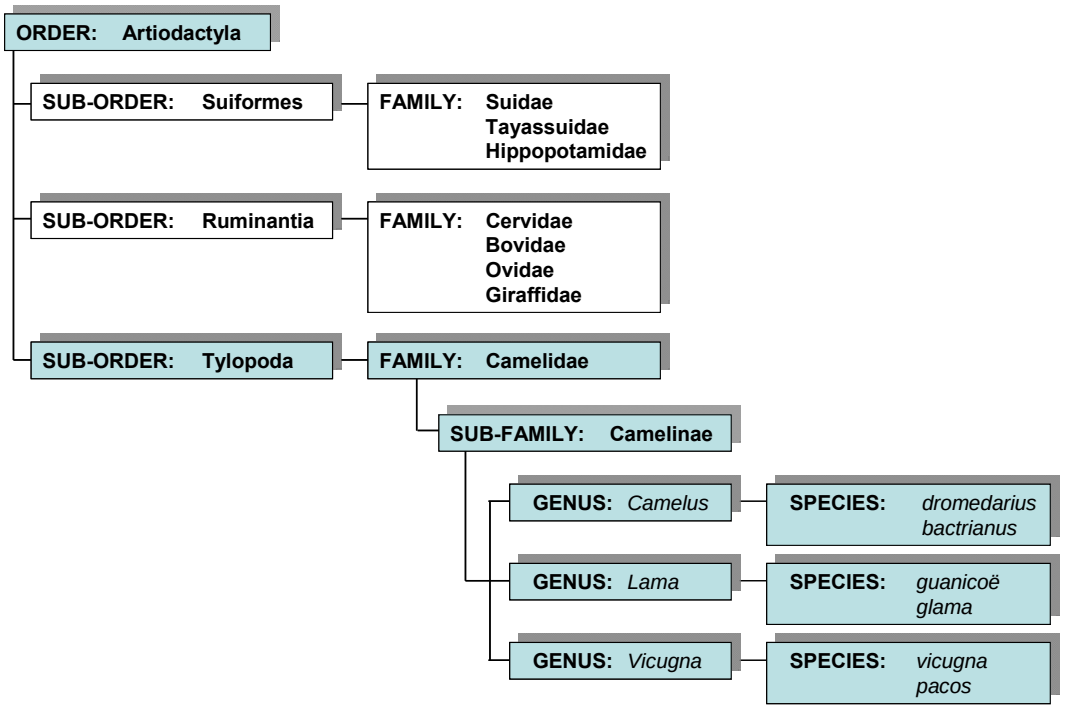

Figure 1-2: Taxonomy of extant camelids

Origin

Tylopods emerged in North America circa (c.) 50 million years ago (m.y.a.) during the middle Eocene epoch (6). While the Camelidae line persisted, members of Xiphodontidae all became extinct by the Oligocene epoch (c. 35 m.y.a.) (7). Mitochondrial DNA analyses suggest that the separation between the Lamini and Camelini tribes took place c. 11 m.y.a (8). Fossil records indicate that Camelinae was the only sub-family that emigrated out of North America. Members of the Lamini tribe migrated through the Isthmus of Panama into South America while those of the Camelini tribe journeyed west across the Bering Strait into Asia. The oldest records of *C. antiquus* and *C. siwalensis* found outside of North America dated from the late Pliocene epoch (c. 4 – 3 m.y.a.) and were recovered from Northern India (7). Meanwhile, the earliest records of *Hemiauchenia*, thought to be the direct ancestor of *Lama* and *Vicugna*, were discovered in Argentina and have been dated from the Pleistocene epoch (c. 2 to 3 m.y.a.) (6). Recent genetic studies using mitochondrial and microsatellite DNA confirmed the alpaca to be the domesticated descendant of the vicuña and the llama to be that of the guanaco (9). Domestication of the alpaca and llama has been shown to have occurred c. 6000 to 7000 years ago while that of the dromedary and Bactrian took place c. 4500 – 5000 years ago (1).

Distribution

The Bactrian camels are typically found in cold regions of Eurasia, e.g. China, east and central Asia, Mongolia and Russia. The dromedaries are dispersed throughout the deserts of Ethiopia, the Sahelian states, Somalia, Sudan, the Mediterranean littoral and northern Kenya. The Bactrian and dromedary are sympatric within east and central Asia. The New World camels are distributed through the High Andes of Ecuador, Peru, Bolivia, Chile, and Argentina. Although *Paleolama* (also a

descendant of *Hemiauchenia*) migrated back to North America, all North American species have become extinct (6). Old and New World camelids are found in locations outside of the aforementioned countries as a result of importation.

Features of IgG isotypes produced by camelids

Physical properties

Antibodies are proteins of the immunoglobulin (Ig) superfamily, which are classified according to their heavy chain isotype, i.e. IgM, IgD, IgG, IgE, and IgA. Isotypes vary both in structure and function. Generally, antibodies are tetramers comprised of two identical heavy (H) chains (μ, δ, ε, α, or γ) associated with two identical light (L) chains (λ or κ). Each H-chain is comprised of an N-terminal variable (VH) domain followed by two or more constant (CH) domains, depending on the isotype. Three genes rearrange to encode the VH domain while only one gene encodes the CH domains, and in some isotypes, the hinge region. Light chains have a VL domain and one C λ or Cκ domain. The antigen-binding site of an antibody is formed by the association of VH and VL domains. The CH domains determine the isotype and effector function of the antibody.

Mammals synthesize one to seven IgG isotypes. In camelids, three IgG isotypes have been described. IgG1 exhibits the typical antibody structure while, IgG2 and IgG3, commonly referred to as heavy-chain antibodies (HCAbs), do not incorporate L-chains (10). The nomenclature of camelid IgGs in no way corresponds to that of other species, but instead was originally based on the decreasing molecular masses of the γ chains of dromedary IgGs: IgG1 (55 kDa), IgG2 (46 kDa) and IgG3 (43 kDa) (10). The current nomenclature of camelid IgGs is based on the biophysical properties of the IgG isotypes, e.g. differential binding to protein A and protein G, a property exploited to chromatographically separate the three isotypes (10). Only IgG1

and the IgG3 HCAbs adsorb to protein G; the IgG2 HCAbs do not. All three IgG species bind protein A. Evidence has been reported that substantiates further categorization of camelid IgGs into sub-isotypes based on variation in hinge length (11-14).

There are obvious distinctions between heavy-chain and conventional IgGs. Firstly, the CH1 domains that typically form covalent bonds with CL domains have been removed, and in some instances have been replaced by extended hinge regions (Figure 1-3*A* and *B*) (10, 12, 14). In addition, the variable domains of these HCAbs (V_HH) have replaced key hydrophobic amino acid residues at the putative VH-VL interface with hydrophilic residues in order to confer solubility (15). Further, the first and second hypervariable loops of the V_HH domain adopt non-canonical loop structures to increase the conformational diversification of these antibodies (16-19). Lastly, a single V_HH domain comprises the antigen-binding site in contrast to conventional immunoglobulins that combine a VH with a VL domain to form the antigen-binding site (Figure 1-3*C* and *D*) (15, 17).

Conventional IgG1 antibodies produced by Old and New World camelids have similar biophysical characteristics; however, pioneering work in the field identified differences in HCAb properties among the species. Hamers-Casterman ***et. al*** (11) showed that dromedary IgG2 HCAb (46 kDa) adsorbed only to protein A while the IgG3 (43 kDa) adsorbed to both protein A and G. In contrast, van der Linden ***et. al*** (20) demonstrated that protein A-specific llama IgG2 (~ 43 kDa) had a smaller apparent molecular mass than IgG3 (~ 46 kDa). Purification of camel IgGs demonstrated that HCAbs constitute up to 75% of serum IgG while llama HCAbs constitute between 25 – 45% of serum IgG (10).

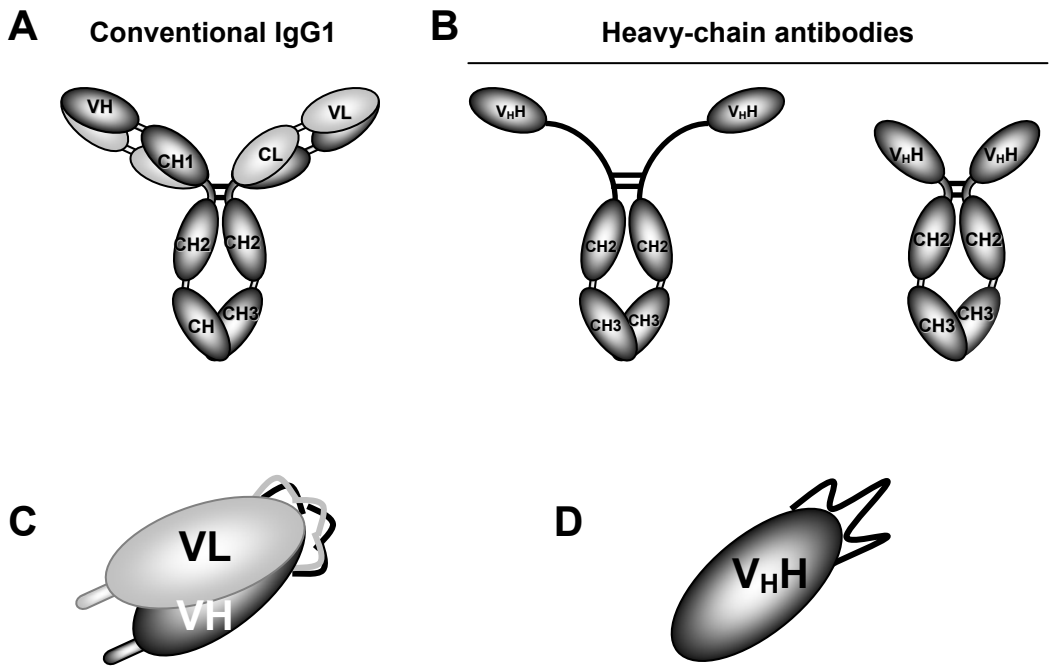

Figure 1-3. Structures of camelid IgGs. **(A)** Conventional IgG1 is a tetramer comprised of homodimeric H- and L-chains. **(B)** HCAbs are devoid of L-chains and the isotypes vary in hinge lengths. **(C)** Antigen-binding domains typically incorporate a VH and VL while HCAbs use only a single VHH to bind separate epitopes **(D)**.

Variable domains

The wealth of information on camelid V genes has been garnered from analysing genomic and cDNA sequences obtained from the dromedary. Genes encoding heavy-chain and conventional antibodies are distinct. Employing Kabat's numbering system (21), V_HH genes can be distinguished from conventional VH genes by the occurrence of hallmark Val37Phe or Tyr, Gly44Glu, Leu45Arg or Cys, Trp47Gly substitutions (15, 22, 23). These substitutions, which are present in germline genes, occur within the framework two region of the V_HH domain. In VH domains, these residues are integral to interactions with VL domains. Molecular evolutionary analyses have revealed that V_HH genes evolved from the conventional VH genes 82–37 m.y.a., before the divergence of the *Camelus* and *Lama*, approximately 45–11 m.y.a. (23). To date, genomic DNA analysis identified 50 VH and 42 V_HH non-redundant germline genes that are expressed in the camels (22, 24). Although over 167 non-redundant cDNA sequences have been described in llamas, so far only one VH and five V_HH germline genes have been characterized (25). Ongoing studies will likely reveal more germline V genes in llamas. The dromedary V_HH genes have been assigned to the VH3 family (11). Seven sub-families have been characterized, five of which have been cloned as rearranged products. Some of the V_HH genes from the remaining two sub-families should produce functional HCAbs as they incorporate appropriate promoter and recombination signal sequences (22).

Similar to conventional V genes, V_HH genes rearrange with diversity (D) and joining (J) genes to produce complete polypeptide domain. So far, there have been one D and six (five in llamas) J gene sequences described in camelids (26, 27). Comparative analyses of rearranged genomic sequences indicate a common use of D and J genes by both VH and V_HH genes suggesting an interspersed organization of VH, V_HH, D, J and CH genes.

Lastly, hypervariability within V_HH domains far exceeds that found in VH domains (22). Both the CDR1 and CDR3 loops in V_HH may be longer than those found in VH domains (11, 15). Whereas conventional CDR3 loops average 11 - 14 amino acids in length in mice and humans, the average length of a CDR3 loop in V_HH domains is 17 amino acids. The use of extended, hypervariable CDR3 to generate antibody diversity has also been noted in bovines (28, 29). The presence of extra cysteine residues within CDR1 and CDR3 may serve as stabilizers by forming interloop disulfide bridges. Additional cysteine residues are also present in the bovine CDR3; however, in contrast to those found in camelid CDR3, these residues form intraloop disulfide bridges (29).

Light chains

To date, genes encoding L-chains have been documented only in the dromedary (30). The production of both λ and κ L-chains has been confirmed based on cDNA sequences. While only one type of IGKC has been obtained there are at least two IGLC. IGLC2 contains a glycosylation site while IGLC1 does not. Southern blot techniques illustrated that IGLC genes recombine with at most 20 VL genes, but only a limited number of VK genes are used by IGKC genes (25). Under SDS-PAGE conditions, L-chains resolve as two distinct bands, 27 kDa and 30 kDa. Further analyses on the gel-purified proteins revealed that glycosylation of λ2-chains (30 kDa) accounted for the retardation in electrophoretic migration. The 27 kDa band contained both λ1- and κ-chains (13, 30).

L-chains have been shown to associate with conventional, camelid γ and μ H-chains (31). Cross-reactive antisera indicate the presence of IgA; however, there have not been any reports of IgD or IgE. Accordingly, there is no information available regarding L-chain association with these isotypes in camelids.

Constant domains

Constant domains of heavy-chain and conventional antibodies are encoded by discrete C γ genes. In keeping with De Genst *et. al*, these genes will be named according to the ImMunoGeneTics nomenclature system (25). Genomic and cDNA sequence analyses have identified at least six different genes that encode IgG1, IgG2 and IgG3 in the *Camelus* and *Lama*. In the dromedary, cDNA sequences have been obtained for five sub-isotypes; IGHG1A, IGHG1B, IGHG2A, IGHG2C and IGHG3 (12-14). In llamas, genomic sequence of a IGHG2B gene substantiates the presence of a sixth sub-isotype (11). The IGHG gene sequences correlating to each sub-isotype are quite variable; however, it is within the hinge region that the most variability is observed. As a result, classification into sub-isotypes is determined by the nucleotide sequence of the hinges (11).

The CH genes are comprised of exons that encode the C domains, hinge and transmembrane regions. In conventional IgG, each H-chain has a constant portion that exhibits a CH1-hinge-CH2-CH3 organization. The functional specialization of immunoglobulins is attributed to the participation of each CH domain in various biological activities (32-34). The CH1 serves two purposes; the random combination with CL drives diversification of the antigen-binding repertoire, and CH1-CL disulfide bridge assist in stabilizing the VH-VL interactions. The hinge is a proline-rich region connecting the CH1 and CH2 domains, and which contains essential cysteines that facilitate dimerization of the H-chains. The flexibility of the hinge regions also serves to modulate effector functions (35). The CH2 domains are generally separated in the dimer by oligosaccharide side chains. This makes them rather accessible, allowing them to bind elements of the complement system (33, 36). Amino acid sequences within the CH2 and CH3 domains are important in binding Fc-receptors (34, 37, 38).

It has been established that HCAb germline C genes encode a CH1, hinge, CH2, CH3, M1 and M2 region (12, 14). However, the CH1 domain is absent from the protein due to post-transcriptional excision of its exon. A conserved AT mutation within the flanking donor splice consensus sequence prevents retention of the CH1 exon in the mRNA. The CH2 and CH3 domains are highly homologous across isotypes. Work by Woolven *et. al* (12) revealed 88 – 98 % and 93 – 96 % identities among the CH2 and CH3 exons of the llama IgGs, respectively. Conversely, the hinges of IgG2a, IgG2b and IgG2c contain 35, 29, or 15 amino acid residues, respectively, while that of IgG3 contains 12 amino acid residues. By comparison, the conventional IgG1a and IgG1b hinges have 19 or 12 amino acid residues. The effector functions of camelid HCAbs have not been described. However, conserved nucleotide sequences within the CH2 exons predict that these domains may become glycosylated, and that they should bind to C1q and FcRs, which suggest that conventional IgG effector functions might have been retained by HCAbs (14).

Repertoire diversification and class-switching of HCAbs

A diverse set of germline V_HH, combined with mechanisms of somatic diversification generate the repertoire of camelid HCAb specificities. Comparisons between germline and cDNA sequences reveal much higher variability within cDNA sequences, implying that somatic hypermutation plays a key role in diversifying the repertoire (22). Extra hypervariable regions are located within extended CDR1 and CDR3. In addition, extra disulfide bridges that link and stabilize CDRs, high incidences of nucleotide insertions/deletions within the hypervariably regions, and gene conversion contribute to diversification of camelid V_HH (22). In cattle, a limited number of VH genes belonging to a single gene family depend on long CDR3s, gene conversion and point mutation to enrich the repertoire of antigen specificities (39, 40).

Based on the current understanding of how antibodies maintain specificity of their V domains while changing effector function of the C domain, we predict that the same events take place in camelid immunoglobulin gene rearrangement. Indeed, one study has identified hapten-specific IgG2 and IgG3 encoded by V_HH genes from the same sub-family (41). In addition, HCAb C genes contain the relevant switch recombination sequence upstream of their CH1 exon. This would enable isotype switching between the HCAbs; however, the μ-γ switch remains an enigma as no gene encoding a μ equivalent of the HCAb has been identified.

Applications of HCAbs

The features of HCAbs afford them novel properties. HCAbs have a lower molecular mass (100kDa) than conventional antibodies (150kDa), which should facilitate tissue distribution. Whereas conventional antibodies tend to aggregate when their L-chains have been removed, HCAbs are soluble. Solubility results from replacement of hydrophobic residues by hydrophilic residues at what would be the light/heavy chain interface (11, 24). HCAbs are bivalent (each V_HH domain binds an epitope), and capable of binding with nanomolar affinities to antigens, comparable to conventional antibodies (15, 42). Affinity maturation is possible as the V_HH genes have been shown to undergo hypermutation. Diversification of V_HH genes enables HCAbs to obtain a wide repertoire of specificities against protein antigens (20, 43). Remarkably, HCAbs induced by complex antigens have been reported to demonstrate distinct specificities from conventional antibodies (20, 27, 41).

Research conducted on HCAbs has focused mainly on their potential application as novel immunological tools. V_HH domains are thermally stable and capable of binding antigen at temperatures as high as 90 ^oC; temperatures at which conventional antibodies lose affinity for their antigens (44). This ability to withstand

high temperatures has been exploited to purify V_HH from VH domains and to design assays to detect caffeine in hot beverages (45, 46). The V_HH domain is comprised of a single polypeptide sequence making cDNA cloning and expression in yeast fairly uncomplicated. The purification process is simple and expression yields are high. In addition, the extended hinges associated with V_HH serve as stable linkers when conjugating to other proteins. Baral *et. al* engineered 'immunotoxins' by conjugating V_HH domains, specific for conserved, cryptic trypanosome antigens, with a human serum trypanolytic protein. These were shown to have therapeutic implications for African trypanosomiasis (47, 48). In addition, several investigations document the use of V_HH-conjugates to effectively target tumour-associated antigens (49-53). Rothbauer *et. al* demonstrated the applications of fluorescence-conjugated V_HH domains ('chromobodies') to conduct *in vivo* targeting and imaging of intracellular antigens in live cells (54).

A fascinating feature of HCAbs is their ability to be competitive enzyme inhibitors (17, 27, 55-58). In one study, a substantial proportion of enzyme-specific HCAbs (approximately 50%) demonstrated inhibitory activities (59). Generally, inhibition is executed by adopting a paratope structure that mimics the carbohydrate substrate, that is, by inserting an extended CDR into the active site of the enzyme (58). The ability to inhibit enzyme activity in this manner has not been observed in conventional antibodies.

Effector functions of conventional IgG antibodies

The purpose of immunoglobulins is not simply to bind to antigens. Depending on the nature of the antigen, binding may bring about several different effector mechanisms, which serve to limit or contain infections. It is the Fc portion (heavy chain or isotype) of immunoglobulins that dictates the effector mechanism that is

initiated. For IgG, these mechanisms include the activation of the C′ pathway, neutralization of microbes and toxins, opsonization and the induction of antibody-dependent cellular cytotoxicity (ADCC). All, except for complement (C′) activation requires the binding of Fc to Fc-receptors (FcγR) on effector cells. Sub-isotypes of IgG specialize in distinct effector mechanisms; thus, it is imperative that the correct isotype be elicited for an immune response to be effective. The IgG sub-isotype that is induced is determined by the cytokine milieu provided by innate and adaptive leukocytes involved in the immune response.

Opsonization

Opsonization by IgG prepares particulates, microbes and infected cells for clearance by phagocytic cells. In humans, IgG1 and IgG3 serve as effective opsonins by binding with high affinities to the FcγRs expressed on phagocytic cells. The high affinity FcγRI, and low affinity FcγRIIA and FcγRIIIB expressed on macrophages and neutrophils bind Fc domains of antigen-bound IgG to signal phagocytosis (60-62). Low affinity FcγRs bind only immune complexes while high affinity FcγRs bind free IgG. Gene targeted experiments identified FcγRI, when associated with the common γ-chain, to be the key receptor involved in FcR-mediated phagocytosis (63). This process involves the mobilization of the cytoskeleton to form pseudopods that engulf the target infected cell or microbe (64, 65). In the phagosomes, destruction is facilitated by the release of reactive oxygen intermediates and enzymes (66).

Antibody-dependent cellular cytotoxicity

IgG can bind to antigens on tumors, infected cells, microbes, and helminths to mediate lysis by cytotoxic cells that express FcγR on their surface. Macrophages, monocytes, eosinophils, neutrophils and especially natural killer cells express on their

surfaces receptors that can bind Fc on antigen-bound IgG to facilitate ADCC (67-70). Natural killer cells are the primary effectors of ADCC. In humans, these cells express the low affinity FcγRIIC and FcγRIIIB (71). The binding of Fc to FcγR initiates cell signaling resulting in the release of lytic enzymes, granzymes, perforin and tumor-necrosis factor at the site of Fc-mediated contact. This may lead to the destruction of the target cells via ADCC, a mechanism that is distinct from C′-induced cell lysis. IgG-dependent ADCC has been implicated as the effector mechanism involved in protective immunity against helminths as well as clearance of bacteria-, virus- and parasite-infected cells (72-75).

Complement-mediated effects

The antibody-dependent activation of C′ proceeds via the classical pathway subsequent to the formation of a stable association with C1 (76, 77). At least two of the globular C1q regions of C1 must be bound. Since IgGs possess only one C1q binding site, two or more IgG molecules must be optimally situated to permit propagation of the C′ pathway. In humans, IgG3 is the most effective in activating the C′ pathway. The C′ system has several roles in mediating IgG-dependent effector functions. Fc domains of IgG forming immune complexes are bound by C3b, which then binds CR1 receptors on erythrocytes (78, 79). In the liver and spleen, these immune complexes are removed by phagocytic cells without the ultimate lysing of erythrocytes. This prevents the deposition of immune complexes in tissues that is responsible for the development of autoimmune diseases such as systemic lupus erythematosus (80, 81). Also, IgG binding to bacteria or viruses can initiate the C′ cascade eventually leading to the formation of a membrane-attack complex and cell lysis, opsonization or the recruitment of inflammatory cells (82-85). Overall, C′ serves to augment and diversify IgG effector functions (86).

Neutralization

Neutralization occurs when IgG binds epitopes on toxins and viruses to prevent attachment to target cells (87-89). In the case of virus neutralization, the mechanism can be executed in several ways (90). The attachment process can be abrogated when IgG binds to a surface protein, which is responsible for binding the host cell receptor (91). Alternatively, IgG neutralization can act downstream of the attachment process by preventing membrane transformation required for binding ancillary molecules on the target cell surface (91). Virus-specific IgGs may also prevent internalization of virions by interfering with the endocytic process (91). Overall, IgG neutralization is concentration-dependent and may be enhanced by the activation of the C′ pathway.

B lymphocytes

Ontogeny

Antibodies are produced by B lymphocytes. Stem cells that are destined to become B lymphocytes are produced in the liver during gestation or in the bone marrow of adult animals. Lymphopoeisis of B cells occurs throughout life in humans and mice or only during gestation in rabbits and ruminants (92-96). In some species, B cell progenitors leave the bone marrow to undergo development in other lymphoid organs. The Bursa of Fabricius in birds and the gut-associated lymphoid tissues (GALT) in rabbits and ruminants function as primary lymphoid organs (40, 97-99). Within these organs, B lymphocytes undergo several tightly regulated developmental stages during which gene rearrangement occurs in order to diversify the antigen-binding repertoire. Presentation of endogenous antigens by stromal cells in the bone marrow serves to test receptors expressed on the surfaces of developing B lymphocytes (100). The role of stromal cells in GALT is not clear.

The expression of terminal deoxynucleotidyl transferase and the recombination-activating genes (RAG1/2) at the pro-B cell stage enables V(D)J gene rearrangement at the H-chain locus (101, 102). Successful rearrangement of these genes is achieved when stable transcripts have been obtained. Subsequently, the rearranged V(D)J exons are transcribed with a μ gene to produce IgM H-chains that are transported with surrogate L-chains to the cell surface of pre-B cells to form the pre-B cell receptor (pre-BCR) complex with the IgαIgβ chains. At this stage, the pre-B cell undergoes the initial checkpoint to ascertain whether the IgM H-chain can transmit signals. Presently, it is not clear whether the signal is ligand-dependent or -independent; however, the non-immunoglobulin portion within the λ5 region of the surrogate L-chain is essential in mediating this signal (103, 104). The pre-BCRs transmit signals into the cells via the IgαIgβ signal transduction molecules, which then determines whether gene rearrangement was successful and, hence, the fate of the B cell (105). A cell is positively selected if it has successfully rearranged its H-chain genes. These cells undergo rapid proliferation and appear as large pre-B cells. Next, the quiescent small pre-B cells undergo rearrangement at the L chain locus. Generally, the κ chain genes are rearranged before λ genes. The L-chains are paired with IgM H-chains and expressed on the surface of immature B cells to form the BCR complex. At this stage, the cell undergoes negative selection. If the BCR binds to self antigens, the cell may be rescued from apoptosis by undergoing receptor-editing of the VL genes. Conversely, cells that do not bind endogenous antigens are permitted to enter the periphery. Approximately 5–10 % of B cells that are produced leave the primary lymphoid organ and enter peripheral circulation.

In addition to antigen receptors, there are several molecules that are expressed on the B cell surface that correlate to the stage of development (106). The cell-surface receptor tyrosine kinase, Kit, and the interleukin-7 receptor are expressed on the stems

cells through to the late pro-B cell stage. These molecules are essential in transducing signals that induce proliferation. The expression of CD25 replaces that of Kit until the small pre-B cell stage. Other molecules that are essential to B cell development include CD45R (B220) and CD19, which are involved in signal transduction of the BCR complex and are expressed from the pro-B cell stage throughout the life of the cell.

Sub-populations and their functions

Subsequent to egress from the central lymphoid organs, B cells enter the periphery and begin a circulatory pattern to different lymphoid organs that depends upon its lineage, receptor specificity, and cytokine milieu within the lymphoid organs (107). There are three lineages of B cells: B-1, follicular (FO) B-2 and marginal zone (MZ) cells (108, 109). B-1 cells are a self-renewing population that is produced before B-2 B cells by the fetal liver (110). B1 cells have been shown to produce the anti-inflammatory cytokine, IL-10 (111). In humans and mice, B-1 cells constitute < 5 % of the B cell population, which is in contrast to rabbits where B-1 cells are the predominant population (112, 113). B1a cells, which express the CD5 antigen, produce natural antibodies that bind large, polymeric bacterial antigens (114). B1b cells are induced by bacterial polysaccharide antigens to produce the antibodies that are essential in conferring long-lasting immunity (114). The positive selection that is responsible for development and maintenance of B-1 cells takes place in the periphery and is thought to be driven by self-antigens (115, 116). These cells are enriched within the pleural and peritoneal cavities, implying a role in defending the body cavities against invading pathogens.

The MZ B cells are long-lived B cells that are located within the marginal sinuses of splenic white pulp. In mice, MZ B cells are rather sessile, while human MZ B cells recirculate (117). These cells have a pre-activated phenotype and expand rapidly in response to blood-borne antigens and bacteria (109, 118, 119). It has been shown that MZ cells are crucial in immunity to *Streptococcus pneumoniae*, *Neisseria meningitides*, and *Hemophilus influenzae*. Although the repertoire of antigen receptors among MZ B cells is quite restricted, these cells recognize T-dependent as well as T-independent antigens, and are often involved in the initial non-adaptive immune response to pathogens. Compared to FO B cells, intrinsic features of MZ B cells, e.g. higher expression of B7 costimulatory molecules and greater signaling through the BCR, make them more efficient at activating naïve T cells (120). Generally, MZ B cells undergo extrafollicular response to antigens; however, recent studies have shown that these cells can initiate germinal center formation within which they undergo somatic hypermutation in response to T-dependent antigens (121).

Follicular B cells display a broad receptor repertoire and are the major population involved in adaptive immunity. In mice, after leaving the primary lymphoid organ, immature B cells enter the spleen exclusively where they go through three distinct transitional stages before developing into mature B cells (122). The subsets of transitional cells are distinguished by the expression of IgM, IgD, CD21, CD23 and CD24 molecules (123, 124). BCR signaling determines whether these cells survive passage through the spleen. Positively selected cells then leave to traffic through lymph nodes as mature, naïve B cells. Within lymph nodes, in response to exogenous antigens, FO B cells present peptides on MHC class II to T cells in order to receive co-stimulation, instructions, and survival signals required to become activated. If the T cell had previously encountered the antigen presented by the B cell, then it expresses CD40 ligand and secretes growth factors, which together drive proliferation

and differentiation of the activated B cell (125, 126). Activated B cells enter the follicles (termed germinal centers) to proliferate, undergo class-switching and affinity-maturation of their receptors, and differentiate into long-lived effector (plasma) or memory B cells (127). Memory B cells recirculate while plasma cells migrate to and reside within bone marrow (128).

Research objectives

Studies being conducted on the unusual HCAbs produced by camelids focus mainly on exploiting their features for biotechnological purposes. Little work has been done to elucidate the functions of these antibodies within the animals that produce them. The purpose of this thesis research was to contribute to the knowledge of camelid HCAbs by illuminating their role in immunity, and by describing the ontogeny of the B cells that produce them. To achieve this, we first had to produce and fully characterize isotype-specific mAbs that distinguished the different IgG species produced by llamas and alpacas. Such reagents had not been prepared previously. These mAbs were subsequently used in various experiments designed to address specific questions about camelid immunity. Firstly, to investigate whether different HCAb classes were induced by distinct pathogens, we compared the IgG profiles elicited during nematode and viral infections. Next, we investigated the functional properties of HCAb isotypes by testing their abilities to neutralize virus and mediate Fc-dependent enhancement of virus infectivity. To evaluate HCAbs in a clinical and physiological context, the mAbs were used to measure IgGs in body fluids. Lastly, to describe the life history of the cells that produce HCAbs, B cells in tissues from fetal, neonatal and adult camelids were phenotyped using flow cytometry and immunohistochemistry analyses.

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CHAPTER 2

Application of Monoclonal Antibodies to Functional and Comparative Investigations of Heavy-Chain IgGs in New World Camelids[1]

[1]Lisa P. Daley, Lucille F. Gagliardo, Michael S. Duffy, Mary C. Smith, and Judith A. Appleton. 2005. Application of monoclonal antibodies in functional and comparative investigations of heavy-chain immunoglobulins in new world camelids. *Clin Diagn Lab Immunol* 12:380-386.

Abstract

Of the three IgG isotypes described in camelids, IgG2 and IgG3 are distinct in that they do not incorporate light chains. These heavy-chain antibodies (HCAbs) constitute approximately 50% of IgG in llama serum and as much as 75% of IgG in camel serum. We have produced isotype-specific mouse monoclonal antibodies (mAbs) in order to investigate the roles of HCAbs in camelid immunity. Seventeen stable hybridomas were cloned, and detailed characterization was performed for three mAbs that were specific for epitopes on the γ chains of llama IgG1, IgG2 or IgG3. Affinity chromatography revealed that each mAb bound its isotype in solution in llama serum. The antibodies bound to the corresponding alpaca IgGs, to guanaco IgG1 and IgG2, and to camel IgG1. Interestingly, anti-IgG2 mAbs bound three heavy-chain species in llama serum confirming the presence of three IgG2 sub-isotypes. Two IgG2 sub-isotypes were detected in alpaca and guanaco sera. The mAbs detected llama serum IgGs when bound to antigen in ELISA, and were used to discern among isotypes induced during infection with a parasitic nematode. Diseased animals, infected with Parelaphostrongylus tenuis, did not produce antigen-specific HCAbs; rather, they produced the conventional isotype, IgG1, exclusively. Our data document the utility of these mAbs in functional and physiologic investigations of the immune system(s) of New World camelids.

Introduction

The discovery of camelid IgGs that violated established rules governing the structure of antibodies was described in 1993 (12). Unlike their conventional IgG1 counterpart, camelid IgG2 and IgG3 do not associate with light chains and are called heavy-chain antibodies (HCAbs) (Fig. 2-1*A*). Genomic and/or complementary DNA

sequences have been obtained for six γ constant (Cγ) genes: Cγ1a and Cγ1b for IgG1, Cγ2a, Cγ2b, and Cγ2c for the IgG2 HCAb, and Cγ3 for the IgG3 HCAb (20,22,35,38). The Cγ2b gene was identified in genomic clones prepared from llamas (38) and has not been described in other camelids. Variation in masses among the IgG2 sub-isotypes is predicted from variability in the lengths of exons encoding the hinge regions. Extended hinge regions, deletion of the CH1 domain, and amino acid substitutions in the V domain that make it more hydrophilic, promote the flexibility and solubility of HCAbs (5,19,21,31,35). These properties may expand the antigen-binding repertoire of the immune system by enabling HCAbs to bind otherwise inaccessible epitopes. Given their unusual physical properties, it seems likely that HCAbs will play a novel role in immune defense.

Investigations of camelid antibodies have focused largely on manipulating or mimicking the architecture of the variable domains of the HCAbs (V_HH) for application to medical therapy and biotechnology (4,18,24,30). Unlike conventional antibodies, HCAbs use a single V_HH to bind an epitope. The absence of the light chains variable domain is compensated by extended complimentarity determining regions (CDR) that provide an adequate antigen-binding surface and demonstrate affinities comparable to conventional antibodies (5,28). An exceptional property of HCAbs, which has fueled research activity, is their ability to inhibit enzymes by inserting an extended CDR into the active site (17,32). This property has not been observed in conventional antibodies. The aggregate physical and binding characteristics of HCAbs, along with the ease of production of recombinant, active V_HH in the yeast system (10,34), have prompted researchers to explore their use as immunological tools.

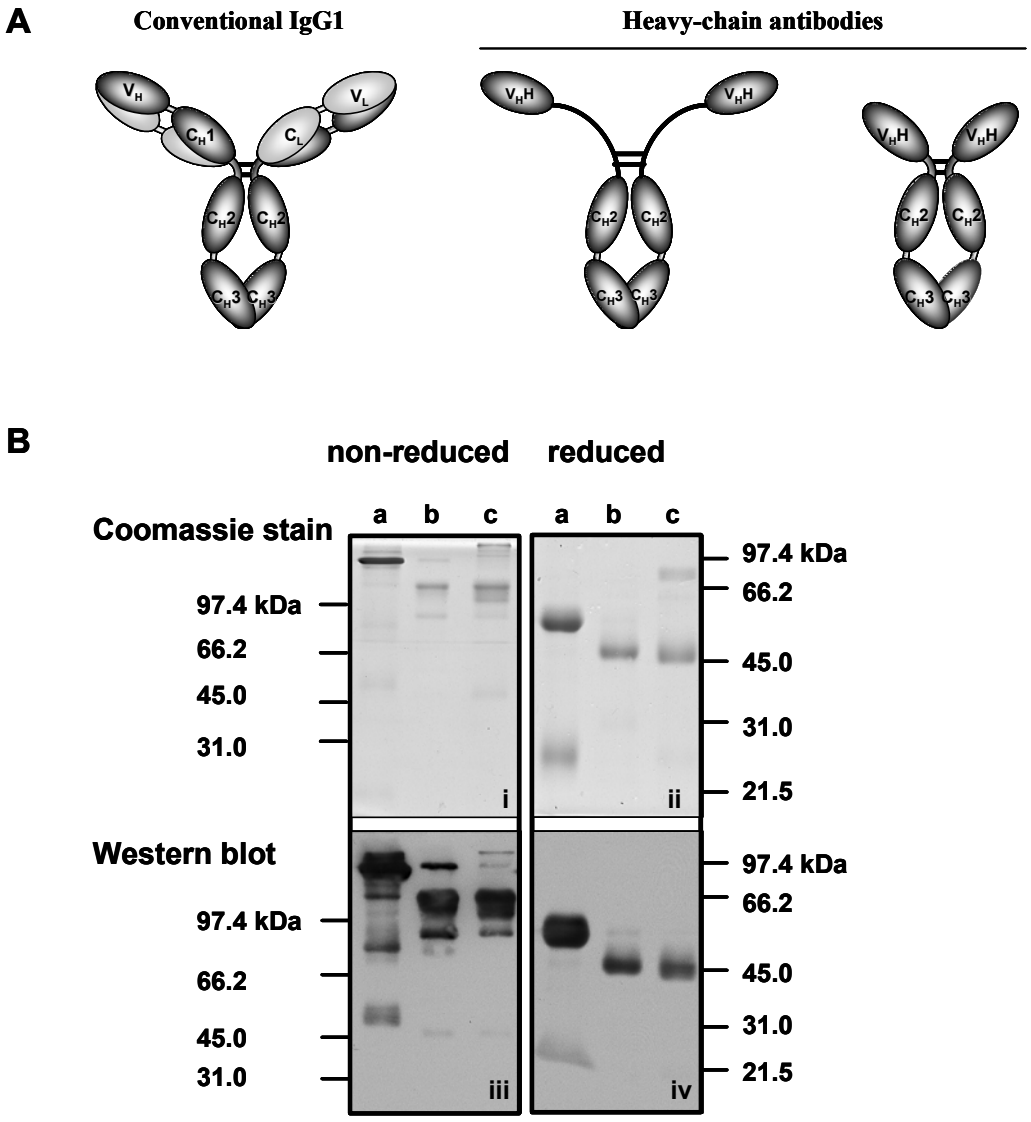

Figure 2-1. (A) Structure of camelid IgGs. **(B) Top:** Coomassie blue-stained SDS-PAGE of purified llama IgG isotypes resolved under non-reducing and reducing conditions. **(a and b)** IgG1 and IgG3 (eluted from protein G); **(c):** IgG2 (eluted from protein A). **Bottom:** Western blot of gels identical to Coomassie stained gels developed with polyclonal anti-llama IgG (H + L). Molecular masses (in kilodaltons) of protein standards run in parallel are indicated.

There are three published reports of the involvement of HCAbs in camelid immunity, specifically, in the response to trypanosome infection and to immunization with bacterial proteins (12,30,33). The production of IgG isotypes in response to infection with parasitic nematodes has not yet been investigated. A limiting factor for investigations into camelid immunity has been a shortage of well-characterized, isotype-specific immunological reagents.

Currently, polyclonal antibodies that react broadly with camelid IgGs are available from commercial sources. Monoclonal antibodies (mAbs) specific for dromedary (*Camelus dromedarius*) IgG1 and IgM have been produced and characterized (1). At this time, analysis of lamoid HCAbs can be conducted only on chromatographically separated immunoglobulin fractions. The application of isotype-specific mAbs in experimental and diagnostic assays will facilitate direct assessment of the composition and specificity of the lamoid IgG response to infection. Results of such assays will engender better understanding of the roles of HCAbs in immune defense.

Here we describe the production and characterization of mAbs specific for conventional and heavy-chain isotypes of llama IgG. Seventeen stable hybridomas were cloned, and detailed characterization was performed for three mAbs that were specific for epitopes on the γ chains of llama IgG1, IgG2 or IgG3 fractions purified on Protein A and Protein G. The antibodies bound to the corresponding alpaca IgGs, to guanaco IgG1 and IgG2, and to Bactrian IgG1. Anti-IgG2 mAbs bound three heavy-chain species in llama serum confirming the presence of three IgG2 sub-isotypes. Two IgG2 sub-isotypes were detected in alpaca and guanaco sera. The mAbs detected llama serum IgGs when bound to antigen in ELISA, and were used to discern among isotypes induced during infection with a parasitic nematode.

Materials and Methods

Animals and sera. BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and housed in the James A. Baker Institute vivarium according to the guidelines of the American Association of Accreditation of Laboratory Animal Care. Blood samples were obtained by jugular venipuncture from selected camelids (one healthy and four diseased llamas, and one healthy alpaca, guanaco, and Bactrian camel) that were brought into the Cornell University Hospital for Animals or examined by the ambulatory service. Serum was separated by centrifugation and stored at -20^{o}C. Infection with *P. tenuis* was confirmed in diseased llamas by observation of nematodes within the central nervous system during necropsy. Sera from llamas in Pullman, Washington, an area non-endemic for *P. tenuis,* were kindly provided by Dr. William Foreyt of Washington State University**.**

Antibodies. Polyclonal goat anti-llama IgG (H+L) conjugated to horseradish peroxidase (HRP) (Bethyl Laboratories Inc., Montgomery, Texas) was used in ELISA and Western blot. Monoclonal mouse antibodies were detected with HRP-conjugated goat anti-mouse antibodies (ICN/Cappel, Aurora, Ohio). Three mAbs - 27E10 (anti-IgG1), 19D8 (anti-IgG2), and 8E1 (anti-IgG3) - were selected for use in the serologic assays. An ELISA to determine the isotype of the mAbs employed rat mAbs to mouse isotypes and HRP-conjugated mouse anti-rat κ chains antibodies (BD PharMingen, San Diego, California).

Purification of llama IgGs. Llama IgG isotypes were purified using affinity chromatography as described elsewhere (12,33). Briefly, serum was first loaded onto a Protein G-Sepharose 4B column (Sigma Chemical Co., St. Louis, Missouri), and the unbound fraction was collected and loaded on a Protein A-Sepharose 4B column

(Sigma Chemical Co.). IgG3 was eluted from Protein G with 0.15M NaCl, 0.58% acetic acid, pH 3.5, and IgG1 was eluted with 0.1M glycine-HCl, pH 2.7. IgG2 was eluted from Protein A with 0.15M NaCl, 0.58% acetic acid, pH 4.5. Fractions were neutralized immediately with 0.1 M Tris-HCl, pH 9.0.

SDS-PAGE and Western blots. Serum and chromatographically separated IgGs were resolved in discontinuous polyacrylamide gels (16). Mini-gels were used, except for the determination of apparent molecular weights where proteins were resolved in standard 15 cm gels. Samples and molecular weight markers (Bio-Rad Laboratories, Hercules, California) were boiled for 7 minutes in sample preparation buffer, with or without 2-mercaptoethanol, before loading the gels. Gels were stained with Coomassie blue or were blotted onto nitrocellulose membranes.

Western blotting procedures were performed at room temperature. Incubation periods were 1 hr unless specified otherwise. Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) and washed with TBS containing 0.05% Tween 20 and 0.1% bovine serum albumin (BSA; Sigma). Primary antibodies were diluted in blocking solution, and conjugates were diluted in blocking solution containing 10% normal goat serum. Antibody binding was detected with a chemiluminescent substrate (ECL reagent, Amersham Pharmacia Biotech, Piscataway, New Jersey) and autoradiography. Films were scanned, and images were prepared using Adobe® Photoshop® and Microsoft® Powerpoint®.

Production of monoclonal antibodies. Mice were injected intraperitoneally with 100-300 μg of purified llama IgGs mixed in complete Freund's adjuvant (Sigma). One mouse per isotype was injected intravenously with purified IgG in Dulbecco's phosphate-buffered saline (DPBS) 30 days post immunization and killed three days

later. Spleen cells were fused with mouse myeloma SP2/0 cells (American Type Culture Collection, Rockville, Maryland) according to Kohler and Milstein (15).

Culture supernates were screened by ELISA for selective binding to one of the three llama IgG isotypes. Plates were coated at 4^{o}C, and other procedures were conducted at room temperature. Well volume was 25 µl. Wells of polyvinyl 96-well microtiter plates (Costar, Cambridge, Massachusetts) were coated overnight with llama IgG1, IgG2 or IgG3 in 10% DPBS (1 µg/ml). Wells were blocked 1 hr with DPBS containing 0.05% Tween 20 and 5% skim milk then incubated 1 hr with supernatants diluted 1:10 in blocking solution. Wells were washed five times with 0.05% Tween 20, DPBS and incubated 1 hr with HRP-conjugated goat anti-mouse (0.6 µg/ml) in blocking solution containing 10% normal goat serum. Binding was detected with 3,3′,5,5′-tetramethylbenzidine substrate (KPL, Gaithersburg, Maryland) and stopped after 30 minutes with 1M H_3PO_4. Optical density readings were taken at 450 nm using a microplate reader (Biokinetics Reader, EL340; Bio-Tek Instruments, Winooski, Vermont).

Hybridomas that secreted mAbs specific for one llama IgG isotype were selected and cloned by limiting dilution. Hybridomas producing mAbs that were selected for further characterization were expanded using the VectraCell system (BioVectra, Oxford, Connecticut). MAbs were purified from culture supernatants by affinity chromatography using Protein G Sepharose-4B (Sigma) on an FPLC system (ÄKTA, Amersham Pharmacia Biotech Inc., Piscataway, New Jersey) according to manufacturer's instructions.

Isotyping the mAbs. A modified version of the ELISA protocol used for screening the hybridomas was developed to determine the isotypes of the mAbs. Bound mAbs were detected with rat monoclonal antibodies to mouse isotypes diluted

1 μg/ml in blocking solution, followed by HRP-conjugated mouse anti-rat κ chains at 2 μg/ml in blocking solution with 10% normal mouse serum.

Pepsin digestion of llama IgG1. Purified IgG1 was dialyzed against 0.2 M sodium acetate buffer, pH 4.5 and adjusted to 2 mg/ml. Crystallized pepsin (Sigma) was dissolved in the same buffer (1 mg/ml), and digestions were performed at a ratio of 1 μg enzyme to 20 μg antibody. After 1, 2, 4, 6, 12, 24, and 48 hrs in a 37^{o}C water bath, digestion reactions were stopped by the addition of 40 μl of 2 M Tris base. Fractions were resolved by SDS-PAGE in 8% acrylamide mini-gels. Gels were either stained with Coomassie blue or Western blotted.

Affinity chromatography of camelid IgGs. Affinity columns were prepared by coupling 10 mg of a mAb (27E10, 19D8 or 8E1) to 1 g CNBr-activated Sepharose-4B (Sigma) in the presence of 0.1 M $NaHCO_3$, pH 8.0. Unbound active sites were blocked with 0.1 M Tris, pH 8.0 for 2 hrs at 4 ^{o}C. The coupled gels were washed alternately with 0.1 M acetate buffer, pH 4.0 and borate buffered saline (BBS), pH 8.5. Columns were loaded with 1 ml llama, camel, guanaco or alpaca serum and washed with BBS. Bound IgGs were eluted from the columns with 0.1M glycine-HCl buffer, pH 2.7, into tubes containing 0.1 M Tris-HCl, pH 9.0, and evaluated by Western blot.

Detection of llama IgGs specific for *P. tenuis* antigens. The ELISA protocol detailed above was modified to detect llama IgGs specific for the recombinant *P. tenuis* aspartyl protease inhibitor, r*Pt*-API. Preparation of the r*Pt*-API has been described elsewhere (7). Plates were coated with 4 μg/ml r-*Pt*-API. Llama sera were diluted 1:100 and added to triplicate wells. Wells were incubated with the 27E10, 19D9 or 8E1 mAbs (5 μg/ml) or HRP-goat anti-llama antibodies (1 μg/ml) diluted in

blocking solution. Bound mAbs were detected with 5 μg/ml HRP-conjugated goat anti-mouse antibodies diluted in blocking solution containing 10% normal goat serum.

Statistical analysis. ELISA data from groups of infected and uninfected llamas were evaluated by analysis of variance. Values were considered statistically significant when $p < 0.05$.

Result

Purification and characterization of llama IgGs. Affinity chromatography, using Protein A and Protein G, was carried out in order to purify the isotypes of llama IgGs. As reported previously (33), llama IgG1 and IgG3 bound to Protein G and Protein A while IgG2 bound only to Protein A. Comparing Coomassie blue-stained gels (Fig. 2-1*B*; Panels i and ii) with Western blots (Fig. 2-1*B*; Panels iii and iv) confirmed that eluted proteins were llama IgGs. Multiple bands were observed in fractions resolved under non-reducing conditions. The majority of the proteins of greater mobility were bound by polyclonal goat anti-IgG (Fig. 2-1*B*, Panel iii), suggesting the occurrence of mild protein degradation during storage of samples. In each non-reduced preparation, a single band migrated between the 66.2 kDa and 45 kDa molecular weight standards (Fig. 2-1*B*; Panel iii), compatible with the masses of the corresponding monomeric heavy chains. The IgG3 fraction eluted from Protein G was contaminated with IgG1 (Fig. 2-1*B*, Panel iii, Lane b). IgG2 co-eluted with an 80 kDa protein from the Protein A column (Fig. 2-1*B*, Panel ii, Lane c). This protein was not detected by the anti-llama IgG conjugate (Fig. 2-1*B*; Panel iv, Lane c).

Under reducing conditions, IgG1 was resolved into light chains (22.7 kDa) and γ1 heavy chains (49.4 kDa) (Fig. 2-1*B*, Panel iv, Lane a). The IgG3 preparation was reduced to one dominant heavy chain species of 42.1 kDa (Fig. 2-1*B*, Panel iv, Lane

b). The IgG2 preparation was reduced to one dominant heavy chain species of 40.9 kDa (Fig. 2-1*B*, Panel iv, Lane c). Contaminants and degradation products were not evident in reducing gels. The three fractions were used to immunize Balb/c mice for isotype-specific mAb production. Because the preparations were known to be heterogeneous, the screening procedure for each fusion was designed to reduce the likelihood that cross-reactive antibodies would be selected.

Production of monoclonal antibodies. Supernatants from fusion plate wells were tested by ELISA against all three preparations of llama IgG. Thirty-four hybridomas were found to secrete antibodies reactive primarily with only one preparation. Seventeen stable cell lines were cloned to purity: five that secreted mAb that bound IgG1, and six each that bound IgG2 or IgG3. Four of the 17 mAbs were mouse IgM and were not pursued. The remaining 13 mAbs were mouse IgG1, IgG2a or IgG2b. The binding of these 13 mAbs with Protein A/G-purified llama IgGs are summarized in Fig. 2-2. Antibodies 27E10, 19D8 and 8E1, were selected for further characterization based on their overall high reactivity with llama IgG1, IgG2 and IgG3, respectively.

Specificities of mAbs for immunoglobulins in Western blot. In order to confirm that the IgG1 epitope bound by 27E10 was in the heavy rather than the light chain, Protein G purified IgG1 was cleaved with pepsin, and the fragments were assayed in Western blot. Digestion of IgG1 for 6 hrs yielded an array of fragments as well as residual whole IgG1 (Fig. 2-3*A*). 27E10 bound whole IgG1 as well as $F(ab')_2$ fragments, but did not bind Fab- or Fc-like fragments (Fig. 2-3*B*). Thus binding to IgG1 by 27E10 appeared to be dependent upon the presence of the hinge region. Further, the antibody did not bind Fab/c-like fragments (Fig. 2-3*B*) or reduced IgG1

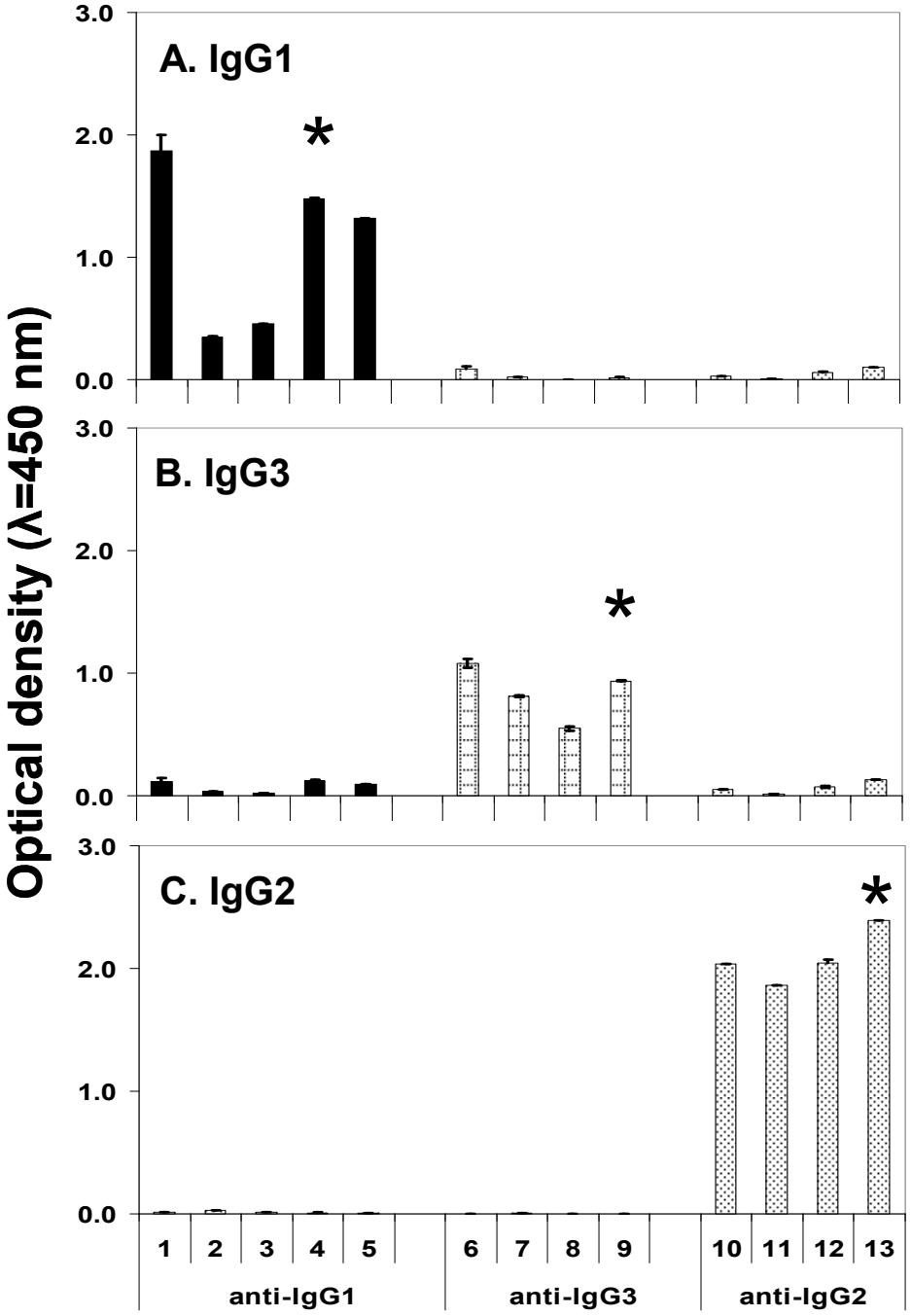

Figure 2-2. Reactivities of mouse mAbs with affinity-purified llama IgGs in ELISA: **(A)** IgG1; **(B)** IgG3; **(C)** IgG2. Twofold dilutions of supernatants were tested in triplicate wells against purified IgGs. Bound mAbs were detected with HRP-goat anti-mouse IgG. Columns represent mean optical densities for three replicate wells ± standard deviation of the mean (I bars). Asterisks denote mAbs selected for further evaluation.

(data not shown) compatible with a requirement for intact disulfide bridges and stable tertiary and quaternary structure in the hinge region. Our data support the conclusion that 27E10 binds within the C_H1/hinge/ C_H2 region of IgG1.

The 19D8 and 8E1 mAbs bound to IgG2 and IgG3, respectively, in Western blots when used at high concentrations (20-80 μg/ml) (data not shown). These concentrations were 20-80 fold that of normal working concentrations for peptide specific antibodies, and some cross-reactivity with other IgG isotypes was evident. We concluded that the epitopes recognized by these antibodies are not well-preserved under the denaturing conditions of SDS-PAGE and Western blotting.

Binding of mAbs to camelid IgGs in solution. To ensure that the mAbs had been induced against epitopes on native IgGs and not to epitopes exposed during the purification process, llama serum was passed over affinity columns prepared by coupling CNBr-activated sepharose with 27E10, 19D8 or 8E1. Each mAb bound the predicted llama IgG isotype (Fig. 2-4*A*). Unexpectedly, three protein bands were observed in the fraction eluted from the anti-IgG2 column, the 40.9 kDa species observed in Protein A/G purified IgG2, plus species of 44.1 kDa and 47.2 kDa (Fig. 2-4*A*, reduced). The 8E1 mAb (anti-IgG3) did not bind any of the IgG2 heavy chains in Western blot, indicating that they were not IgG3 antibodies that contaminated the IgG2 preparation (Fig. 2-5*A*). These HCAbs will be referred to as IgG2a, IgG2b and IgG2c according to their decreasing molecular weights in order to be consistent with the published reports.

Binding of the anti-llama IgG mAbs to immunoglobulins in sera of other camelid species was documented by similar experiments with mAb affinity columns (33). The three mAbs reacted with the homologous isotypes in alpaca serum (Fig. 2-4*B*). Similarly, the 27E10 (anti-IgG1) and 19D8 (anti-IgG2) mAbs bound to guanaco

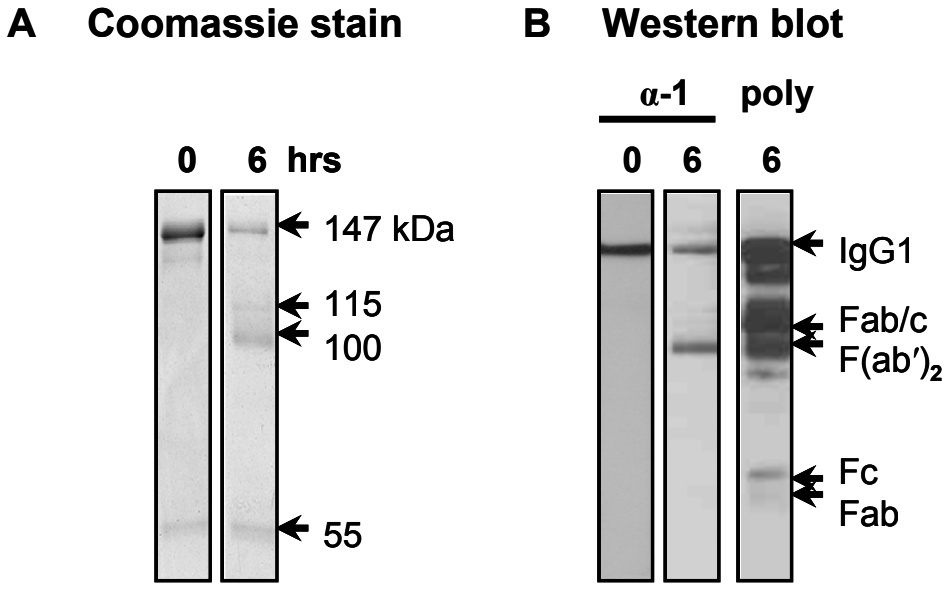

Figure 2-3. Binding of anti-llama IgG1 mAb (27E10) to fragments of llama IgG1. **(A)** Coomassie stained gel of llama IgG1 digested 0 or 6 hrs with pepsin in acetate buffer, pH 4.5. Estimated molecular masses (in kilodaltons) are indicated. **(B)** Western blot showing reactivity of 27E10 (α-1) with llama IgG1, digested 0 or 6 hrs with pepsin, developed with HRP-goat anti-mouse antibodies. poly: HRP-goat anti-llama IgG (H+L) against pepsin-cleaved IgG1.

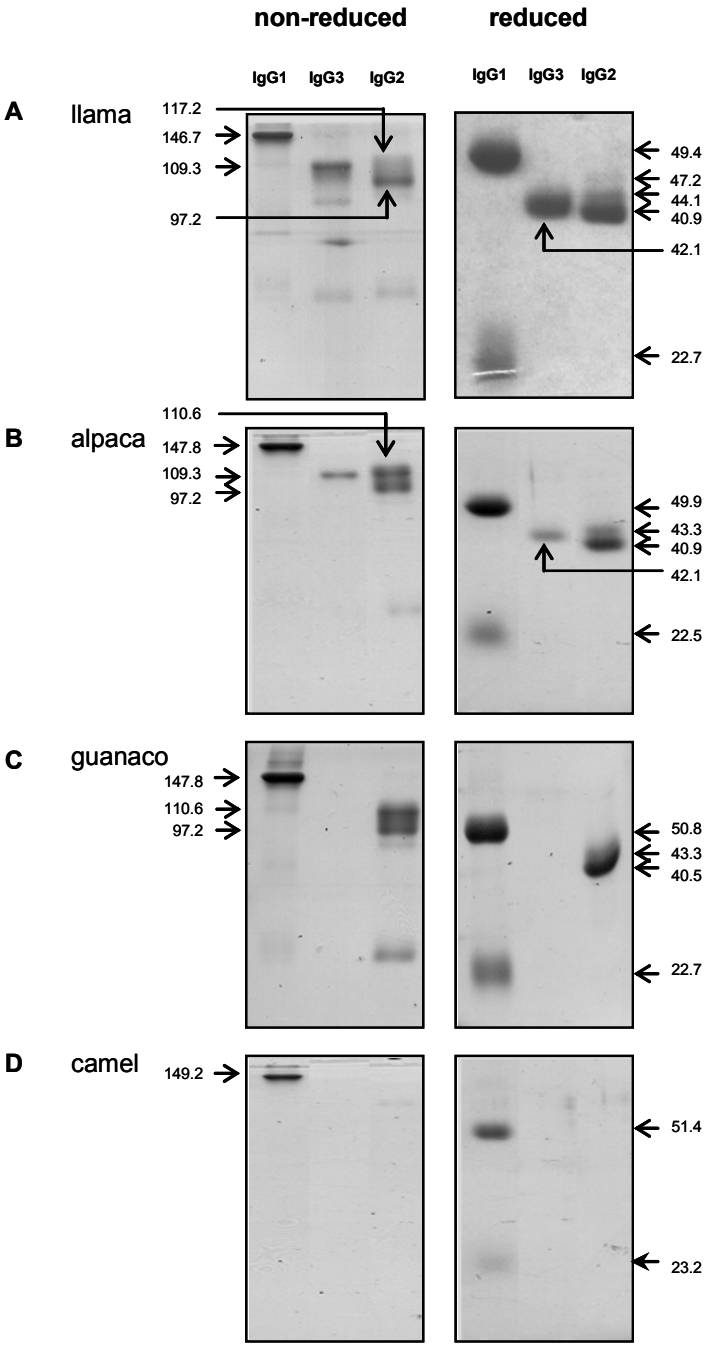

Figure 2-4. Specificities of mAbs for native camelid IgGs. The anti-llama IgG1, IgG3 and IgG2 mAbs, 27E10, 8E1, 19D8, respectively, were coupled with CN-Br activated Sepharose-4B and used to purify IgGs from camelid sera. **(A)** Llama serum; **(B)** alpaca serum; **(C)** guanaco serum; **(D)** camel serum. Eluted IgG fractions were resolved by SDS-PAGE under non-reducing and reducing conditions and stained with Coomassie blue. Estimated molecular masses (in kilodaltons) are indicated.

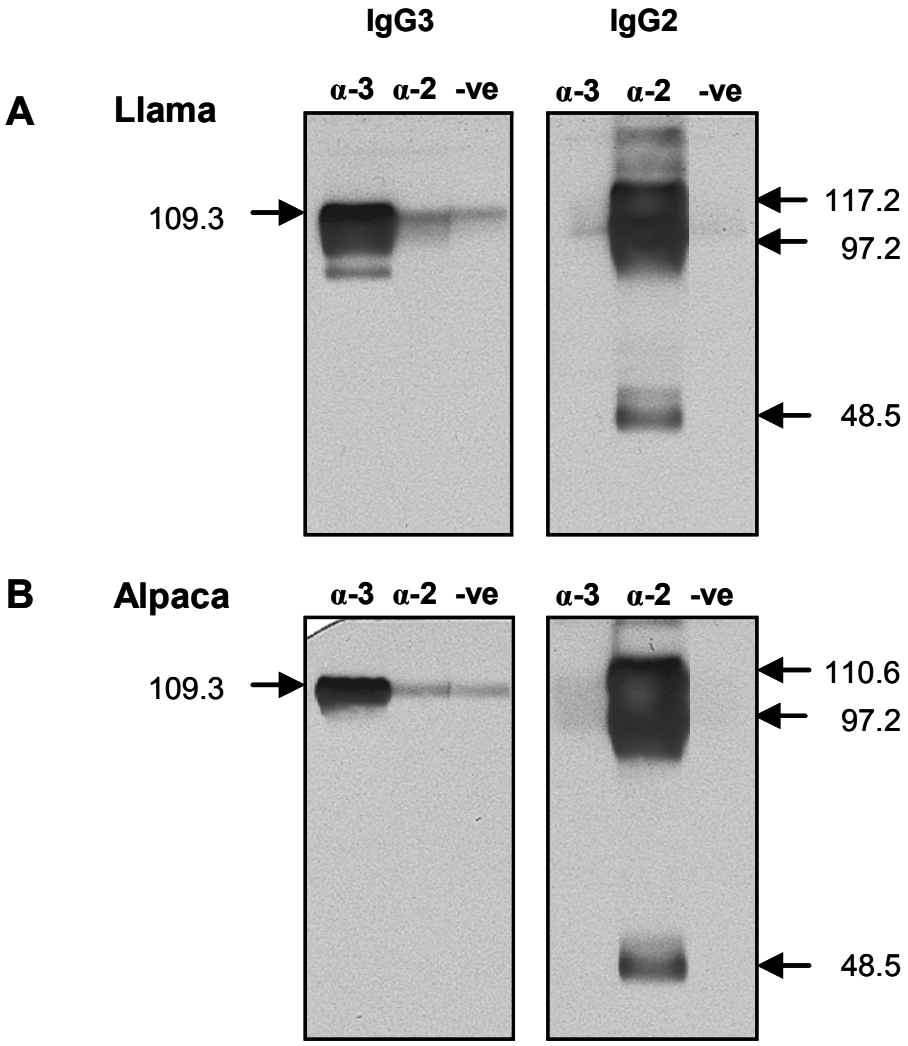

Figure 2-5. Reactivities of HCAb-specific mAbs with eluates from the anti-IgG3 and anti-IgG2 affinity columns in Western blot. Affinity-purified HCAbs from llama **(A)** and alpaca **(B)** sera were resolved by SDS-PAGE, blotted onto nitrocellulose, and developed with anti-IgG2 and anti-IgG3 mAbs (20 μg/ml) or normal mouse serum. **α-3,** anti-IgG3 (8E1) mAbs; **α-2,** anti-IgG2 (19D8) mAbs; -ve, negative control (normal mouse serum). Estimated molecular masses (in kilodaltons) are indicated.

IgG1 and IgG2; however, 8E1 (anti-IgG3) did not bind a guanaco IgG3 (Fig. 2-4*C*). In contrast with llama, passage of alpaca and guanaco sera over the anti-IgG2 column yielded only two IgG2 species: one of 43.3 kDa and another of 40.5 kDa (guanaco) or 40.9 kDa (alpaca). Development of a Western blot of the alpaca IgG2 fraction with 8E1 (anti-IgG3) indicated that neither protein was IgG3 (Fig. 2-5*B*). The anti-IgG1 mAb (27E10) bound to camel IgG1, but neither of the HCAb-specific mAbs bound to the homologous isotypes in camel sera (Fig. 2-4*D*). In summary, the mAbs bind epitopes on native llama and alpaca IgG1, IgG2 and IgG3, guanaco IgG1 and IgG2, and camel IgG1.

Apparent molecular weights of the camelid γ and light chains isolated as described above were calculated based on their migration in SDS-PAGE under reducing conditions (Table 1).

Evaluation of llama IgGs induced during *P. tenuis* infection. Sera from llamas resident in areas endemic or non-endemic for *P. tenuis* were tested by ELISA for the presence of parasite-specific antibodies of each IgG isotype. Polyclonal goat anti-llama IgG conjugate was tested in parallel. All sera tested yielded high OD readings with the polyclonal reagent. Sera from infected/diseased animals could not be distinguished from that of a healthy llama from an endemic area, nor from sera of animals from non-endemic areas (Figure 6). The apparent lack of specificity may be due to binding of the conjugate to IgM as a result of its specificity for light chains. Although the recombinant protein antigen bears a His tag and was affinity-purified by Ni-chelation chromatography (7), it was produced in *E. coli* and may carry trace amounts of bacterial protein. The polyclonal conjugate may bind light chains of low affinity, *E. coli*-specific, or other non-specific, IgM that is likely present in the sera of all llamas. Although there are other possible explanations for the lack of

Table 2-1. Molecular masses (kDa) of camelid IgGs estimated by migration in SDS-PAGE[a].

	IgG1[b]		IgG2			IgG3
	H	**L**	**a**	**b**	**c**	
Llama	49.4	22.7	47.2	44.1	40.9	42.1
Alpaca	49.9	22.5		43.3	40.9	42.1
Guanaco	50.8	22.7		43.3	40.5	42.1[c]
Camel	51.4	23.2		42.9[c]		39.0[c]

[a]10 % acrylamide, 15 cm gels.
[b]Calculated from Western blot of sera resolved under similar conditions.
[c]H, heavy chain; L, light chain.

discrimination by the polyclonal reagent (discussed below), our results diminish enthusiasm for its use in ELISA.

In order to determine the isotype composition of the antibody response to a parasitic nematode, the ELISA was modified to incorporate the isotype-specific mAbs. Serum from the uninfected llama residing in an area endemic for *P. tenuis* lacked IgG specific for r*Pt*-API (Fig. 2-6*A*). In contrast, all sera collected from diseased/infected llamas contained high concentrations of IgG1 specific for the r*Pt*-API. Trace concentrations of IgG2 were detected in two of these animals. Because of the high prevalence of *P. tenuis* infection in endemic areas, it is impossible to assert with confidence that any llama has not been exposed to the parasite. For this reason we tested sera from healthy llamas obtained from a region free of *P. tenuis* (Washington, USA). These sera yielded variable results. Four of seven llama serum samples had antigen-specific antibodies; however, instead of IgG1 being quantitatively dominant, IgG2 and/or IgG3 were detected in similar concentrations in each sample. Statistical analysis of the ELISA data did not detect significant differences between *P. tenuis* infected and uninfected llama sera. As described above, the antibodies may be specific for bacteria and binding contaminating *E. coli* proteins in the antigen preparation. Alternatively, the antibodies may be specific for r*Pt*-API but induced by similar antigens produced during infection with other parasitic nematodes that are prevalent in Washington. Nematode API show high amino acid sequence similarities across genera and are known to be highly immunogenic (7). Overall, the results show that ELISA employing isotype-specific reagents will improve and enrich the information afforded by such assays, yet the selection of the target antigen remains critical for interpretation of the data.

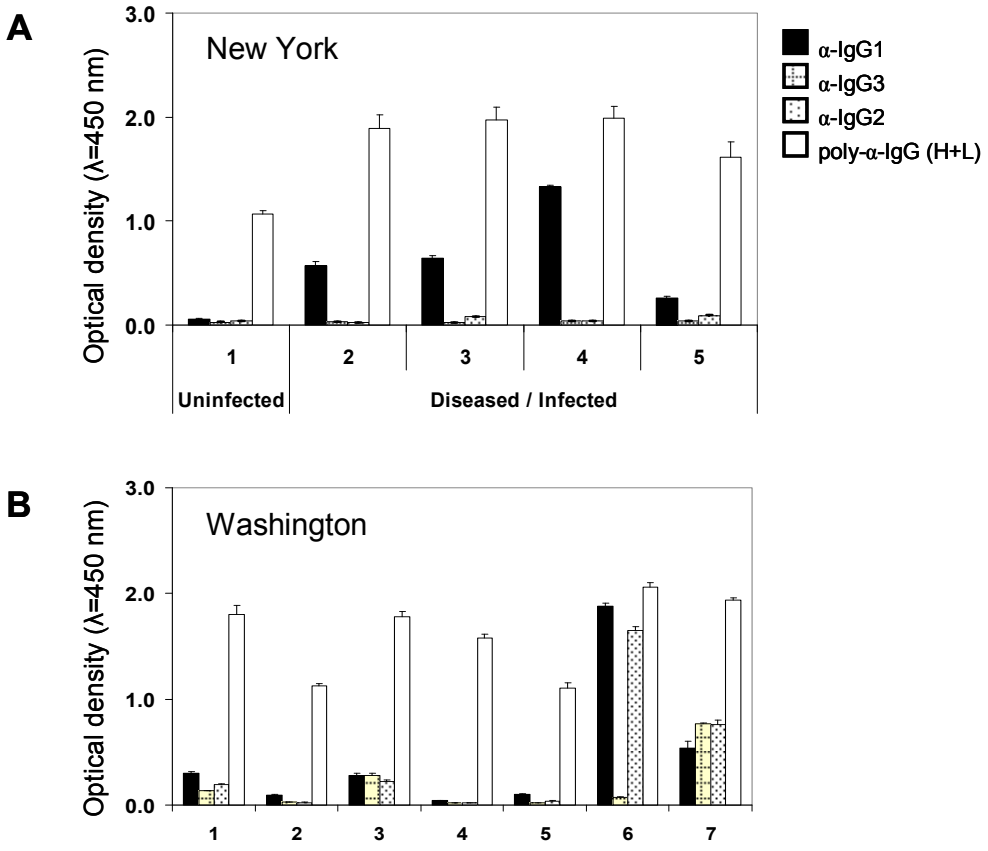

Figure 2-6. Detection of rPt-API-specific IgG isotypes in llama sera by ELISA. (A) Llamas from a P. tenuis-endemic area (New York, USA). Sera from diseased llamas, confirmed to be P. tenuis-infected, contained predominantly IgG1 specific for rPt-API. No antigen-specific IgG isotypes were detected in the uninfected llama serum. Polyclonal goat anti-llama IgG (H + L) did not discern infected from uninfected llamas. (B) Llamas from P. tenuis-free area (Washington, USA). Cross-reactive IgG1 as well as IgG3 and/or IgG2 were detected in sera from uninfected llamas. The polyclonal goat antibodies yielded consistently high readings for all seven sera. Columns represent mean optical densities. of sera diluted 1:100 (tested in triplicate) ± standard deviation (T bars).

Discussion

We prepared mAbs specific for IgG1, IgG2 and IgG3 of llamas. Each mAb bound its soluble, native IgG and, when used to prepare affinity columns, afforded an improvement over previously described Protein A/G affinity methods for separation and purification of the isotypes (12). In addition, the mAbs were effective in ELISA for detection of antigen-specific antibodies. Finally, an IgG2-specific mAb bound three discrete llama heavy chains, confirming the existence of three IgG2 isotypes in the llama, as had been inferred from DNA analyses (35,39).

Camelids emerged in North America during the Eocene epoch some 45-40 million years ago (m.y.a.); however, the Camelini and Lamini divergence did not occur until 11-9 m.y.a. (36,38). Subsequently, the cameloids migrated across the Bering Strait, while the lamoids migrated towards South America. To date, the one-humped dromedary (*Camelus dromedarius*) is native to the Arabian Desert while the two-humped Bactrian (*Camelus bactrianus*) is found in Asia. The lamoids include guanacos (*Lama guanicoe*), llamas (*Lama glama*), alpacas (*Lama pacos*), and vicuñas (*Vicugna vicugna*), and are indigenous to South America. Domestication of llamas and alpacas is estimated to have occurred 5500 to 6000 years ago (37). The camelids that remained in North America became extinct and llamas from South America were reintroduced in the late 1880s.

Microsatellite and mitochondrial DNA analyses have revealed genetic similarity between vicuña and alpaca and between guanaco and llama, suggesting that the alpaca was domesticated from the vicuña and the llama from the guanaco. It has been proposed that alpaca should be reassigned to the genus *Vicugna* (*Vicugna pacos*) (37). Most modern day alpaca and llama carry both guanaco- and vicuña-like DNA reflecting a high incidence of hybridization between the domestic species. It has been estimated that as many as 80% of modern day alpaca are hybrids (37). Although a

likely aim of hybridization was to improve fiber quality in larger animals, fiber quality in domestic species has declined. Efforts are underway to improve genetic purity and fiber quality in alpaca (37).

We evaluated binding of the mAbs to IgGs of Old and New World camelids. Vicuña are both wild and endangered; we did not have access to sera from that species. The anti-IgG1 mAb bound its isotype in all camelids tested, including *C. bactrianus*. Among the New World camelids, IgG3 was not detected by the appropriate mAb in guanaco. Only two IgG2 sub-isotypes were detected in alpaca and guanaco, compared with three in llama. Hybridization in the New World camelids confounds any attempt to interpret these results in evolutionary terms. Although we tested sera from several animals in this study, each alpaca and llama was defined as such by phenotype rather than genotype. Expanded testing on larger numbers of llama and alpaca including animals of defined genotype will be important for validation of the specificities of mAbs against IgG2 and IgG3.

The importance of immunoglobulins to the health and well-being of camelids is documented by the high mortality among neonates that fail to receive adequate colostral antibody (11). The contribution of HCAbs in passive transfer of immunity to neonates is unknown. Furthermore, little is known of the functional contributions of camelid IgGs to immune defense. Camelids have specific pathogens, suffer from common diseases of ruminants, and, as in the case of foot and mouth disease, are resistant to some pathogens (2,6,8,25,27). Camelid HCAbs may have distinct roles in fighting infections. Their more discrete antigen-binding domains, extended CDRs, and increased tissue accessibility, enable HCAbs to bind epitopes otherwise inaccessible to conventional antibodies. In addition, HCAbs have been shown to inhibit enzymes (17,32). The significance of these properties in immune defense, and the nature of the stimuli that induce production of HCAbs, are unknown.

Parelaphostrongylus tenuis is a cause of morbidity and mortality in llamas and alpacas (9,26). Disease results from physical trauma and cellular infiltration into the parenchymal tissues of the central nervous system induced by migrating worms. We found that infections of varying duration stimulated the production of antigen-specific IgG1 in four animals. HCAbs were not prominent in these immune responses. All four animals were ill and eventually died or were euthanized, implying that the immune response induced was not protective. However, our conclusions are based on the results obtained with a single protein antigen, and it will be important to corroborate the data obtained for *Pt*-API with other antigenic proteins and glycans.

Studies in other species have shown that the specificity and sensitivity of a serologic test may be increased when designed to detect a particular IgG isotype. For example, detection of IgG4 antibodies specific for recombinant antigens of *Fasciola hepatica, Angiostrongylus cantonensis,* or *Loa loa* increases the sensitivity and specificity of assays for these infections in humans (13,14,23). Similarly, by using isotype-specific reagents, we reduced high background readings obtained using pan-Ig antibodies, and demonstrated that *P. tenuis*-infected llamas mounted an anti-*Pt*-API response that was overwhelmingly IgG1. The utility of this finding was complicated by our observation that four sera from llamas in a non-endemic area had antigen-specific IgG1, IgG2, and in some cases, IgG3. Although these animals were dewormed every six months, they were reared conventionally and were likely exposed to other parasitic nematodes (William Foryet, personal communication). *Ostertagia ostertagi* is a common parasitic nematode of llamas and produces an API that is 74% identical in amino acid sequence to *Pt*-API. Infection with this or some other nematode may have induced the antibodies detected. The difference in isotype representation in sera of the diseased vs. non-endemic llamas may be due to the site of parasite residence (e.g. CNS for *P. tenuis* vs. gastrointestinal tract for *O. ostertagi*) or

other influences. To address the issue of cross-reactivity, we are seeking other, potentially unique, *P. tenuis* antigens for application in serologic tests.

In summary, we have produced and characterized mAb reagents specific for heavy-chain and conventional IgGs of llamas. The mAbs are readily applied in serologic assays and affinity chromatography, and should be useful in quantitative assessments of IgG isotypes in blood, colostrum, and other body fluids. Their application in identifying IgG isotypes induced during protective and non-protective immune responses to different types of pathogens will improve our understanding of immune defense in New World camelids and should aid in the design of effective vaccines.

Acknowledgements

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CHAPTER 3

Participation of Camelid Heavy-Chain Antibodies in Immune Responses to Nematode and Viral Pathogens[2]

[2]Lisa P. Daley, Michelle A. Kuztler, Barbara W. Bennett, Mary C. Smith, Amy L. Glaser and Judith A. Appleton. Participation of camelid heavy-chain antibodies in immune responses to nematode and viral pathogens. Manuscript in preparation.

Abstract

Naturally occurring IgGs devoid of light chains are produced by Camelids. These heavy-chain antibodies (HCAbs) have largely undefined effector functions. In order to describe the contributions of HCAbs to immune defense, HCAbs and conventional IgG1 were assayed in sera from animals infected with a nematode, *Parelaphostrongylus tenuis* or with West Nile virus (WNV). IgG1 dominated the responses to nematode and viral proteins. Nematode antigens induced IgG2, but not IgG3 HCAbs, while WNV infection induced only low levels of HCAbs. In contrast, IgG3 was readily induced by vaccination with WNV. IgG2 HCAbs were produced during the anamnestic response. When purified IgGs were tested in plaque-reduction neutralization titer (PRNT) tests, IgG3 demonstrated PRNT activities comparable to conventional IgG1. At the highest concentrations tested, IgG2 demonstrated only sub-neutralizing activity. We further investigated whether the IgGs altered virus infectivity of alpaca cells that expressed Fc-receptors. IgG3 and IgG1 enhanced WNV infection of macrophages at concentrations below those that effectively neutralized virus on Vero cells. Our data document for the first time a dichotomy in the HCAb response to viral and nematode pathogens, and a distinction in their virus-neutralization properties. This information contributes to the elucidation of the immunological role of these atypical IgGs, and has implications for diagnosis, prophylaxis, and therapy.

Introduction

It has long been established that antibodies combine homodimeric heavy (μ, δ, ε, α or γ) and light chains (λ or κ) to form their characteristic tetrameric structures. However, camelids produce antibodies that do not conform to this rule. In addition to the conventional IgG1 isotype, the IgG2 and IgG3 isotypes produced by these animals

do not incorporate L-chains into their structures (1, 2). These unusual IgGs, termed heavy-chain antibodies (HCAbs), lack the CH1 domain of the H-chain, which would normally form a disulfide bridge with the CL domain to stabilize the heavy/light-chain association (3, 4). This deletion results from a conserved mutation in the flanking donor splice consensus sequence that causes post-transcriptional excision of the CH1 exon. In addition, the VH of HCAbs (V_HH) incorporates mutations that encode hydrophilic amino acid residues at the putative heavy/light chain interface that confer solubility (5). To increase diversification of the combining sites, the first and second hypervariable loops adopt non-canonical loop structures (6-9). V_HH domains may possess extended CDR3s, similar to bovine immunoglobulins, which serve to increase the surface area for binding antigens (10). HCAbs are bivalent, undergo antigen-driven somatic hypermutation, and exhibit nanomolar binding affinities (6, 11). The composition of HCAbs within serum may be as much as 45 % in *Lama* spp. and up to 75 % in *Camelus* spp., compatible with a significant contribution by these antibodies to immune defense (2).

Despite being discovered over fourteen years ago, very little work has been done to elucidate the immunological functions of HCAbs. For example, it is not known how production of camelid IgGs is regulated. In mice and humans, distinct IgG isotypes are elicited by different types of pathogens, and the functions of the isotypes are relevant to defense against each pathogen. There are characteristic IgG isotype profiles associated with type 1 and type 2 immune responses that are induced by viruses and helminth parasites, respectively. Unique biophysical properties bolster the hypothesis that HCAbs have discrete effector functions. The effector function of an immunoglobulin depends upon the CH2 and CH3 domains. In llamas, the CH2 and CH3 exons are 88 – 98% and 93 – 96 % identical, respectively, across the IgG isotypes. However, IgG3, like IgG1, binds protein G while IgG2 does not (2, 12).

This differential binding of IgG2 and IgG3 to protein G, an FcγRIII orthologue, may correlate with effector functions. Compared with classical antibodies, HCAbs are small in structure, having apparent molecular masses of approximately 100 kDa versus 150 kDa for IgG1, and the V_HH domains (approximately 15 kDa) are the smallest, natural antigen-binding fragments known to date (2, 13). The smaller size may render HCAbs more diffusible than conventional IgG1, allowing them to bind epitopes that would normally be inaccessible. The structural features of HCAbs suggest that these isotypes may have distinct roles in camelid immunity. Research into camelid antibody functions have been greatly hindered by the lack of antibody reagents. To address this, we produced and characterized mAbs that bind specifically to IgG1, IgG2 or IgG3 produced by llamas and alpacas (14).

In this study, we compared the contributions of IgG1, IgG2 and IgG3 in immune responses elicited during infections with two pathogens: the parasitic nematode, *P. tenuis*, and WNV. Furthermore, we investigated the effector functions of virus-specific heavy-chain and conventional antibodies. Our data indicate that IgG2 is distinct from IgG3, both in function and in regulation.

Materials and Methods

Animals and sera. In all cases, camelid blood was obtained by jugular venipucture into vacutainers containing sodium heparin. Blood samples from llamas infected with *P. tenuis* were obtained from diseased animals that were brought into the Cornell University Hospital for Animals or examined by the ambulatory service. Infection with *P. tenuis* was confirmed in diseased llamas by observation of nematodes within the central nervous system during necropsy. Serum was separated by centrifugation and stored at -20 ^oC. Sera from llamas in Pullman, Washington, an area non-endemic for *P. tenuis,* were kindly provided by Dr. William Foreyt,

Washington State University. These animals may have been exposed to *P. andersoni* and almost certainly gastrointestinal nematodes endemic to western Washington. Sera from alpacas that were naturally infected with WNV were obtained during a surveillance study conducted in New Jersey. Sera from WNV-vaccinated alpacas were collected as part of a vaccine study (15). Both sets of samples were kindly provided by Dr. Michelle Kutzler, Oregon State University.

For macrophage cultures, blood was obtained from two mature female alpacas owned by the Cornell University Veterinary School. Animals were housed according to the guidelines of the American Association of Accreditation of Laboratory Animal Care.

WNV vaccination. The vaccination protocol has been described elsewhere (15). Briefly, alpacas were vaccinated intramuscularly with 1 ml formalin-inactivated WNV vaccine adjuvanted with MetaStim™ (West Nile Innovator, Fort Dodge Animal Health). Alpacas received three vaccinations at 3-week intervals. Some animals received an additional dose of vaccine 1 yr after the third immunization and are referred to as "vaccinated/boosted". Sera from these animals were collected 4 wks after the fourth immunization. The animals were monitored for local and systemic reactions to vaccination.

Antibodies. Polyclonal goat anti-llama IgG (H + L) conjugated to horseradish peroxidase (HRP) (Bethyl Laboratories Inc.) was used in ELISA. Monoclonal mouse antibodies (mAbs) were detected with HRP-conjugated goat anti-mouse IgG (H + L) (ICN/Cappel). The 27E10 (anti-IgG1), 19D8 (anti-IgG2), and 8E1 (anti-IgG3) mAbs have been described elsewhere (14).

Recombinant, WNV envelope (E) and nonstructural (NS1 and NS5) proteins. Recombinant proteins were provided by Dr. Amy Glaser, Animal Health Diagnostic Center, Cornell University and the preparation protocol will be described elsewhere. Standard coupled reverse transcriptase polymerase chain reaction (RT-PCR) techniques were used to amplify the NS5, NS1 and E coding sequences from WNV RNA, using primers for the genes of interest. WNV NS5 amplification primers: forward, GACGATATCAGGTG GGGCAAAAGGACGCACC, and reverse, CTAGTCGACCAGTACTGTGTCCTCA ACCAA; WNV NS1 amplification primers: forward, ACGATCTGCAGATAAGCA TTCACTTGTGACTGCAC, and reverse, ACGTGATATCCACTGGGTGTGCCATA AACATC; WNV E amplification primers: forward, AGCTGATATCTAACTGCCTT GGAATGAGCAAC, and reverse, ACGATCTCGAGAGCTATGGACCTATCAC.

PCR amplified DNA fragments were digested with *Eco*RV and *Xho*I and cloned into the pTriEx-1 plasmid vector (Novagen) containing the honey bee mellitin signal sequence. Plasmids containing inserts were selected based on restriction endonuclease digestion and sequence analysis. The resulting plasmids were designated as pTriEX-WNVNS5, pTriEX-WNVNS1, pTriEX-WNVE. Baculovirus recombinants were prepared by co-transfection of plasmid DNA and BacVector 3000 DNA (Novagen) and used to infect Sf9 cells. Virus purified from plaques was assayed for expression of the respective proteins by indirect immunofluorescent assay using polyclonal anti-WNV murine ascites (NS5) or mAbs against E or NS1 (CDC). Virus expressing WNV proteins was amplified in Sf21 cells.

Cells were lysed and expressed proteins were purified using nickel affinity chromatography (BioRad). Eluted proteins were dialyzed at room temperature in phosphate buffered saline (PBS) containing 20% glycerol and stored at -20 °C in 50% glycerol, PBS.

Affinity chromatography of camelid IgGs. IgG isotypes were purified using affinity chromatography (mAbs 27E10, 19D8 or 8E1) as described elsewhere (14). Purified IgG fractions were dialyzed against Dulbecco's phosphate buffered saline (DPBS) and evaluated for homogeneity by ELISA before use in subsequent assays. Briefly, microtiter plates were coated with mAbs (5 μg/ml) specific for each isotype, 28G4 (anti-IgG1), 16A4 (anti-IgG2) and 2B11 (anti-IgG3). Wells were blocked with 5% skim milk, incubated with affinity-purified IgG (1 μg/ml) and bound IgGs were detected with HRP-goat anti-llama IgG (0.1 μg/ml).

Detection of llama IgGs specific for *P. tenuis*. The ELISA described in Daley *et. al* (14) was modified to detect camelid IgGs specific for *P. tenuis* third-stage larvae (L3). Soluble antigens were prepared by lysing 100 L3 worms by freeze-thawing three times followed by sonication and removal of particulates by centrifugation. For all steps, well volumes were 25 μl and plates were incubated 1 hr at room temperature unless otherwise specified. Plates were coated overnight at 4 ^{o}C with 4 μg/ml soluble L3 antigens in 10 % DPBS then blocked with DPBS containing 2% skim milk and 0.05% Tween 20. Plates were washed three times with DPBS containing 0.05% Tween 20 after each incubation step, and subsequent dilutions were prepared using blocking solution. Llama sera were diluted 1:100 and added to triplicate wells. Wells were incubated with the 27E10, 19D8 or 8E1 mAbs (5 μg/ml) or HRP-goat anti-llama IgG (0.1 μg/ml) as a positive control. Bound mAbs were detected with 5 μg/ml HRP-conjugated goat anti-mouse IgG diluted in blocking solution containing 10% normal goat serum. The assay was developed with 3,3,5,5'-tetramethyl-benzidine (TMB, KHL) and reactions were terminated with 1M H_3PO_4. Optical density (O.D.) readings were taken at 450 nm wavelengths with a microplate reader (Biokinetics Reader, EL340; Bio-Tek Instruments).

Determination of endpoint, serum neutralization titers. The protocol used to determine endpoint, serum neutralization (SN) titers for WNV infected and vaccinated alpacas has been described elsewhere (15). In brief, virus-neutralization antibody titers were obtained by incubating two-fold serial dilutions of serum (heat-inactivated 30 m, 56 ^{o}C) for 1 hr with equal volume of WNV (102, 50% tissue culture infective dose) and 3% guinea pig complement (C′). Incubations were conducted at 37 ^{o}C in 5% CO_2 and samples were tested in duplicates. Mixtures were added to equal volumes of Vero cell suspensions in wells of microtiter plates, incubated 6 d, and cells were examined for cytopathogenic effects (c.p.e.). Geometric mean titers were determined for duplicate samples and endpoint titers were calculated as the inverse of the highest serum dilution that inhibited c.p.e. of virus. A value < 8 was considered as negative.

Detection of camelid IgGs specific for WNV proteins. The ELISA described above was modified to detect IgGs specific for WNV. Plates were coated with 5 μg/ml recombinant viral proteins (E, NS1 or NS5). Sera were diluted 1:100 and added to triplicate wells. Wells were incubated with isotype specific mAbs or HRP-goat anti-llama IgG. Bound mAbs were detected with HRP-conjugated goat anti-mouse IgG.

Plaque-reduction neutralization assay. WNV-NY1999 stock were grown in Vero cells and frozen at -80 ^{o}C until use. Plaque-reduction neutralization titers (PRNT) were determined for heat-inactivated (60 ^{o}C, 5 m) alpaca serum or affinity-purified IgGs. Incubation periods were 1 hr at 37 ^{o}C in 5 % CO_2. Two-fold serial dilutions of serum or affinity-purified IgGs were incubated with an equal volume of WNV ($1 – 2 \times 10^3$ plaque-forming units (p.f.u.) per ml) with or without guinea pig C′

(5 %; Colorado Serum Company). Vero cell monolayers were grown in 6-well culture plates. Inoculum (100 µl) was added to each well, incubated 1 hr., then monolayers were overlaid with medium containing 1% (w/v) low-melting point agarose and incubated 4 days. Plates were stained on day 3 with Neutral red and plaques were enumerated on day 4. Plaque-reduction was calculated as a percentage of plaques obtained in the absence of serum or antibodies. A reduction of 90% (PRNT90%) or greater was set as the positive threshold.

Preparation of monocyte-derived cell cultures. A protocol described by Davis *et. al* (16) was modified to separate mononuclear cells from alpaca blood. Heparinized blood was diluted 1:3 in sterile PBS and layered onto density gradients (upper gradient - Histopaque® 1077; lower gradient - Histopaque® 1119; Sigma) in 50 ml polypropylene tubes. Tubes were centrifuged 700 × g in a swinging bucket rotor with the brake off for 1 hr at ambient temperature. Supernatant was aspirated within 0.5 cm of desired layers and discarded. Mononuclear cells were collected from the plasma-upper gradient interface. Granulocytes (mostly neutrophils) settled at the interface between the upper-lower gradients. Red blood cells pelleted at the bottom of the centrifuge tubes. Cells were washed three times by suspending in PBS and centrifuging (250 × g) for10 m at 10 ^{o}C. Viability was estimated with trypan blue in a hemocytometer, and cellular composition was evaluated by flow cytometry.

Mononuclear cells were cultured according to the protocol described by Saldarriaga *et. al* (17) with few modifications. Pelleted cells were resuspended to a final concentration of 2×10^6 cells/ml (containing approximately 4×10^5 monocytes/ml) in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal calf serum (Atlanta Biologicals), 0.5 mg/ml gentamycin sulfate (Cellgro), 0.25 µg/ml fungizone (Gibco), 100 U/ml

penicillin (Gibco), 0.1 mg/ml streptomycin (Gibco), 2 mM L-glutamine(Gibco), and 50 μM β-mercaptoethanol (Sigma). Cell suspensions (1 ml/well) were added to 24-well tissue culture plates, and incubated at 37 °C with 5% CO_2 to allow for adhesion. After 24 hours, non-adherent cells were removed. Cultures were maintained by replacing medium every four days. Seeding wells at a density of 4×10^5 mononuclear cells generally yielded 1×10^5 adherent macrophages per well at the end of the culture period.

α-Naphthyl acetate esterase assay. To confirm that adherent cells were macrophage-like, 8-day old cultures were incubated 5 m with cold Trypsin-EDTA (Sigma) to release cells from the surface. Cells were washed twice in PBS, centrifuged (500 × g, 5 m) onto glass microscope slides, and air-dried overnight at room temperature. Staining was performed with the α-naphthyl acetate esterase kit (Sigma) following manufacturer's instructions. Briefly, cells were fixed for 30 s with a citrate-acetone-formaldehyde solution at room temperature, and then rinsed thoroughly for 1 m in running deionized water. Slides were incubated in staining solution 30 m at 37 °C in darkness then rinsed 2 m in running deionized water. Coverslips were mounted with Glycergel (DakoCytomation). Images were obtained using a BX51 microscope fitted with DP-12 digital camera system (Olympus).

Antibody-dependent enhancement assays. Virus ($1 - 2 \times 10^4$ p.f.u.) was incubated for 1 hr with affinity-purified IgG with or without added C′. Macrophages were incubated with mixtures (m.o.i. = 0.1), or with medium, or virus only as controls. Samples were tested in triplicate and incubations were done at 37 ^oC in 5 % CO_2. After 3 hr, media were aspirated from individual wells, and cells were washed three times with warm (37 ^oC) PBS to remove unbound virus and antibodies. Fresh medium

was added to each well and plates were incubated an additional 36 hr. Supernatant and cells were harvested from each well for estimation of released and cell-associated viruses by plaque-assay. Cells were lysed by three cycles of freeze-thawing to release cell-associated virus. Plaque assays were performed by inoculating Vero cell monolayers with 200 µl of serially diluted supernatant or lysed cells. After 1 hr, monolayers were overlaid with 1 % low melting point agarose, incubated for 3 d, stained, and plaques enumerated on day 4 as described above.

Statistical analysis. ELISA data from groups of infected and uninfected llamas were evaluated by analysis of variance. Significance was assessed using Bonferroni's and Tukey's tests. Values were considered statistically significant when $p < 0.05$.

Results

IgG isotypes induced during natural nematode infections

Recently we showed that only classical IgG1 antibodies could be detected in *P. tenuis*-llama serum using a worm-specific aspartyl protease inhibitor as capture antigens (14). Although highly immunogenic, this antigen represented just one of the complex mix of worm proteins that the animals may become exposed to during infection. As a result, heterogeneous soluble antigens prepared from infective third stage larvae (L3) was substituted in the ELISA to evaluate IgG isotypes that were induced by worm proteins. As before, IgG1 dominated the response to L3 antigens in *P. tenuis*-infected llamas (n = 4); however, IgG2 HCAbs were also induced. IgG3 levels remained low (Fig. 3-1*A*). IgG1 levels were significantly greater than IgG2 ($p < 0.05$) and IgG3 ($p < 0.01$); however, the difference between IgG3 and IgG2 levels was not statistically significant. As a comparison, we assessed sera from animals (n = 4)

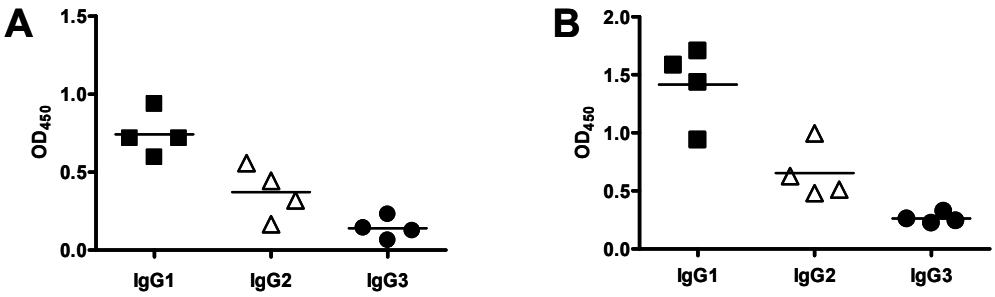


Figure 3-1: Worm antigens induced IgG2 HCAbs and conventional IgG1 in llamas during natural infections. **(A)** IgG isotypes induced during *P. tenuis* infection in four animals. **(B)** Cross-reactive IgGs induced by heterologous worm infections in four animals. Soluble *P. tenuis* L3 antigens (4 μg/ml) were used in ELISA to detect worm-specific serum IgGs. Symbols represent the mean optical density (OD; $\lambda = 450$) value for each isotype obtained from individual alpacas. Horizontal bars represent mean OD values per group.

that had been exposed to heterologous nematode infections. Interestingly, these animals had also produced predominantly IgG1 and IgG2, but few IgG3 cross-reactive antibodies that bound *P. tenuis* L3 antigens (Fig. 3-1*B*). These observations were congruent with the conclusion that IgG3 HCAbs were not efficiently induced by nematode infection.

IgG isotypes induced during natural virus infection

To determine whether distinct isotypes were induced in response to virus, we assessed the IgG isotypes that were induced during natural infection with WNV. Sera from alpacas (n = 4) that demonstrated serum-neutralization (SN) titers ranging from 181 to 1448 (Table 3-1) were tested in ELISA using recombinant WNV envelope (E) and two non-structural (NS1 and NS5) proteins. The IgG profile was similar for all the antigens tested in that, primarily IgG1 antibodies were detected; IgG2 and IgG3 levels were equally low, except for one animal that had relatively high levels of anti-NS1 IgG3 (Fig. 3-2*A*-*C*). These samples were acquired during a serological surveillance program for WNV in camelids therefore, there were no clinical records available that documented the nature of their exposure to WNV. Interpretation of the immune response data is confounded by the fact that the time of exposure is unknown.

IgGs induced by WNV vaccine

We tested serum samples obtained from alpacas that had been administered three doses of a formalin-inactivated WNV vaccine under controlled, experimental conditions (15). All the animals had measurable serum neutralization titers (Table 3-1) and produced IgG antibodies specific for WNV E and NS1 proteins, but not NS5 after two doses of vaccine (Fig. 3-3). In contrast to the anti-nematode response, IgG3 was induced together with IgG1 in response to vaccination. With respect to the

Table 3-1: Summary of the camelid serum neutralization titers obtained against West Nile virus

	Serum neutralization titer						
	days post-vaccination						days post-infection
	21	***44***	***63***	***155***	***365***	***393***	***ND***
Vaccinated (group 1)							
Mr. Nitro	N	512	2048	181			
Bottom Line	N	724	1448	181			
Dancin Boots	N	45	1024	91			
Crayola	N	256	2048	128			
Conrad	N	1024	4096	362			
Bull's eye	N	362	1024	91			
Wild Thang	N	1024	1024	91			
Novio	N	181	1024	128			
Jag	N	724	2048	128			
Vaccinated (group 2)							
#199					N		
#344					N		
#500					N		
#758					N		
#296					N		
#327					N		
#494					N		
#818					N		
Vaccinated/boosted							
#319						2896	
#723						2896	
#550						>4096	
#740						>4096	
Naturally infected							
Veronica							181
Colonel Fenwick							724
Macho Negro							1024
Prisa							1448

ND, not determined; N, negative

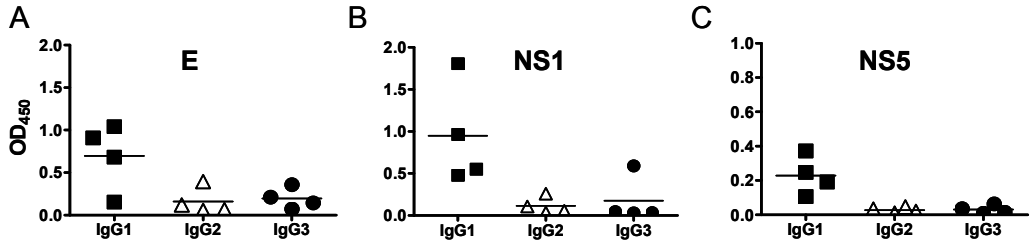


Figure 3-2: Conventional IgG1 dominates the IgG response to natural infection with West Nile virus. WNV-specific IgG isotypes were detected in ELISA using envelope (E), and non-structural proteins (NS1 and NS5). Bound IgGs were detected using peroxidase-conjugated goat anti-sera. Horizontal bars represent mean OD values per group.

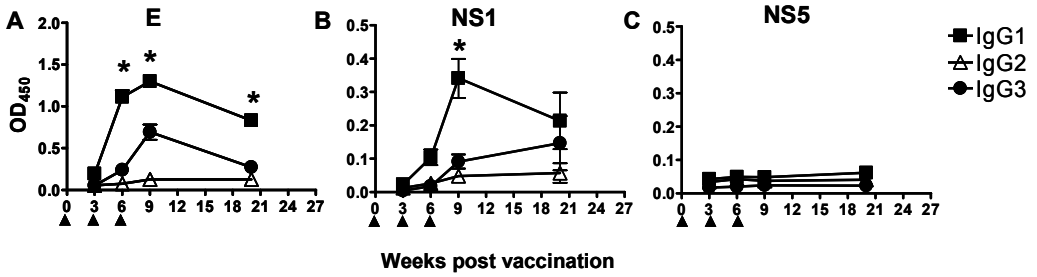


Figure 3-3: WNV vaccination induced IgG3 HCAbs and conventional IgG1 in alpacas. Alpacas (n = 8) were vaccinated three times (arrow heads) with killed, adjuvanted WNV. Antibodies specific for **(A)** WNV envelope (E), or **(B and C)** non-structural proteins (NS1 and NS5) were detected by ELISA. Mean OD_{450} values at 6, 9 and 12 weeks post-vaccination were compared by ANOVA with values obtained at week 3 for each IgG isotype. Significance (*, $p < 0.05$) was assessed using Bonferroni's test. Bars represent standard error of the mean. Absence of antibodies to NS5 is compatible with the fact that the vaccine does not incorporate this protein.

immunodominant E protein, IgG1 and IgG3 levels became significantly higher ($p < 0.05$) than IgG2 by week six of the experiment (Fig. 3-3*A*). The IgG response to NS1 proteins was similar in kinetics but weaker than that for the E protein (Fig. 3-3*B*). A weak IgG2 response to NS1 was detected. As expected, there were no IgG responses induced against the NS5 protein as this is a virus-specific RNA polymerase, which was absent from the vaccine (Fig. 3-3*C*). It took three vaccinations to elevate serum neutralization titers of alpacas to equal those of horses administered only two doses (15). The data show that IgG2 is not efficiently induced by vaccination with killed virus.

The anamnestic response in vaccinated alpacas

IgGs specific for WNV E protein were still detectable one year after vaccination (Fig. 3-4*A*); however, the anti-NS1 IgG response had diminished completely (Fig. 3-4*B*). Administration of vaccine at this time elicited an anamnestic response, including IgG1 and IgG3 specific for the E and NS1 proteins. These results document that all the isotypes are induced by re-vaccination. In addition, a marked increase in IgG2 HCAb levels was detected against the E protein.

The host cell receptor-binding domains of WNV are located within the E protein. In other species, antibodies elicited against this protein have been reported to be highly effective at neutralizing virus. We next sought to address whether the IgG isotypes induced against WNV could neutralize.

Evaluation of virus-neutralizing activities of alpaca IgGs

To quantify neutralizing activities of WNV-induced antibodies, we compared the PRNT of sera from naturally infected with vaccinated/boosted alpacas. Sera from vaccinated/boosted alpacas were 8-fold more potent in PRNT(90%) assays than sera

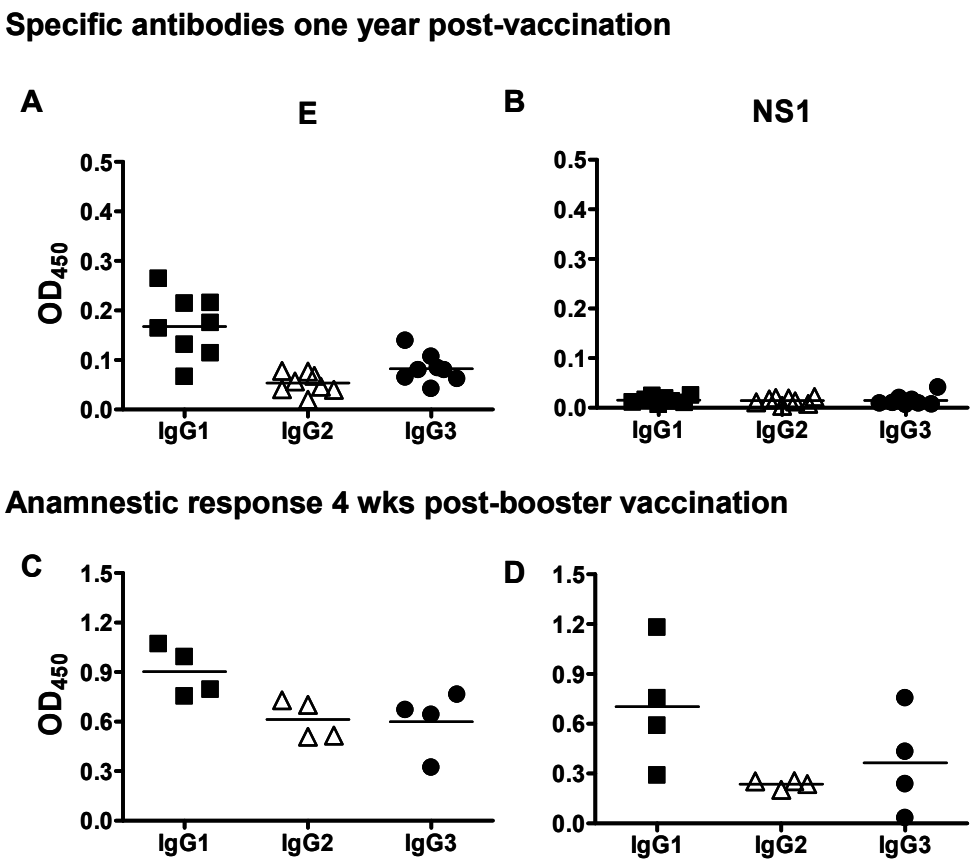


Figure 3-4: Revaccination with WNV one year after primary immunization induced IgG3 HCAbs and IgG1 in alpacas. **(A and B)** Anti-WNV E, but not anti-NS1, antibodies were detected in alpacas (n = 8) one year post-vaccination. Alpacas (n = 4) that were revaccinated at this time produced IgG2 along with increased concentrations of IgG1 and IgG3 against WNV E **(C)** and NS1 proteins **(D)**. ELISA was conducted as described in Fig. 3-3. Horizontal bars represent mean OD values per group.

from naturally infected alpacas (Fig. 3-5). To determine whether IgGs participated in neutralization, we purified IgG1, IgG2 and IgG3 from sera using affinity columns prepared with isotype-specific mAbs. Antibodies were purified from sera of eight individual alpacas. Homogeneity of each IgG preparation was confirmed by sandwich ELISA (data not shown). Before assaying the purified IgGs in PRNT assays, we confirmed that they had not deteriorated during the purification process by testing them in ELISA using WNV E proteins. Binding activities were preserved with one exception (Fig. 3-6). The binding of IgG3 from naturally infected alpacas improved substantially after purification (Fig. 3-6*A* vs. Fig. 3-2). One explanation for this change would be that IgG1 and IgG3 were induced against similar epitopes on WNV E protein, and once separated, IgG3 no longer had to compete for binding. It is possible that, by virtue of its larger size IgG1 sterically hinders access of IgG3 to binding sites on the E protein. Confident that the affinity-purified IgGs had retained their binding properties, we proceeded to test them in plaque-reduction assays.

Neutralization assays were carried out by incubating WNV with two-fold dilutions of purified IgG1, IgG2 and IgG3, with or without C′, before inoculating Vero cell monolayers. Conventional IgG1 and IgG3 HCAbs induced by either natural exposure or vaccination demonstrated potent neutralizing activities (Fig. 3-7). Neutralization was C'-independent at concentrations greater than 30 μg/ml for these isotypes from both groups of animals. The role of C' was evident when IgGs produced by natural exposure were tested at concentrations lower than 30 μg/ml (Fig. 3-7*A*). IgG3 and IgG1 induced by hyper-immunization neutralized independently of C' even at 3 μg/ml (Fig. 3-7*B*). Conversely, IgG2 induced in these animals demonstrated sub-neutralizing activities (< 90% plaque-reduction), which were notably enhanced by the presence of C' (Fig. 3-7*B*). In all the cases, WNV-induced IgG1 and IgG3 efficiently neutralized virus while IgG2 demonstrated sub-neutralizing properties.

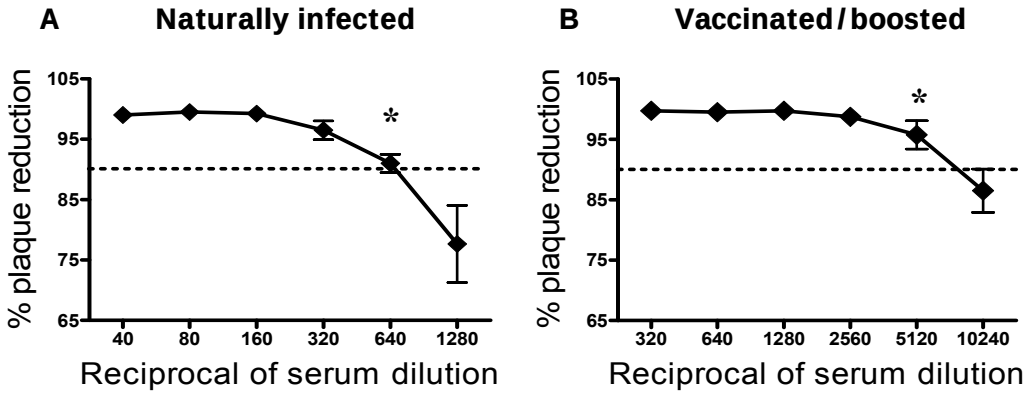


Figure 3-5: Detection of neutralizing antibodies in sera from naturally infected (**A**) and vaccinated alpacas (**B**). Standard PRNT were conducted with sera in the presence of C'. Sera were collected at onset of clinical signs from naturally exposed alpacas and one month post-boosting from vaccinated/boosted alpacas. N = 4 alpacas per group. * indicates endpoint titer. Dotted lines indicate positive threshold.

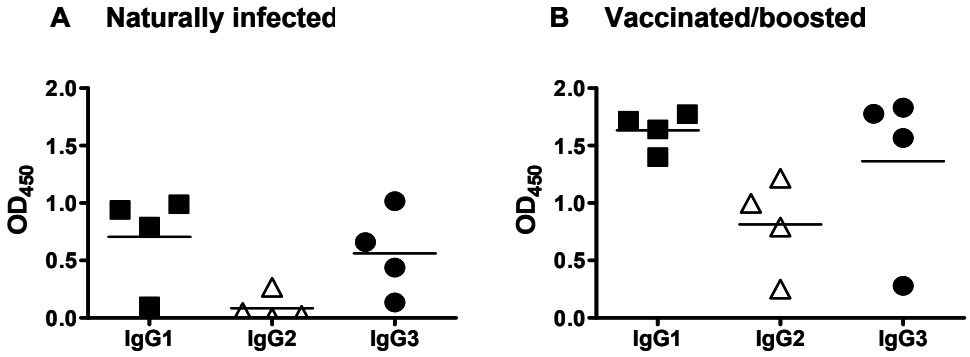
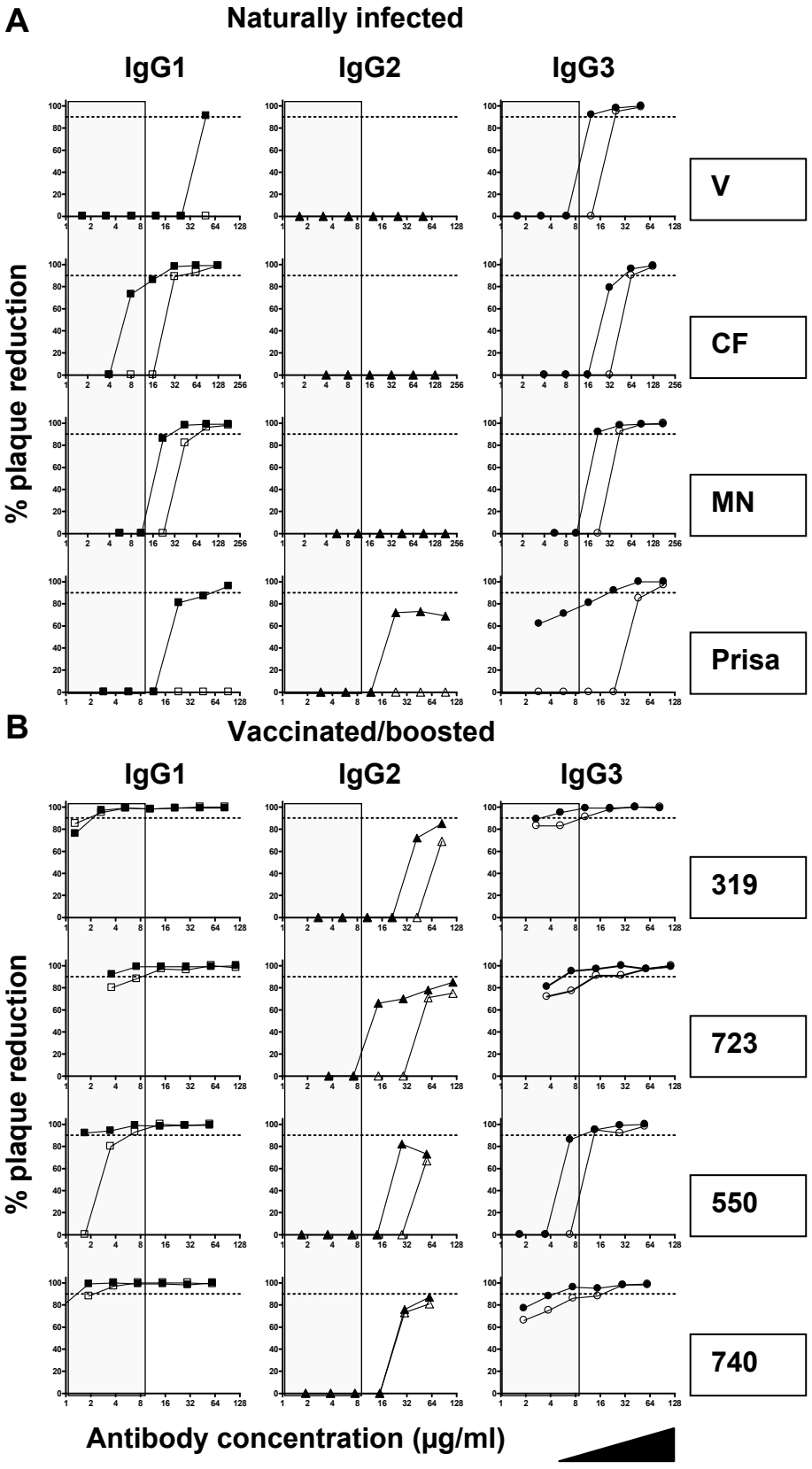


Figure 3-6: Affinity-purification of IgGs did not impair epitope binding. IgGs affinity-purified from alpaca sera described in Fig. 3-5 were assayed for binding to WNV E protein in ELISA. Horizontal bars represent mean OD values per group.

Figure 3-7: IgG3 and IgG1 demonstrated comparable neutralizing activities for WNV while neutralization by IgG2 was inefficient. The PRNT assays were conducted with affinity-purified IgGs from naturally infected **(A)** and vaccinated **(B)** alpacas. Filled symbols indicate values obtained with C′, open symbols, without C′. Shaded areas represent concentrations at which IgGs where assessed for enhancement of WNV infection of alpaca macrophages (Fig. 3-9).


A
Naturally infected
IgG1
IgG2
IgG3
% plaque reduction
V
CF
MN
Prisa
B
Vaccinated/boosted
IgG1
IgG2
IgG3
% plaque reduction
319
723
550
740
Antibody concentration (µg/ml)

Effect of IgG on WNV infection of macrophages

Antibody-dependent enhancement of viral infection is characteristic of flaviviruses. In humans, dengue virus replicates primarily in Fc-receptor bearing monocytes and macrophages (18). These cells are infected more efficiently in the presence of dengue virus-specific antibodies (19). To assess the effect of anti-WNV IgGs on infectivity, we conducted experiments using macrophages prepared from alpaca peripheral blood.

Cells were separated over density gradients to eliminate erythrocytes in alpaca blood that cannot be lysed using conventional methods (Fig 3-8A). The cells obtained from the upper band were enriched in mononuclear cells (Fig. 3-8*B*), and the separation process was effective in eliminating polymorphonuclear cells (Fig. 3-8*C*). Within 8 days of culture, adherent monocytes had differentiated into macrophage-like cells that expressed cytoplasmic esterases specific for α-naphthyl acetate (Fig. 3-8D). Cells obtained from the polymorphonuclear cell layer of the density gradients did not react with α-naphthyl acetate (Fig. 3-8E).

The ability of WNV to infect and replicate productively in alpaca macrophages was evaluated. A multiplicity of infectivity (m.o.i.) of 0.1 established infection and resulted in considerable WNV replication; however, an m.o.i. = 10 proved too high resulting in a one step infection of all the cells, viral replication and subsequent cell death (Fig. 3-8*F*). Titers of virus released within five days of established infections were low (20,000 p.f.u./ml), suggesting that alpaca macrophages are not highly permissive to WVN infection (Fig. 3-8*F*; left panel). The results observed were not affected by inoculation time (Fig. 3-8*F*) or the addition of C′ (not shown).

To assess the effects of WNV-specific IgGs on the early events of virus infectivity, WNV was incubated with IgG (10, 2, 0.4 and 0.08 μg/ml, and 50 μg/ml; n = 1), with or without added C′, prior to inoculating macrophages. Cells (1×10^5)

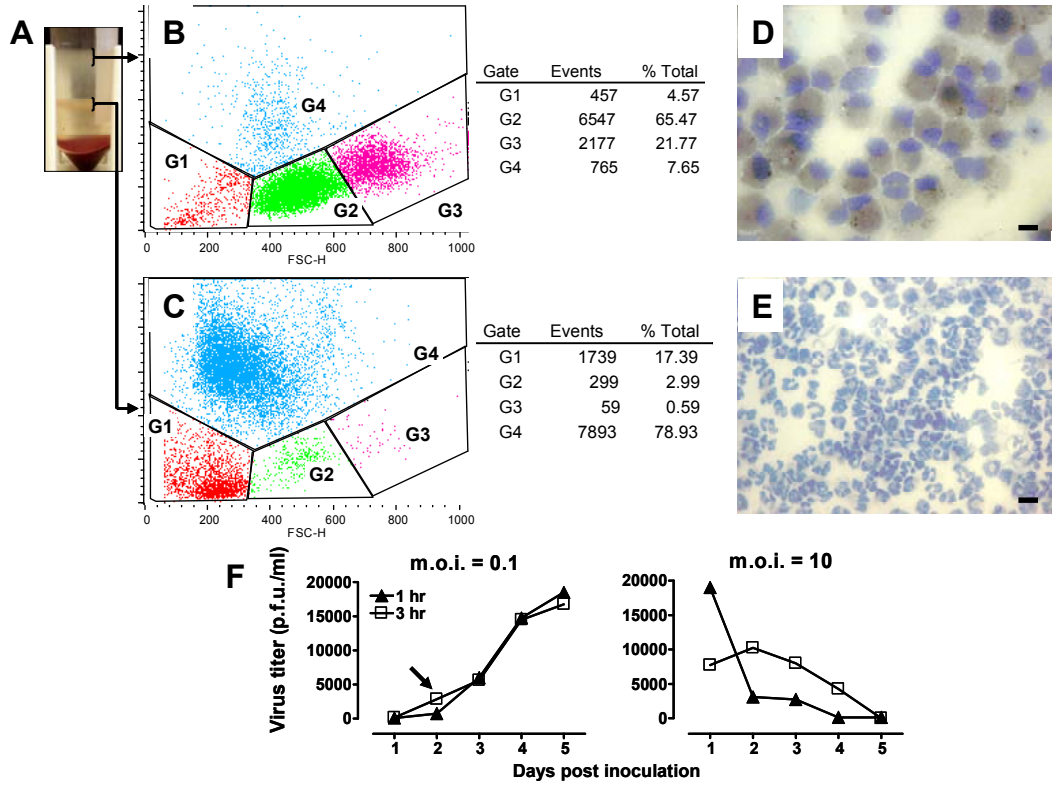


Figure 3-8: Purification and assessment of alpaca macrophages.
(A) Density gradients (upper gradient - Histopaque 1077; lower gradient - Histopaque 1119) were loaded with alpaca blood diluted 1:3 in PBS, and centrifuged 750 × g.
(B, C) Flow cytometric analysis of cell fractions isolated by density gradients. Scatter plots are shown for the low density (B) and high density (C) fractions. Gates were set around red and dead cells (G1), lymphocytes (G2), monocytes (G3) and granulocytes (G4).
(D, E) Cytospin preparations of adherent cells from low density fraction after 8 days in culture (D), or cells from the high density fraction (E). Macrophages stained positive (black) for α-naphthyl acetate esterase but granulocytes did not. Scale bar represents 10 μm.
(F) Macrophages (1×10^5) were incubated for 1 hr (filled symbols) or 3 hr (open symbols) with WNV (m.o.i. = 10 or 0.1), rinsed with PBS and cultured at 37 ^{o}C for 5 days. Supernatant was collected at daily intervals and virus titrations were obtained by plaque assay. Arrow indicates m.o.i. (0.1), inoculation period (3 hr) and time point of supernatant collection used in subsequent assays.

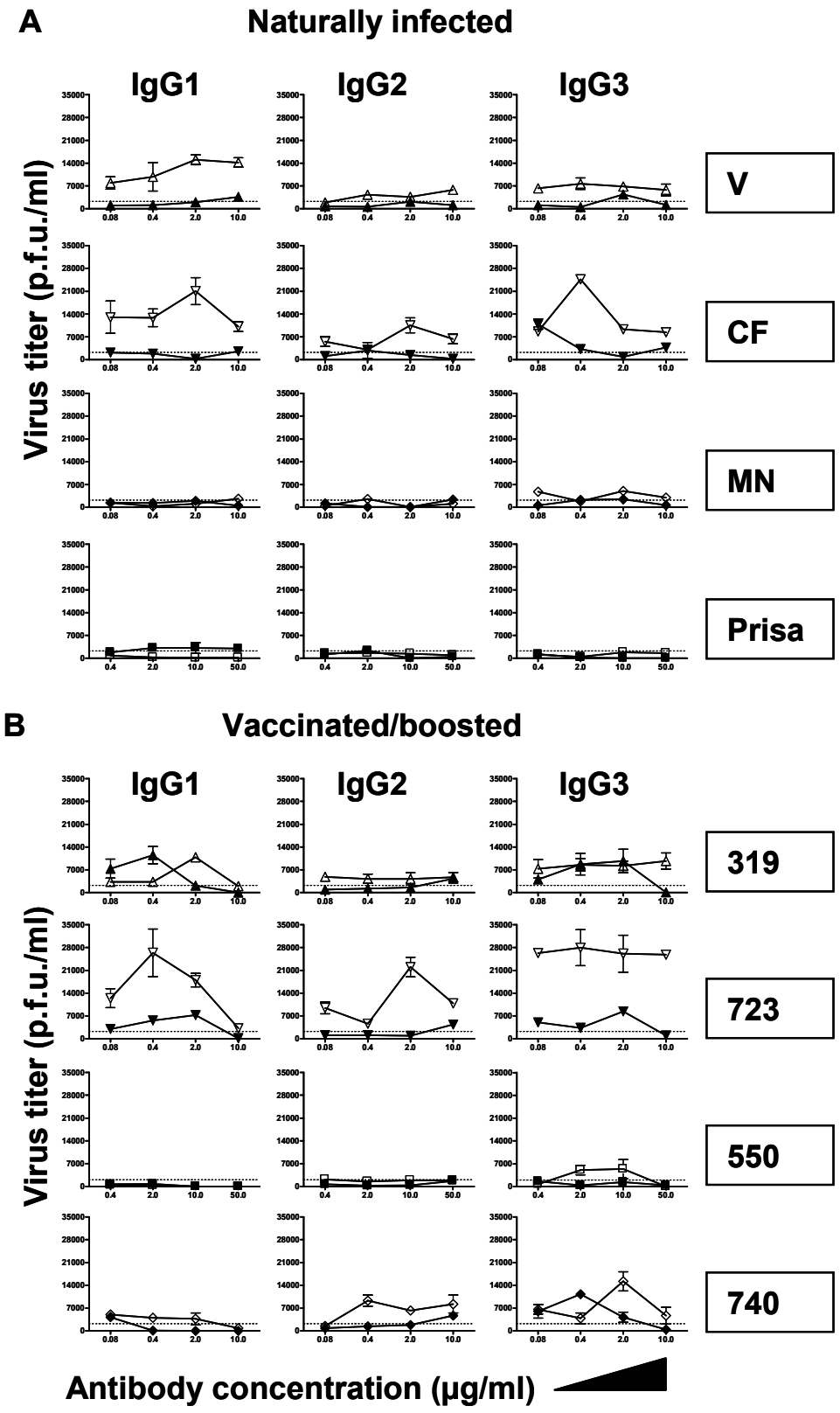
were inoculated for 3 hr with 1×10^4 p.f.u. of WNV (m.o.i. = 0.1), and virus titers were obtained 36 hours after. Enhancement of infectivity was observed with all three isotypes, but was variable among the preparations tested (Fig. 3-9). Variability was apparent even among animals with similar isotype results on Vero cells. In some instances virus replication was enhanced more than ten-fold by IgG1 and IgG3 (animals V and 723). In other cases, little or no enhancement was observed (Prisa and 550) (Fig. 3-9). Nevertheless, antibodies from seven of the eight animals showed enhancement with at least one isotype. Inclusion of C′ reduced the enhancement effects in nearly all the preparations assayed. Titrations of cell-associated viruses reflected those of released viruses but were considerably reduced (Fig.3-10).

Overall, the data are compatible with the conclusion that WNV infection of camelid macrophages is limited; however, infectivity can be dramatically enhanced by low concentrations of WNV-specific IgG antibodies. Enhancement process can be abolished in a C′-dependent manner.

Discussion

The role of HCAbs in immunity is largely unknown. This study is the first to document that IgG2 and IgG3 HCAbs have discrete roles in immune responses to nematode and viral infections. In response to nematode infections, it is the IgG2 heavy-chain isotype that is produced together with conventional antibodies. Conversely, viral infections induce IgG3 HCAbs that compete with conventional IgG1 for epitopes on immunodominant E proteins. In humans, Th1 cells produce INFγ that drives IgG1 and IgG3 production in response to infection with viral pathogens while parasitic nematode infections elicit a Th2 response. This response is associated with the production of IL-4, IL-5, and IL-13, the induction of IgE and discrete IgG isotypes (IgG1 in mice and IgG4 in humans), basophilia, mastocytosis and eosinophilia.

Figure 3-9: Antibody-dependent enhancement of WNV replication in alpaca macrophages. Virus was opsonized with IgGs purified from naturally infected **(A)** or vaccinated/boosted animal sera **(B)** prior to inoculating alpaca macrophages (Fig. 3-8). Supernatants were collected 24 hr post-inoculation and the titrations of virus released from infected cells were obtained by plaque assays. Filled symbols represent assay conducted with C′, open symbols, without C′. Stippled lines represent virus obtained from macrophages inoculated with WNV only.


A
Naturally infected
IgG1
IgG2
IgG3
Virus titer (p.f.u./ml)
V
CF
MN
Prisa
B
Vaccinated/boosted
IgG1
IgG2
IgG3
Virus titer (p.f.u./ml)
319
723
550
740
Antibody concentration (µg/ml)

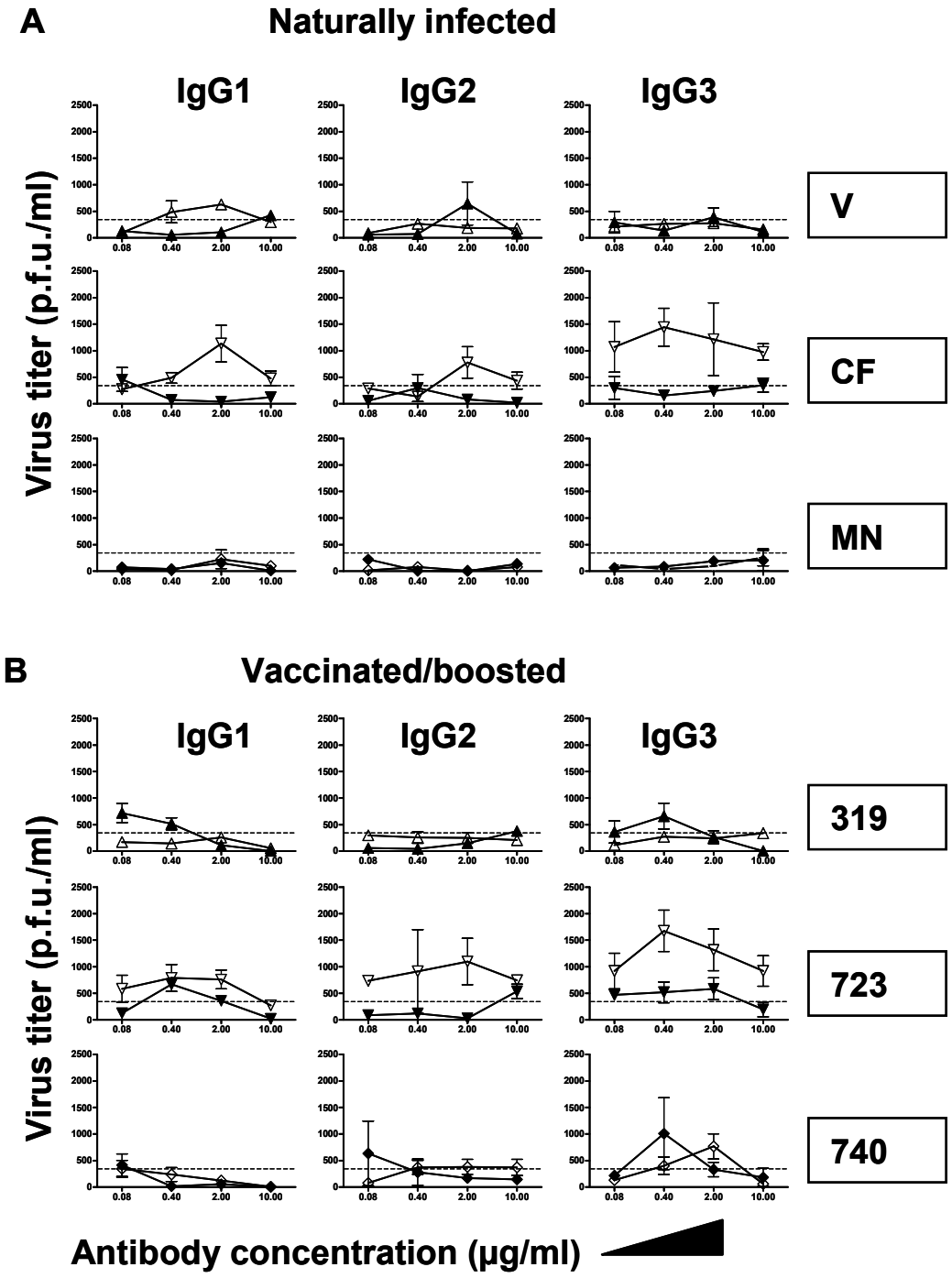


Figure 3-10: Cell-associated virus titers were low but reflected titers calculated for virus released in the supernatant. Macrophages were infected with WNV as in Fig. 3-9. Cell lysates were prepared by three cycles of freeze-thawing and virus titrations were obtained by plaque assays. Filled symbols represent assay conducted with C′, open symbols, without C′. Stippled lines represent virus obtained from macrophages inoculated with WNV only. In both groups n = 3.

The Th1/Th2 paradigm has been characterised in the mouse and human, but does not appear to be enforced in other species, e.g. ruminants. Disparities in the biophysical features of the unusual IgG2 and IgG3 produced by camelids predicted that these isotypes should display dissimilar effector functions. Our data demonstrate that antigens that would normally generate Th1- and Th2-driven responses produced IgG2 and IgG3 HCAbs, respectively, in infected camelids. Clearly, synthesis of the two heavy-chain isotypes in camelids is influenced by distinct immune regulators.

A successful humoral immune response against virus infection is determined by the ability of the elicited antibodies to bind virus and neutralize infectivity. West Nile virus is a member of the Flaviviridae family of viruses and is related to clinically important viruses such as those that cause dengue, yellow fever and Japanese encephalitis (20). Its 11 kb RNA genome encodes a single polyprotein that is cleaved by both viral and host enzymes to yield three structural and seven non-structural proteins (21). The E protein of WNV is comprised of three structural domains (I, II and III). Domain III adopts an immunoglobulin-like structure and it is believed that receptor binding sites are located within this domain. Antibodies raised against DIII are the most efficient at neutralizing WNV (22-24). In our study, alpaca IgG3 HCAbs were induced against WNV E proteins and demonstrated inhibitory effects comparable to IgG1 when assayed for WNV neutralization using a fibroblast cell-line. In addition to IgG1 and IgG3, hyperimmunization produced E-specific IgG2 HCAbs; however, this isotype failed to meet the threshold (PRNT90%) even when tested at concentrations exceeding 100 μg/ml. Altogether, IgG3 HCAbs demonstrated effector properties that were consistent with virus neutralizing antibodies.

Mononuclear cells are important sites of replication for flaviviruses. Because these cells bear FcR, they phagocytose antibody-coated viruses and other particles, usually leading to destruction of the complex. It has been reported that monoclonal

antibodies that are highly efficient in neutralization assays using fibroblast cells can enhance infection of FcR-bearing cells by dengue virus (25). To ascertain whether anti-WNV IgGs were able to neutralize virus infection of mononuclear cells, experiments were performed with macrophages prepared from alpaca blood. We adapted a protocol for culturing bovine macrophages for this purpose. The protocol that we developed worked quite well and we were able to culture monocytes that differentiated into large, vacuolated cells of dendritic morphology. Within 8 days of culture, cells were confirmed to be macrophages using conventional cytologic methods. The cells were used in infection assays at day 14 of culture. When infected with WNV alone or in the presence of C′, viral replication in alpaca macrophages was low. In other studies, equine monocytes/macrophages have been shown to be susceptible to WNV replication attaining titers of 3.2-6.6 log10 p.f.u./ml (26). Here, we demonstrated that replication of WNV in alpaca macrophages is less efficient.

Interestingly, IgG1 and IgG3 induced by natural infection enhanced WNV infectivity of alpaca macrophages. However, in the presence of C′, enhancement was abolished. In contrast, IgG1 and IgG3 induced by hyperimmunization also enhanced; however, C′ only reduced virus production and did not abolish it. The starting IgG concentration (10 μg/ml) used in these experiments was selected because it was the concentration at which neutralization of WNV infectivity of Vero cells declined, and a dependency on C′ was evident. At these diluted concentrations, antibodies enhanced infectivity in macrophages, but C′ abrogated this effect. Thus, the neutralization data were correlated with the enhancement data.

It has been established that IgG can mediate antibody-dependent enhancement (ADE) of West Nile virus infection (27). Although there are several mechanisms that have been suggested, the most widely accepted mechanism for ADE of viruses involves Fc-receptors (28). Virus attachment to target cells is facilitated when Fc

portions of antibodies complexed with viruses bind to FcγRs. It has been shown that dengue virus replication mediated by FcR-dependent ADE can lead to suppression of anti-viral gene expression (29). In our system, clearly WNV replication in macrophages is mediated by an ADE mechanism. WNV-specific IgGs may serve as a bridge that strengthens the interaction of the virus with cells to bring about entry. Likewise, the virus may take advantage of the phagocytic pathway to avert detrimental cellular responses. In other species, macrophages express FcγRI, FcγRII and FcγRIII on their surface. In dengue virus infections, human FcγRI and FcγRII have been shown to mediate ADE *in vitro* (30). FcγRs have not been described in camelid leukocytes; however, amino acid motifs are present within the camel IgG2 CH domains that are essential for binding of the human FcγRI. Also, camelid IgG1 and IgG3 bind to protein G, an orthologue of FcγRIII. It is likely that ADE of WNV in alpaca macrophages is mediated by an FcγR-dependent mechanism. We intend to confirm the presence of FcγRs using flow cytometric methods.

Other mechanisms of ADE of viruses involve activation of C′ by the classical pathway (31). In our system, C′ served to augment antibody neutralizing but not enhancing activities. This observation concurs with similar reports documenting the protective effect of C′ activation during WNV infection in mice (32, 33). The non-structural glycoprotein, NS1, has also been shown to produce antibodies that neutralize WNV to prevent lethal infection in mice (34). The antibodies assessed in our study were polyclonal in nature and showed reactivity with NS1 proteins in ELISA. It is likely that anti-NS1 antibodies contribute to the neutralizing effects of camelid IgGs.

In aggregate, our data provide evidence supporting distinct immune functions for the IgG2 and IgG3 HCAbs. IgG2 participates in anti-nematode responses while IgG3 are effective neutralizing antibodies that have relevance in anti-viral immune

responses. Both heavy-chain isotypes are induced by vaccination. These findings enrich our understanding of camelid immunity, have implications for therapeutic and diagnostic approaches to infectious diseases in camelids, and provide a foundation for efforts to improve the health and well-being of llamas and alpacas.

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CHAPTER 4

Contributions of Conventional and Heavy-Chain IgGs to the Alpaca Immune System during Fetal, Neonatal and Adult Life[3]

[3]Lisa P. Daley, Stephen R. Purdy, William C. Davis, and Judith A. Appleton. Contributions of conventional and heavy-chain IgGs to the alpaca immune system during fetal, neonatal and adult life. Manuscript in preparation.

Abstract

In addition to conventional immunoglobulins, camelids produce antibodies that do not incorporate light chains into their structures. These so-called heavy-chain antibodies (HCAbs) have incited great interest in the biomedical community as they have considerable potential for biotechnological and therapeutic application. Comparatively little is known about their immunological functions and the B lymphocytes that produce them. Focusing on the alpaca, we set out to clarify the life histories of B lymphocytes that synthesize heavy-chain or conventional IgGs and to determine the contribution of these isotypes to maternal immunity that is conferred on neonates via colostrum. While conducting these studies, we were able to document the presence of a second conventional IgG isotype and showed that it is synthesized by cells that localize to the marginal zone of the spleen in adult alpacas. In addition, we identified for the first time a third heavy-chain isotype, most closely related to the previously defined IgG3. Cells staining positive for this heavy-chain isotype were detected in adult, ileal Peyer's patches, in contrast none of the other four isotypes were detected at this site. All five isotypes were present in colostrum and were passively transferred to crias. The concentrations of heavy-chain isotypes in cria blood declined more rapidly than conventional IgGs, suggesting that they have a shorter half-life. Our findings are compatible with specialized functions for IgG sub-isotypes in the alpaca.

Introduction

The camelids are the only mammals known to produce functional IgG isotypes that do not incorporate L-chains into their structures (1, 2). The term heavy-chain antibody (HCAb) has been assigned to these unusual IgG isotypes. There are three IgG isotypes reported to be produced by camelids: IgG1 is a conventional isotype,

IgG2 and IgG3 are HCAb isotypes. It must be emphasized that the nomenclature of camelid IgGs in no way correlates with that of the rodent or human isotypes. In fact, at the time of discovery in dromedaries, these IgG isotypes were named according to decreasing apparent molecular masses of their H-chains as revealed by SDS-PAGE under reducing conditions, and subsequently by their differential binding to protein A and protein G (1, 3-5). While all three isotypes will bind to protein A, protein G will only bind the conventional IgG1 and the IgG3 HCAb. This difference has been exploited to separate the isotypes from serum in order to calculate concentrations; however, this method does not yield pure fractions.

Distinct V and C genes encode HCAb and conventional IgGs. In fact, approximately 42 germline genes encoding HCAb V domains (VHH) and 50 encoding conventional V domains (VH) have been reported for the dromedaries (6). The hallmark of VHH genes is that they encode extended CDR3 loops as well as amino acid substitutions at five distinct positions within the framework two region (5, 7). In addition, cDNA sequences point to the existence of at least six functional IGHG genes; IGHG1A and IGHG1B correspond to conventional IgG1a and IgG1b isotypes, IGHG2A, IGHG2B, IGHG2C putatively encode IgG2a, IgG2b and IgG2c, respectively, and a single IGHG3 correlates to IgG3 (3, 5, 8, 9). Sequences for the IGHG2B gene has only been obtained in cloned genomic DNA from llamas and apparently is not produced by camels (3). The IGHG genes encoding HCAbs have a distinguishing mutation within the splice consensus sequence of the CH1 that results in the exclusion of this domain from the protein structure (8). All the IGHG genes encode IgGs with distinct hinge regions and as such these proteins have been classified into sub-isotypes (10). Interestingly, the VHH and VH rearrange with the same set of J and D genes, which is suggestive of a clustered or interspersed arrangement (11). In the dromedary, genomic and cDNA sequences have been

obtained for a conventional IgM, and cross-reactive antiserum indicates the presence of IgA; however, at this time none of this evidence is available for llamas. Neither is there any information on IgD and IgE isotypes produced by the camelids. Of particular interest is the fact that an IgM HCAb remains elusive.

The bio-physical attributes of HCAbs are consistent with those of conventional antibodies with some important exceptions. The H-chains of HCAbs have lower apparent molecular masses than those of conventional IgG due to the absence of CH1 domains. Also, being devoid of L-chains, their unencumbered structures likely make HCAbs more tissue-diffusible and allow them to bind epitopes otherwise inaccessible to their conventional counterparts. Like conventional Ig, HCAbs are bivalent antibodies; however, just a single VHH comprises the Ag-binding platform. In fact, the extended CDR3 loops provide an increased Ag-binding surface to compensate for the loss of the VL, and coincidently, this loop contains many hypervariable regions that contribute to the high affinity of the HCAbs for antigens (12, 13). The HCAbs undergo somatic hypermutation (1, 14), and remarkably, when induced against enzymes, these antibodies preferentially bind epitopes within clefts housing the catalytic sites (14-16). The aggregate physical features of HCAbs, and the ease in which their VHH domains can be expressed in bacterial and yeast systems, make them attractive biotechnological and therapeutic tools (17-19).

The HCAb isotypes constitute approximately 50 % of serum IgGs compatible with a significant role in camelid immunity (1). Studies of HCAbs remain primarily molecularly based. Little research has been done to elucidate the immunological and physiological relevance of these antibody isotypes. The exact concentrations of HCAb isotypes in body fluids are unknown. The contribution of HCAbs to maternal antibodies to newborn health and well-being has not been investigated. Information on the B lymphocytes that produce these unusual antibodies is lacking. And, at this

time, published reports investigating immune regulators of HCAb expression remain sparse. These investigations have been hindered chiefly by the dearth of available isotype-specific reagents. However, we have recently produced and fully characterized mAbs that bind specifically to the IgG isotypes produced by llamas and alpacas (20).

This study describes the application of IgG isotype-specific mAbs in physiological assessments of camelid HCAbs and the B cells that produce them. Here we report for the first time that *Lama* spp. produce not one, but two types of IgG3 HCAb. In addition, we now have the reagents to distinguish the two conventional IgG1 isotypes produced by llamas and alpacas. The concentrations of lamoid IgG isotypes within body fluids have also been described, and lastly, the efficiency of these mAbs in immunohistochemical applications has allowed us to illustrate the distribution of $HCAb^+$ and conventional B cells within different lymphoid tissues.

Materials and Methods

Reagents

MAbs specific for llama/alpaca IgG1 (24F1, 26G11, 27E10, and 28G4), IgG2 (10E6, 13A9, 16A4, 16H10 and 19D8) and IgG3 (1C11, 1D1, 2B11, 3E6 and 8E1) have been described elsewhere (20). LH41A and LT97A bind undetermined cell surface epitopes expressed by llama/alpaca B and T lymphocytes, respectively (Washington State University Monoclonal Antibody Center) (21). TH14B binds MHC II expressed by llama/alpaca cells (WSU Monoclonal Antibody Center). Horse-radish peroxidase (HRP)-goat antibodies to llama IgG (H + L) (Bethyl) were used in ELISA and Western blots. Mouse antibodies were detected using HRP-goat anti-mouse IgG (Cappel). HRP-streptavidin was used in immunohistochemistry (SAv, 10 µg/ml; Pierce Biotechnology Inc.). Phycoerythrin (PE) anti-mouse IgM (clone II/41,

0.6 μg/ml; eBioscience), SAv-peridin chlorophyll-a protein (PerCP, 0.6 μg/ml; BD Pharmingen), and fluorescein (FITC)-goat anti-camelid IgM (5 μg/ml; Triple J Farms) were used in flow cytometry.

SDS-PAGE and Western blots

IgGs were resolved in discontinuous, 15 cm polyacrylamide gels under reducing and non-reducing conditions as described elsewhere (20). Samples and molecular weight markers (Bio-Rad Laboratories) were boiled 7 minutes in sample preparation buffer, with or without 2-mercaptoethanol, before loading. Gels were either stained 1 hr with Coomassie blue or transferred to nitrocellulose membranes.

Western blots were developed at room temperature. Each incubation step was conducted for 1 hr unless specified otherwise. Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) and washed with TBS containing 0.05% Tween 20 and 0.1% bovine serum albumin (BSA; Sigma). HRP-conjugated goat anti-llama IgG (H+L) was diluted in blocking solution containing 10% normal goat serum. Antibody binding was detected with a chemiluminescent substrate (ECL reagent, Amersham Pharmacia Biotech, Piscataway, New Jersey) and autoradiography. Films were scanned and images were prepared using Adobe® Photoshop® and Microsoft® Powerpoint®.

Affinity purification of alpaca IgGs

IgG isotypes were purified by affinity chromatography (20). Columns were prepared by coupling 10 mg antibodies (26G11, 27E10, 19D8, 1D1 or 8E1) to 1 g CNBr-activated Sepharose-4B (Sigma) in the presence of 0.1 M $NaHCO_3$, pH 8.0. Unbound active sites were blocked with 0.1 M Tris, pH 8.0 for 2 hrs at 4 ^{o}C. The coupled gels were washed alternately with 0.1 M acetate buffer, pH 4.0 and borate

buffered saline (BBS), pH 8.5. Alpaca sera or colostrum were diluted 1:5 in BBS before loading columns. Samples were always loaded onto the 8E1 column before loading onto the 1D1 column. Unbound proteins were washed from columns using BBS. Bound IgGs were eluted with 0.1M glycine-HCl buffer, pH 2.7, into tubes containing 0.1 M Tris-HCl, pH 9.0, and characterized by Western blot.

Identification of IgG isotypes contained in protein G eluate

Fourteen mAbs that were generated against llama IgG1, IgG2 or IgG3 were employed in ELISA to identify the isotypes that bound protein G. Individual 96-well microtiter plates (Costar) were coated with 1 μg/ml of the protein G eluate containing unknown IgG isotypes, or affinity-purified llama IgG1, IgG2 or IgG3 diluted in 10% DPBS and incubated overnight at 4 ^{o}C. The working volume was 25 μl/ml unless stated otherwise. Subsequent incubations were conducted at room temperature and wells were washed with 0.05% Tween 20, DPBS after each step. Wells were blocked with 50 μl of 2% skim milk, 0.05% Tween 20, DPBS 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 10% normal goat serum, blocking buffer. The assay was developed with 3,3,5,5'-tetramethyl-benzidine (TMB, KHL) and reactions were terminated with 1M H_3PO_4. Optical density (O.D.) readings were obtained at 450 nm wavelengths.

Collection of alpaca blood and lacteal fluids

Samples collected from fourteen healthy alpaca dam-cria pairs (Cas-Cas-Nac farm, Perkinsville, Vermont) were evaluated. Lacteal secretions were collected at parturition (colostrum) and 24 hrs later (transitional milk). Blood samples were

collected from dams and crias by jugular venipuncture in Becton-Dickinson vacutainers, centrifuged to separate sera, and sera were stored at -20 ^{o}C until use.

Calculation of IgG concentrations

The ELISA described above was modified to calculate IgG concentrations in lacteal fluids and sera. Fluids were assayed directly for all isotypes except for $IgG3_{1D1}$. In that instance, samples (n = 3) were depleted of $IgG3_{8E1}$ over 8E1-sepharose affinity columns then reconstituted to original volume prior to use in ELISA.

Microtiter plates were coated with 5 μg/ml mAbs in 10% DPBS and incubated overnight at 4 ^{o}C. Wells were blocked with 2% skim milk, 0.05% Tween 20, DPBS then incubated with serial dilutions of sera or lacteal secretions. Affinity-purified IgGs were used as standards. Bound alpaca antibodies were detected with 0.1 μg/ml HRP-goat anti-llama IgG diluted in 10% normal goat serum, blocking buffer. The assay was developed with TMB, terminated with 1M H_3PO_4, and O.D. values were obtained at 450 nm wavelengths. The O.D. values of the standards were plotted against respective IgG concentrations, and the line equation was used to extrapolate the IgG concentrations of the samples tested.

Immunohistochemistry

Lymphoid tissues were collected from animals submitted to the Cornell University Hospital for Animals necropsy service. Tissues were collected from two fetuses (5 and 6 months of gestation) and seven adult male and female animals (2 – 14 years of age). Tissues were embedded in tissue freezing medium (Electron Microscopy Sciences, Ft. Washington, Pa.) and snap-frozen on dry ice. Sections (7 μm) were prepared (Cryocut 1800; Reichert-Jung, Buffalo, N.Y.), mounted on glass

slides, and stored at –80 °C. Slides were warmed to room temperature then fixed in 100% acetone for 10 m. Endogenous peroxidase activity was blocked with phosphate-buffered saline (PBS) containing 0.3% H_2O_2 and 1% NaN_3. As a general blocking step, sections were incubated in PBS containing 5% skim milk and 10% NGS. Subsequent procedures were conducted at room temperature and slides were washed in three changes of PBS following each incubation step. Sections were incubated with mAbs diluted in the general blocking solution. Bound antibodies were detected with HRP-conjugated goat anti-mouse IgG and color development was carried out using 3-amino-9-ethylcarbazole (AEC; Sigma). Sections were counterstained with Gill's no. 2 hematoxylin (Vector Labs), rinsed in tap water, mounted with Glycergel (DAKO Corp., Carpinteria, Calif.), and photographed images were obtained using a BX51 microscope fitted with a MagnaFIRE SP digital camera system (Olympus, Melville, N.Y.).

Flow cytometric analysis

Blood or single cell suspensions (prepared by mechanically disrupting tissues) were layered onto density gradients (upper gradient - Histopaque® 1077; lower gradient - Histopaque® 1119; Sigma) in polypropylene tubes. Tubes were centrifuged with brake off in a swinging bucket rotor at 700 × g for 1 hr at 26 °C. Supernatant was aspirated within 0.5 cm of the mononuclear cell layers (at the plasma-upper gradient interface) and discarded. Mononuclear cells were transferred to fresh 15 ml polypropylene tubes and washed three times with PBS for 10 m at 250 × g.

Cells were stained for flow cytometric analysis as described by Davis *et. al* (21). Briefly, cells were treated with 10% normal mouse serum in PBS for 20 m on ice, stained with fluorescent antibodies for 1 hr at room temperature, washed with PBS, and analyzed with a FACSCaliber flow cytometer (Becton Dickenson,

Mansfield, Mass.). For data acquisition, 10^4 events were collected within a gate encompassing lymphocytes. Phenotypic analysis was performed using CellQuest Software (BD Pharmingen).

Results

Discovery of a new HCAb and isolation of two IgG1 proteins produced by alpacas

For other studies, affinity-columns were prepared and used to deplete serum of IgG antibodies. Despite confirming that individual IgG isotypes had been completely removed from serum (by eventually obtaining no peaks during the elution steps), a substantial amount of protein was obtained when the unbound fraction was loaded onto a protein G column. This protein G eluate was resolved by SDS-PAGE under reducing conditions and found to contain four distinct protein bands; three of which were detected in Western blot by polyclonal antibodies to llama IgG (H+L) (Fig. 4-1*A*). A fourth weak band having the slowest electrophoretic mobility was not detected by the anti-IgG antibodies. This protein band migrated just above the 66.2 kDa molecular mass marker under both reducing and non-reducing conditions, which was identical to the electrophoretic mobility of albumin in alpaca serum resolved under these conditions (data not shown).

To ascertain which IgG isotypes remained in the protein G eluate, an ELISA was conducted using a panel of 14 isotype-specific mAbs (20). Four mAbs reacted with epitopes within the protein G eluate; three that were raised against llama IgG1 (24F1, 26G11 and 28G4) and one that was raised against llama IgG3 (1D1) (Fig. 4-1*B*). The 26G11 (that was monospecific for epitopes within the protein G eluate) and 1D1 mAbs were subsequently used to prepare affinity columns, and together with 27E10, 19D8 and 8E1 columns, were used to separate IgGs from alpaca serum.

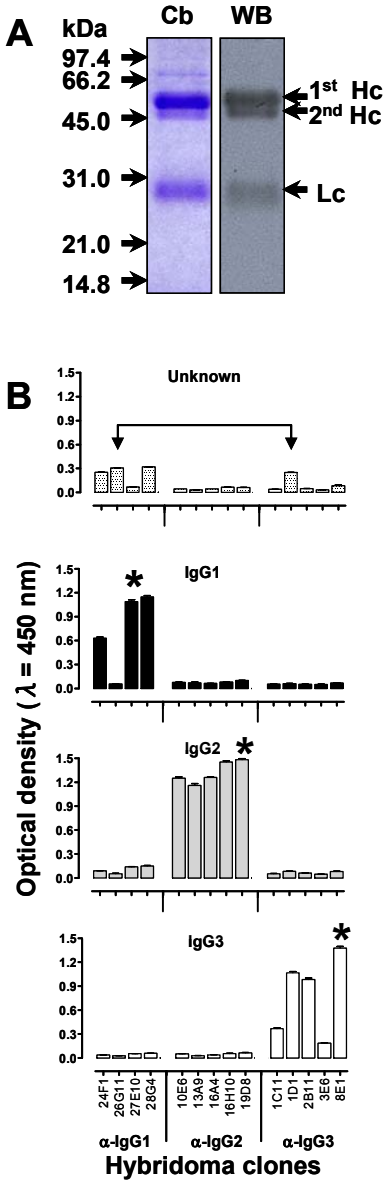

Figure 4-1: Identification of novel IgGs in alpaca serum. Affinity columns were prepared by coupling anti-IgG1 (27E10), IgG2 (19D8) or IgG3 (8E1) mAbs to Sepharose. Serum was passed over all three columns before loading onto a protein G column. **(A)** Protein G eluate was resolved by SDS- PAGE under reducing conditions. Gels were stained with Coomassie blue **(Cb)** or blotted onto nitrocellulose membranes for development with HRP-goat anti-llama IgG (H+L) **(WB)**. **Hc;** heavy chain; **Lc;** light chain. **(B)** Protein G eluate was analyzed in ELISA with a panel of mAbs specific for llama IgG isotypes (30). 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 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 by anti-IgG1 (27E10), IgG2 (19D8) or IgG3 (8E1) mAbs that were used to prepare affinity columns.

The eluates from all five affinity-columns were characterized by SDS-PAGE (Fig. 4-2*A* and *B*) and detected by polyclonal anti-llama IgG antibodies in Western blot (Fig. 4-2*C* and *D*). Apparent molecular masses were calculated for each of the proteins resolved under reducing conditions (Table 4-1). Eluates from the 27E10, 19D8 and 8E1 columns resolved similarly to previously published results (20). The 26G11 column bound one conventional IgG, with H-chains (49.9 kDa) that resolved separately from L-chains (23.1 to 25.5 kDa) under reducing conditions (Fig. 4-2, *A* and *C*). Interestingly, the L-chains of $IgG1_{26G11}$ went undetected by the polyclonal goat anti-llama (H+L) (Fig. 4-2*C*). When the disulfide bridges were intact, this 26G11-bound IgG had a retarded electrophoretic mobility compared with the 27E10-specific IgG1 (Fig. 4-2, *B* and *D*). It has been established that two IgG1 sub-isotypes are produced by camelids based on DNA analyses (5), so it was not surprising that we could isolate an additional isotype from alpaca serum. Hereafter, these isotypes will be distinguished as $IgG1_{27E10}$ (50.9 kDa) and $IgG1_{26G11}$ (49.9 kDa) (Table 4-1) as amino acid sequences have to be obtained to tether these proteins to published IgG1a and IgG1b gene sequences (5).

Surprisingly, the IgG eluted from the1D1 column resolved in a manner similar to $IgG3_{8E1}$ as a single HCAb species under reducing conditions (Fig. 4-2, *A* and *C*). Because this HCAb was bound by protein G and detected by commercial polyclonal antisera against llama IgG as well as our anti-IgG3 mAb, we have assigned it to the IgG3 isotype. We report for the first time that a second type of IgG3 with an apparent molecular mass of 44.3 kDa is produced by alpacas, which will be referred to as $IgG3_{1D1}$ (Table 4-1).

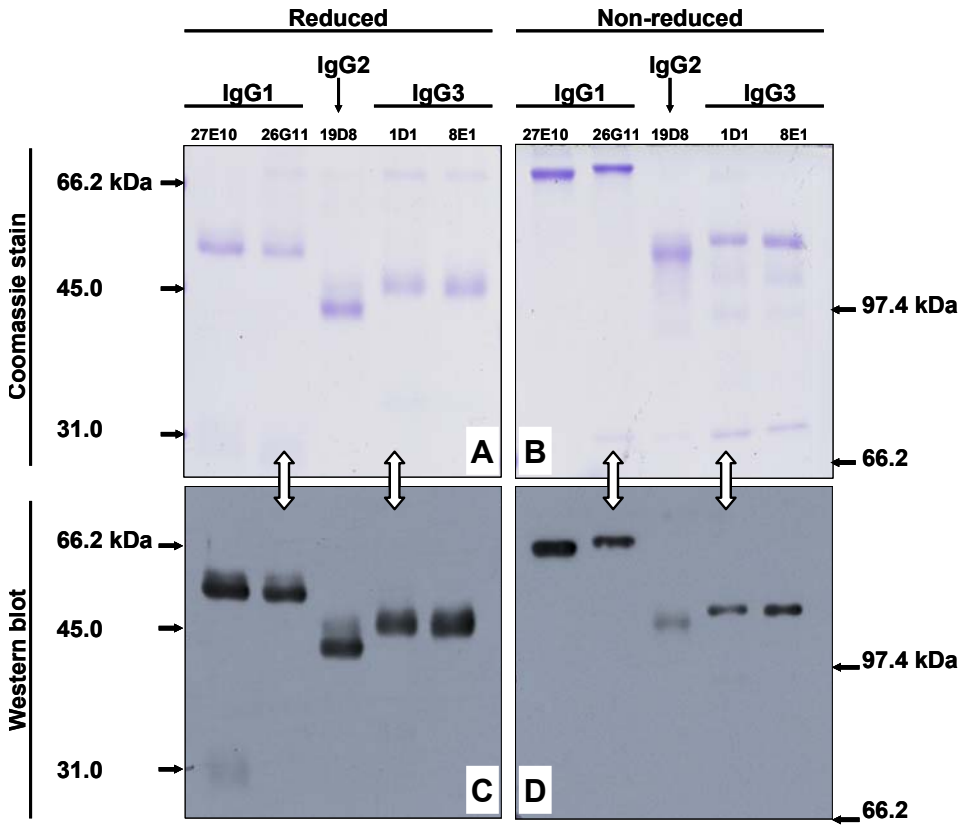

Figure 4-2: Six IgG isotypes were isolated from alpaca serum. Affinity columns were prepared by coupling anti-IgG isotype mAbs to Sepharose. Serum was loaded onto these columns and bound proteins were eluted and characterized by SDS-PAGE under reducing **(A and C)** and non-reducing conditions **(B and D)**. Gels were stained with Coomassie blue **(A and B)** or blotted onto nitrocellulose membranes for development using HRP goat anti-llama IgG (H+L) **(C and D)**. Arrows highlight lanes containing novel IgG proteins isolated from alpaca serum.

Table 4-1: Apparent molecular masses of IgG proteins purified from alpaca serum

Isotype	Specific mAb	Molecular mass (kDa)	
		Hc	Lc
IgG1	27E10	50.9	27.0 - 24.0
	26G11	49.9	25.5 - 23.1
IgG2	19D8	43.4	
		40.5	
IgG3	1D1	44.3	
	8E1	43.4	

Concentrations of IgG isotypes in lacteal secretions and serum

In addition to the three mAbs we previously described as being specific for llama IgG1, IgG2 and IgG3 (20), we have discovered two additional mAbs that recognized a novel IgG3 HCAb as well as a second conventional IgG1 in alpaca serum. These five mAbs were subsequently employed in assays to describe the distribution of IgG isotypes in alpaca body fluids.

IgG is the predominant isotype in both camelid and ruminant colostrum. We set out to investigate the contribution of HCAbs in alpaca lacteal secretions. Capture ELISAs were conducted to compute IgG concentrations in colostrum, transitional milk and serum (19). In the case of colostrum, because 1D1 reacted with both IgG3, samples (n = 3) were depleted of $IgG3_{8E1}$ prior to use in ELISA to calculate $IgG3_{1D1}$ concentrations.

All of the IgG isotypes were present in alpaca colostrum (Fig. 4-3*A*). Combined, the HCAbs represented 38.5% of the total colostral IgG. The predominant isotype in colostrum was the conventional $IgG1_{27E10}$ (43 %) with a mean concentration of 84.4 mg/ml (± 14.11 SE; n = 14), ranging between 18.3 - 176.8 mg/ml. The mean $IgG1_{27E10}$ concentration was significantly greater than the mean concentration of all the other IgG isotypes ($p < 0.001$) (Fig. 4-3*A*). The conventional $IgG1_{26G11}$ and the IgG3 HCAbs were equally represented in colostrum; $IgG1_{26G11}$, 18.5 % (n = 14), $IgG3_{8E1}$, 16.3 % (n = 14) and $IgG3_{1D1}$, 13.6 % (n = 3). The mean concentration of $IgG1_{26G11}$ was 36.3 mg/ml (± 6.4 SE; ranging from 6.7 - 74.5 mg/ml). $IgG3_{8E1}$ had a mean concentration of 31.9 mg/ml (± 5.7 SE; ranging from 2.2 to 68.0 mg/ml) while $IgG3_{1D1}$ was 26.7 mg/ml (± 7.3 SE; ranging from 14.1 – 39.4 mg/ml). The IgG2 HCAbs were the least represented (8.6 %; n = 14) in colostrum, with a mean concentration of 16.9 mg/ml (± 3.17 SE; ranging from 3.6 – 42.7 mg/ml). In total, colostrum had a mean IgG concentration of 196.2 mg/ml (± 25.9 SE, n = 14) ranging

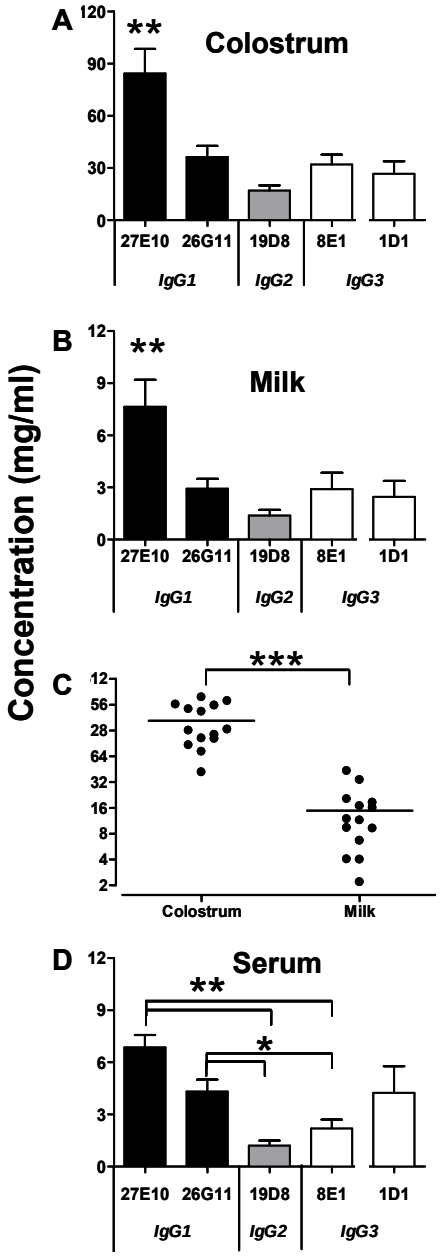

Figure 4-3: Distribution of IgG isotypes in lacteal secretions and serum of alpaca dams. IgG concentrations were measures in ELISA using affinity-purified IgGs as standards. As the 1D1 mAb bound both IgG3 isotypes, the samples were depleted of $IgG3_{8E1}$ prior to assay by 1D1 in ELISA. IgG concentrations are reported for **(A)** colostrum, **(B)** milk, and **(D)** serum. **(C)** Total IgG concentrations were calculated by tallying the values for each isotype. Mean IgG concentrations were compared by ANOVA and significant differences (*, $p < 0.5$; **, $p < 0.01$; ***, $p < 0.001$were assessed using Tukey's test. In all groups, n = 14, except for 1D1 values where n = 3.

from 44.9 – 401.4 mg/ml. Overall, all the IgG isotypes present in serum are consistently represented in alpaca colostrum. This is in contrast to the ruminants where colostrum contains predominantly one IgG isotype.

Colostrum is highly enriched with antibodies; however, these antibodies decline significantly in concentration over time. We found that, coincidental with ~ 90 % decrease in total IgG concentrations (Fig. 4-3*C*), all the isotypes decrease accordingly in dams' milk 24 hr post-parturition (Fig. 4-3*B*). That is, the mean concentration of IgG declined to 17.3 mg/ml ($\pm$ 3.1 SE; n=14) within 24 hr post-parturition, which was equivalent to IgG levels in serum (Fig. 4-3*D*). Again, the mean concentration of $IgG1_{27E10}$ (7.6 mg/ml $\pm$ 1.6 mg/ml SE) was significantly higher ($p < 0.001$) than $IgG1_{26G11}$ (2.9 mg/ml $\pm$ 0.6 mg/ml SE), IgG2 (1.4 mg/ml $\pm$ 0.3 mg/ml SE) and $IgG3_{8E1}$ (2.9 mg/ml $\pm$ 1.0 mg/ml SE). The mean concentration of the novel $IgG3_{1D1}$ was 2.5 mg/ml $\pm$ 0.9 mg/ml SE; n = 3). $IgG1_{26G11}$ and the IgG3 HCAbs were equally represented in 24 hr milk while IgG2 was the least represented.

To evaluate whether IgG distributions in lacteal fluids reflected those in sera, the concentrations of IgG isotypes present in dams' sera at 24 hr post parturition were calculated. The mean concentration of total IgGs in serum was (18.8 $\pm$ 1.5 mg/ml SE; n=14). $IgG1_{27E10}$ was the predominant isotype present in serum (n = 14) (Fig. 4-3*D*). The mean concentration of $IgG1_{27E10}$ (6.9 mg/ml $\pm$ 0.7 mg/ml SE) was significantly greater than $IgG1_{26G11}$ ($p < 0.01$; 4.3 mg/ml $\pm$ 0.7 mg/ml SE) as well as the IgG2 and $IgG3_{8E1}$ HCAbs ($p < 0.001$; 1.2 mg/ml $\pm$ 0.3 mg/ml SE and 2.2 mg/ml $\pm$ 0.5 mg/ml SE). However, in contrast to lacteal fluids, the mean $IgG1_{26G11}$ concentration was significantly greater than $IgG3_{8E1}$ ($p < 0.05$) as well as IgG2 HCAbs ($p < 0.001$). The mean concentration of the novel $IgG3_{1D1}$ was 4.2 mg/ml $\pm$ 1.5 mg/ml SE; n = 3). Collectively, the data demonstrate that all the IgG isotypes present in serum, including the HCAbs, were proportionately transferred to lacteal fluids.

Half-life ($t_{1/2}$) of maternal IgGs in cria serum

We assessed the kinetics of IgG concentrations in sera from newborn crias from 24 hr post-partum to 3 m of age. As previously described (23-26), maximum concentrations of maternal IgG were obtained at 24 hr post-partum in cria sera (22.2mg/ml $\pm$ 4.8 mg/ml SE, n = 14) (Fig. 4-4). There were no correlations between individual IgG concentrations in colostrum and that in cria serum at 24 hr post-partum. An $r^2 = 0.49$ value was observed between total IgG concentrations in colostrum and cria serum; however, this was not statistically significant. Maternal HCAbs declined much more rapidly than conventional IgG1 in cria serum as concentrations fell by 50 % within 14 d as opposed to 25 d and 20 d for $IgG1_{27E10}$ and $IgG1_{26G11,}$ respectively (Table 4-2). The half-life of maternal $IgG3_{1D1}$ in cria sera was not calculated. In contrast to the other isotypes, a notable increase in the $IgG1_{27E10}$ and $IgG3_{8E1}$ concentrations were observed in cria sera after 42 d of birth (Fig. 4-4 *A* and *D*), indicating that cria B cells had begun to secrete measurable quantities of antibodies.

Distribution of B cells within adult lymphoid tissues

In order to describe the distribution of HCAb-producing cells, we examined tissues from adult (n = 7) secondary lymphoid organs using flow cytometric and immunohistochemical methods. Only the 27E10, 19D8 and 8E1 mAbs were used in flow cytometry as we had not discovered the second IgG1 and IgG3 antibodies at the time these experiments were conducted. The data shown were results from one animal and are representative of results obtained with tissues from all seven.

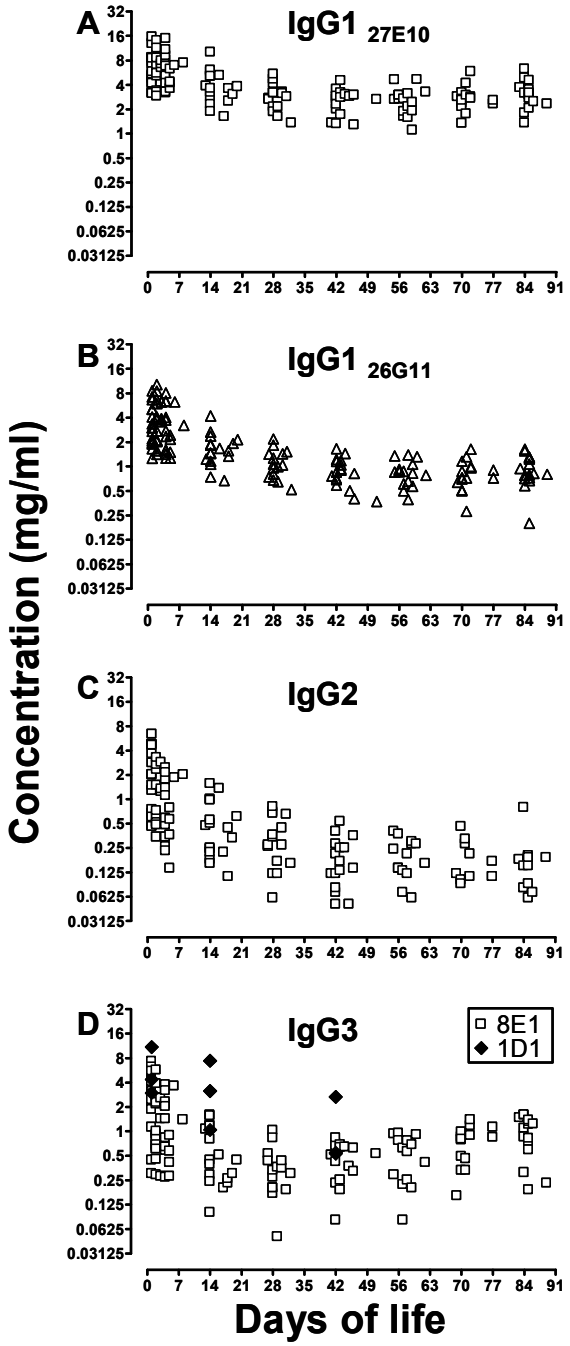

Figure 4-4: IgG concentrations in neonatal cria sera. Concentrations of IgG1 **(A and B)**, IgG2 **(C)** and IgG3 **(D)** were obtained by ELISA as described in Fig. 4-3. In all groups, n = 14, except for 1D1 values where n = 3.

Table 4-2: Half-life ($t_{1/2}$) of maternal IgG isotypes in neonatal sera.

Isotype	Specific mAb	Mean conc. (mg/ml) Day 1 of birth	$t_{1/2}$
IgG1	27E10	6.34	25
	26G11	3.18	20
IgG2	19D8	1.12	14
IgG3	8E1	1.46	14
	1D1	5.88	ND

In all groups n=14, except for 1D1 values where n = 3

Twenty nine percent of cells in liver stained positive for B cell receptors (Fig. 4-5). IgM^+ B cells predominated while $IgG1_{27E10}{}^+$ and $IgG3_{8E1}{}^+$ cells were present in equivalent numbers. $IgG2^+$ B cells were the least represented. Few B cells for any isotype were identified by immunohistochemical staining, a result that was consistent with the flow data (data not shown).

The LH41 mAb detected B cells within bone marrow, which were identified as conventional (IgM^+ and $IgG1_{27E10}{}^+$) and $HCAb^+$ B cells (Fig. 4-6). There were twice as many B cells expressing $IgG1_{27E10}$ (8.9%) compared to $IgG2^+$ and $IgG3_{8E1}{}^+$ cells (4.1%). Combined, the percentage of IgG^+ cells (17.2%) was greater than IgM^+ cells (14.6%) (Fig. 4-5). Thus, both conventional and HCAb-producing plasma cells resided within bone marrow.

B cells were found dispersed along with T lymphocytes and MHC II^+ throughout the lamina propria of the ileum (Fig. 4-7). These B cells expressed IgM and all isotypes of IgG. The B cells present in ileal Peyer's patches stained positive only with the 1D1 mAb (Fig. 4-7; bottom row, center panel). In flow cytometric analyses, $IgG1_{27E10}{}^+$B cells were more prevalent than $IgG2^+$ and $IgG3_{8E1}{}^+$ B cells (16.3 % vs. 4.2%, respectively) (Fig. 4-5).

Flow cytometric analyses demonstrated that B cells within the spleen and mesenteric lymph nodes were predominantly IgM^+ (Fig. 4-5). $IgG1_{27E10}{}^+$ and $IgG3_{8E1}{}^+$ cells were equally represented while $IgG2^+$ cells were rare. In spleen, B cells were dispersed primarily within the red pulp compartment along with MHC II^+ cells. Follicular B cells ($LH41^+$) were shown to be confined to the marginal zones (Fig. 4-8; top row, left panel). B cells present in red pulp were predominantly IgM^+ while follicular B cells expressed all the IgG isotypes except IgG2. Remarkably, marginal zone B cells expressed exclusively the $IgG1_{26G11}$ isotype (Fig. 4-8; middle row, right panel).

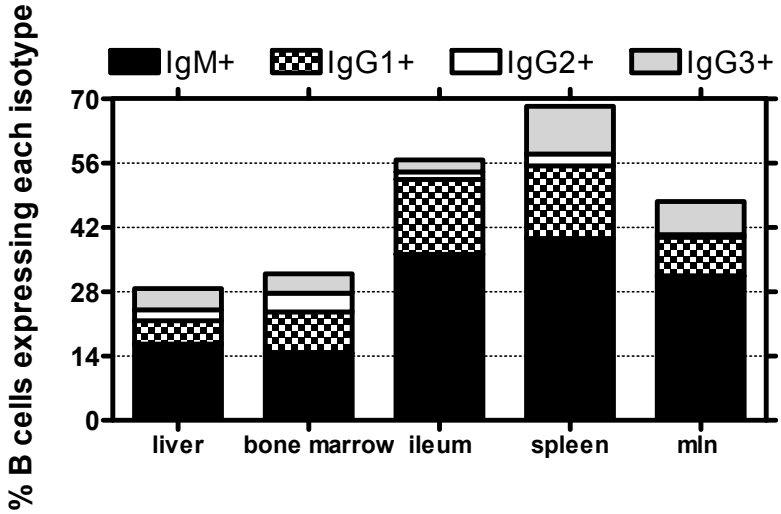

Figure 4-5: Distribution of B cells within the different lymphoid compartments. Single cell suspensions were prepared from different alpaca tissues. Cells were incubated with fluorochrome-conjugated antibodies and characterized by FACS analysis. Data are from one animal representative of n = 3. mln; mesenteric lymph node

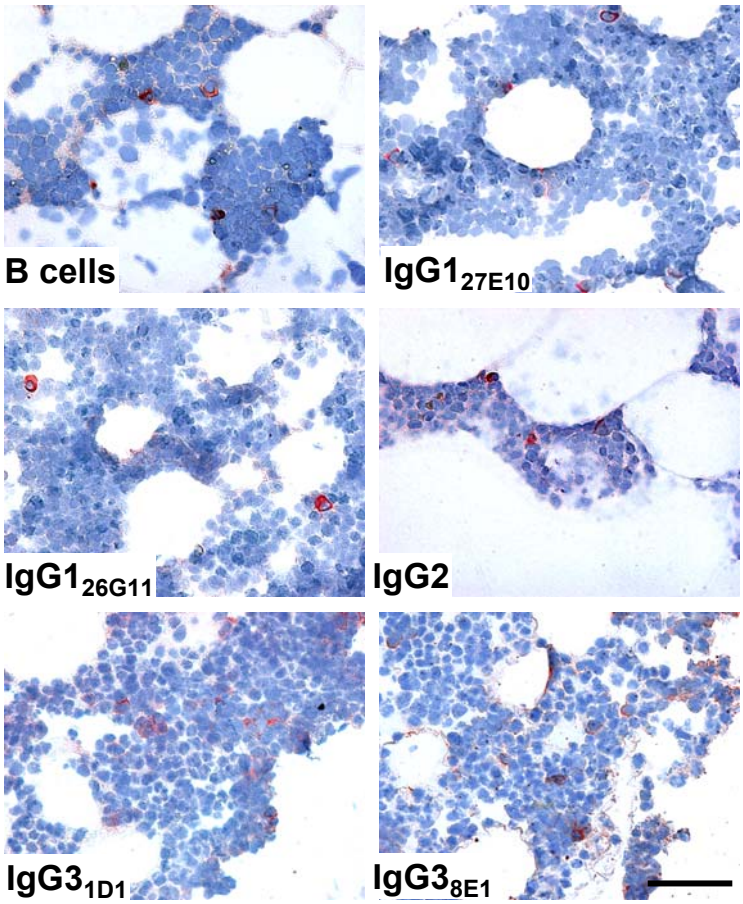

Figure 4-6: All IgG isotypes are detected in plasma cells resident in bone marrow. Immunohistochemical staining for different B cell antigens was performed as described in Methods. Positive cells are red. Scale bar = 50 μm.

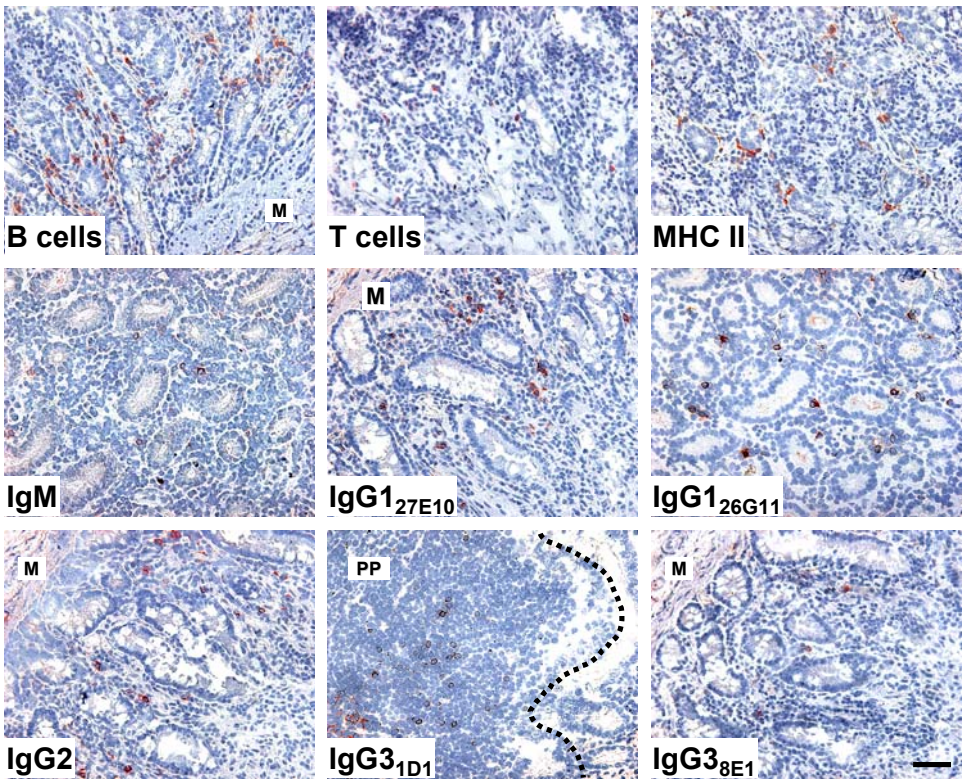

Figure 4-7 Distribution of B cells in ileal lamina propia and Peyer's patches. Immunohistochemical staining for surface markers and IgGs was performed as described in Methods. **M**; muscularis. Broken line delineates Peyer's patches **(PP)**. Positive cells are red. Scale bar = 50 μm.

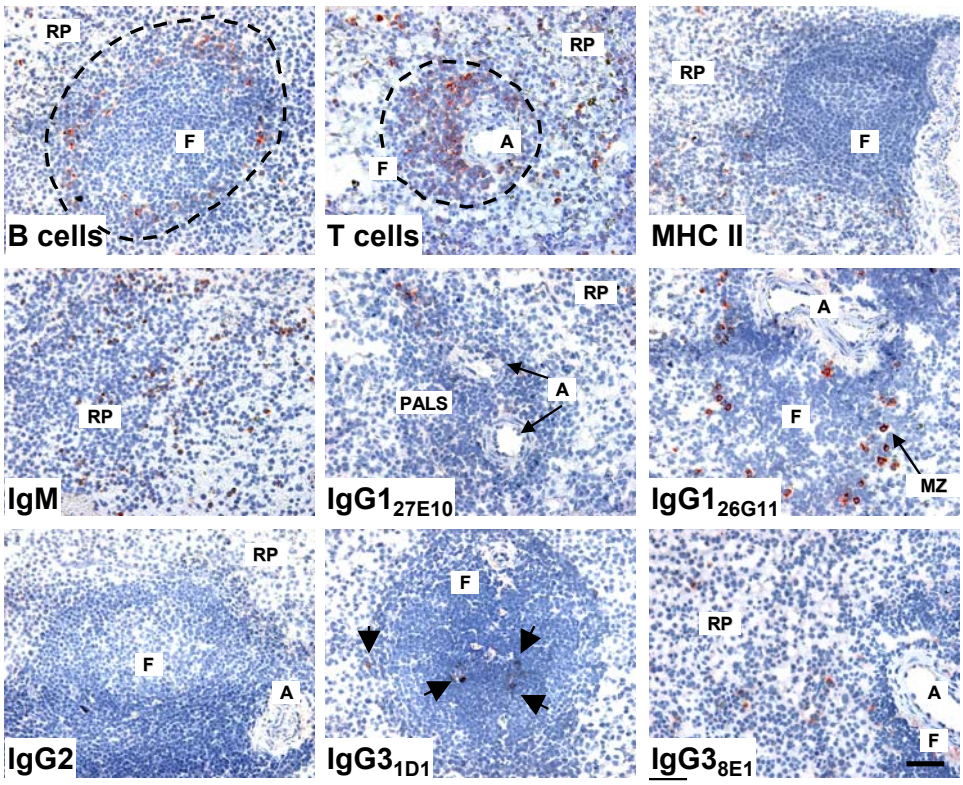

Figure 4-8: Distribution of B cells in the adult spleen. Immunohistochemical staining was performed as described in Methods. **F:** follicle; **RP:** red pulp; **MZ:** marginal zone, **A:** arteriole; **PALS:** periarteriolar lymphoid sheath. Positive cells are red. There were no follicular B cells except for $IgG3_{1D1}^{+}$ cells (arrow heads). Scale bar = 50 μm.

As expected, T cells were located within periarteriolar lymphoid sheaths (Fig. 4-8; top row, middle panel). Within the mesenteric lymph nodes, IgM^+ cells were present mainly within extrafollicular compartments and appeared to be excluded from germinal centers (Fig. 4-9; top row, left panel). $HCAb^+$ cells were present within germinal centers of lymph nodes; however, similar to IgM^+ cells, $IgG3_{8E1}{}^+$ B cells were observed mostly within extra-follicular areas (Fig. 4-9; bottom row, right panel).

Overall, $HCAb^+$ cells were distributed in conventional B cell compartments juxtaposed to T cells suggesting that these cells are poised to execute characteristic B cell functions. However, $IgG3_{8E1}{}^+$ B cells in mesenteric lymph node were found in extrafollicular regions while only $IgG3_{1D1}{}^+$ cells were found in Peyer's patches. Marginal zone B cells expressed $IgG3_{26G11}$.

Development of B cells in fetal tissues

To further describe the life history of B cells, we examined lymphoid tissues from alpaca fetuses at 5 and 6 m of gestation using immunohistochemical methods. Images from the 6 month-old fetus are shown; however, results were similar at both gestation periods.

$LH41^+$ B cells were detected in fetal liver; however, these cells were rare and found mainly within endothelial walls or in close proximity to hepatic blood vessels (Fig. 4-10*A*). $LH41^+$ B cells were more prevalent in the fetal bone marrow compared to the liver (Fig. 4-10*B*). Follicles were observed within the ileum at mid-gestation and were found to contain $LH41^+$ B cells (Fig. 4-10*C*). None of the B cells present in 6 m fetal liver, bone marrow and ileum stained positive for IgM or IgG antibodies. T cells were detected within the thymus at this gestation period (Fig. 4-10*D*).

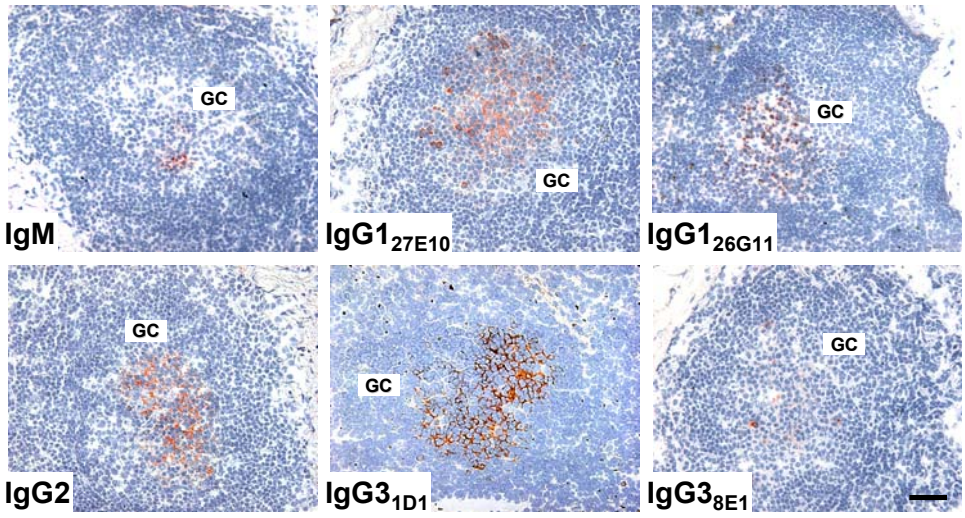

Figure 4-9: Distribution of germinal centers B cells in the mesenteric lymph node. Immunohistochemical staining was performed as described in Methods. Stippled lines delineate follicle and germinal center (**GC**). Positive cells are red. Scale bar = 50 μm.

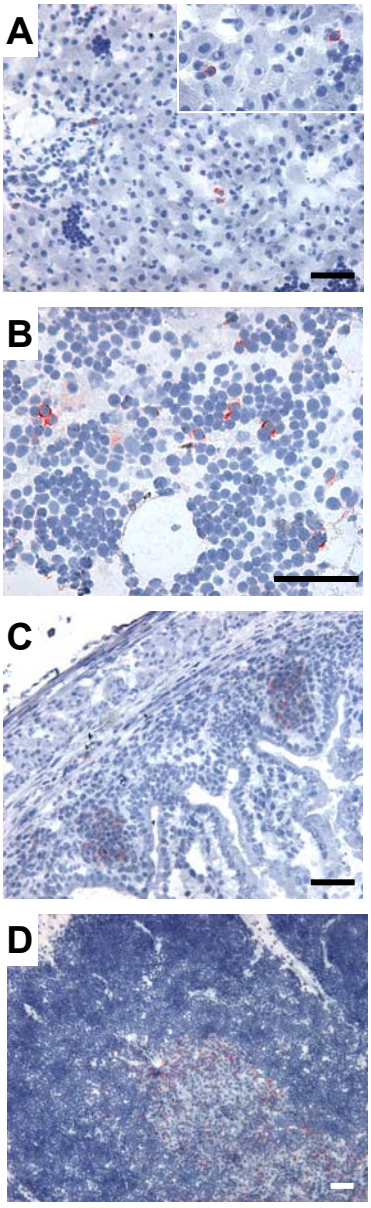

Figure 4-10: B and T lymphocytes were detected in fetal tissues at mid-gestation (6 months). Immunohistochemistry was conducted on **(A)** fetal liver, **(B)** bone marrow, **(C)** ileal, and **(D)** thymic tissues collected as described in Methods. Tissue sections were incubated with LH41 that identifies B cells **(A,B,C)** or LT97 **(D)** that binds T cells. Positive cells are red. Scale bar = 50 μm.

The architecture of fetal spleen was fully developed at mid-gestation as the white pulp follicles were very distinct. This organ was the most active site for lymphocytes of all tissues examined. The LT97 mAbs identified T cells within periarteriolar lymphoid sheaths contiguous with B cell aggregates that stained positive with LH41 (Figs. 11; top row, left and centre panels). MHC II^+ APC formed aggregates between the PALS and adjacent blood vessels (Figs. 11; top row, right panel). Follicular B cells expressed predominantly IgM and $IgG1_{27E10}$ (Figs. 11; middle row, left and centre panels); however, HCAbs were also being expressed at this stage of gestation (Figs. 11; bottom row). All the reagents identified positive cells in extra-follicular regions of the spleen. In the lymph nodes, B cells expressing both conventional IgG1 isotypes were located within the follicles (Figs. 12; middle row); however, $HCAb^+$ B cells expressed predominantly the $IgG3_{8E1}$ isotype (Figs. 12; bottom row, right panel).

Our observations are compatible with the conclusion that at mid-gestation, B cell lymphopoeisis occurs in fetal bone marrow, and that the spleen functions as the primary organ within which development occurs for both conventional and $HCAb^+$ B cells.

Discussion

Other than Tylopods, *Camelus* and *Lama* spp., only primitive chondrichthyan fishes are known to produce functional Ig lacking L-chains (27-29). The camelid HCAbs emerged as a result of duplication of the conventional genes while the emergence and evolution of chondrichthyan HCAbs occurred independently of antecedent genes (11). In camelids, HCAbs constitute as much as 45 – 75 % of IgGs in serum, yet very little is known about their immunological significance.

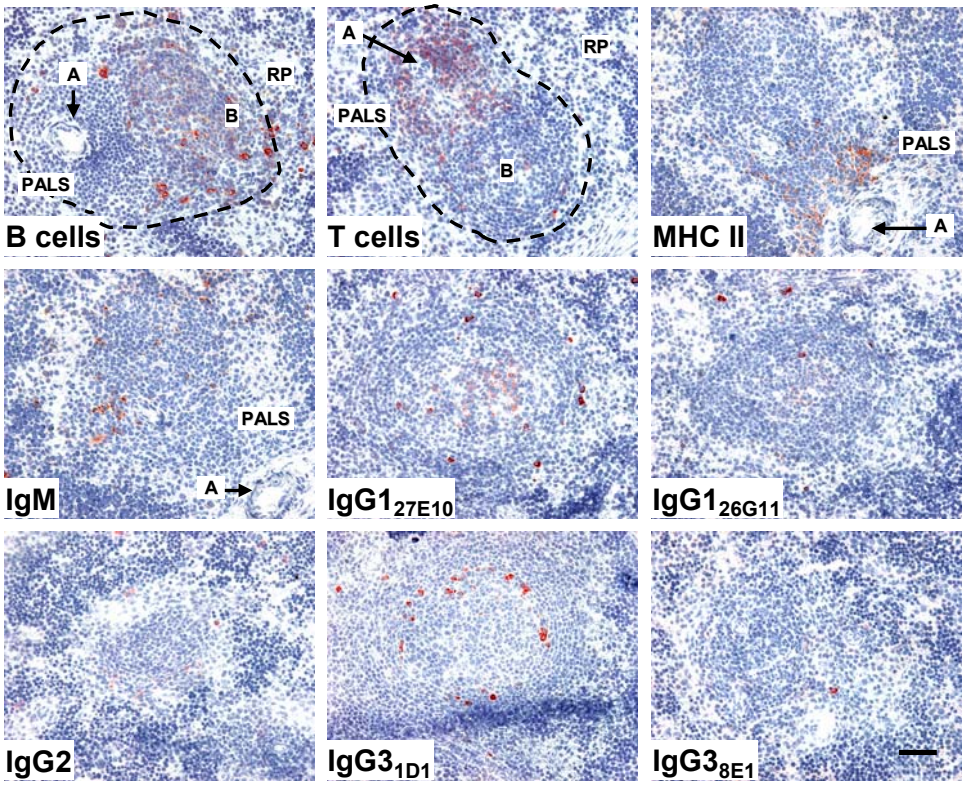

Figure 4-11: Distribution of cells in fetal spleen. Immunohistochemical staining was conducted as described in Methods. Stippled lines delineate white pulp including follicle. **RP:** red pulp; **PALS:** periarteriolar lymphoid sheath; **B:** B cell zone; **A:** arteriole. Positive cells are red. Scale bar = 50 μm.

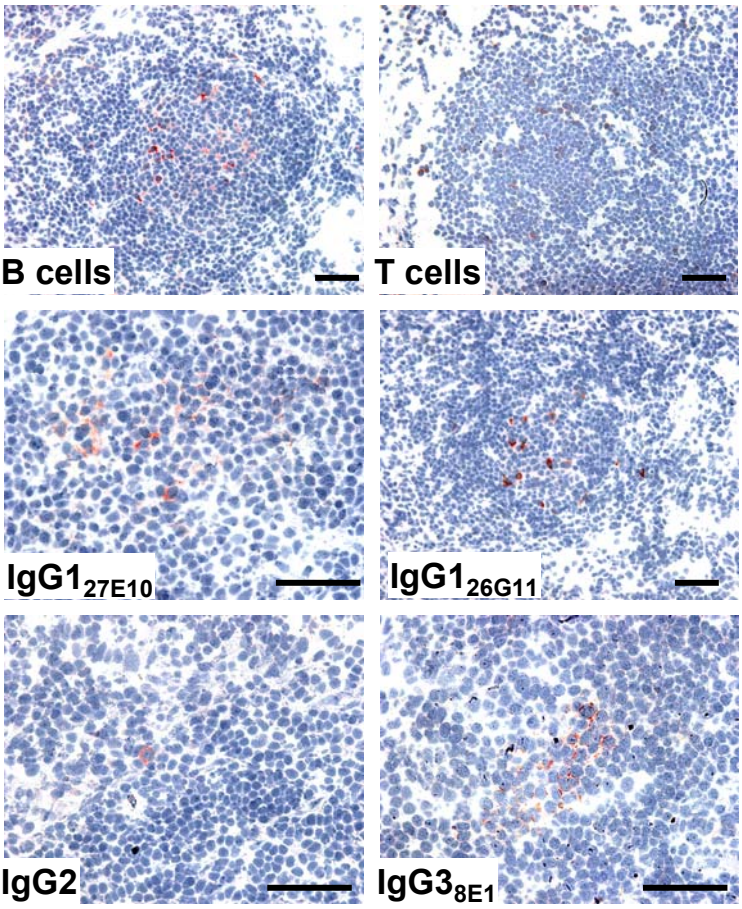

Figure 4-12: Distribution of B cells in fetal lymph node. Immunohistochemical staining was conducted as described in Methods. Stippled lines delineate follicles. Positive cells are red. Scale bar = 50 μm.

We have produced and characterized mAbs generated against llama IgGs that cross-react with identical alpaca isotypes, which have allowed us to investigate just how these antibodies contribute to camelid immunity. We report the discovery of a novel IgG3 that brings the total of HCAbs produced by *Lama* spp. to six. Using the isotype-specific mAbs that we generated against llama IgGs, it is now possible to investigate the immunological significance of HCAbs in camelid immunity and compare them with the functions of the conventional IgG1 sub-isotypes. Presently, our reagents allow us to distinguish the two types of IgG1 and IgG3, but not the different types of IgG2. The remaining anti-IgG2 mAbs will need to be characterized further to see if any of them are discriminating.

Whereas the concentrations of soluble $IgG1_{27E10}$ antibodies predominated in serum, IgG_{26G11} concentrations were comparable to both IgG3 antibodies while IgG2 concentrations were consistently low. In colostrum, the composition of IgGs reflected that of a more concentrated serum, and indeed all the isotypes were similarly represented. It is established that, like ruminants, colostral IgG is crucial to the survival of neonatal crias. Camelids are born severely hypogammaglobulinemic as the thick epitheliochordial placenta obstructs passive transfer of antibodies *in utero* and crias rely on maternal IgGs from colostrum (30, 31). Failure of passive transfer of immunity by this route correlates well with morbidity and mortality in camelids (24, 30). While others have focused on the relevance of total IgG uptake in the crias, our study examined the contributions of individual IgG isotypes to the passive transfer of immunity from dam to cria. Here, we confirm for the first time that, in contrast to ruminants, where the role of colostral IgG2 appears inconsequential as its level is < 6 % that of IgG1 (32), all isotypes are represented in colostrum as they are in serum in alpacas.

Our calculations of total IgG concentrations in dam colostrum (196.2 mg/ml $\pm$ 25.9 SE) and day 1 cria sera (22.2 mg/ml $\pm$ 4.8 mg/ml SE) corroborates those of previous studies (23); however, here we show that all the IgG isotypes were represented in lacteal secretions and transferred to the neonate. Recent studies implicate the FcRn, detected in mammary gland tissues, as being involved in transcytosis of IgG into colostrum (33). Because this receptor is predicted to bind both conventional and heavy-chain IgGs, based on conserved Fc contact residues (33, 34), we presume that HCAbs may also exploit these receptors to facilitate their transcytosis into colostrum as well as their uptake into neonatal serum.

B cell development was observed as early as 5 m of gestation. The appearance of $HCAb^+$ B cells within fetal tissues coincided with that of conventional B cells. Since no IgM-HCAb equivalent has been described to date, and structural incompatibilities make isotype-switching from conventional IgM to a HCAb isotype implausible, it is appealing to postulate that a distinct lineage of B cells in camelids express BCR comprised of IgG HCAb. In fact, Zou *et. al* (35) have shown conclusively that HCAb expression can instigate B cell development exclusive of IgM expression. In alpacas, it appears that while lymphopoeisis may occur within bone marrow, it is the spleen that functions as the site of development for both HCAb-producing and conventional B cells.

Two pathways have been described for B cell development in mammals: classical and alternative. The latter proceeds with the rearrangement of light chain genes independently of heavy chain gene rearrangement (36-39). In human and mouse, the fetal liver and bone marrow serve as primary lymphoid organs for B cells maturation (40). In the ruminants, it is the fetal spleen that serves as the site of B cells maturation (41). This function is subsequently transferred to the ileal Peyer's patches of gut-associated lymphoid tissues (GALT) during the late stages of gestation (42-44).

In the sheep, IgM^+ B cells appear initially in the fetal spleen by the end of the first trimester of gestation (41), and within the jejunum and ileum by days 65 and 68, respectively (45). However, lymphoid follicle formation commences by day 75 in the jejunum and day 97 in the ileum (46). The ileal B cells subsequently leave this site to disseminate to the secondary lymphoid organs (47, 48). Our investigations provide evidence that camelid B cell development was more analogous to that of the ruminants.

It is not until after 42 days of birth that optimal levels of endogenous IgGs were detected in cria serum. The data correlate with those from dromedary studies (2, 25). We observed that maternal HCAbs were cleared more quickly than conventional IgGs, as it took 14 days for the HCAb isotypes to decline to 50% that of the highest concentrations obtained at day 1 post-partum, compared with 20 – 25 days for the conventional IgG isotypes. While the levels of maternal IgG1 isotypes remained high in cria serum, a noticeable increase in the $IgG1_{27E10}$ and $IgG3_{8E1}$ levels could be detected. This event may signify the maturity of the neonate's immunity in response to environmental factors as well as vaccination. Newborn crias are administered vaccinations against clostridium, rabies and tetanus at two and four months of age.

In adult alpacas, the distribution of HCAb-producing B cells among and within secondary lymphoid tissues was similar to conventional B cells. Cells that were positive for HCAbs were found in all the lymphoid tissues examined, including mucosal tissues. The presence of $HCAb^+$ cells within germinal centers provides evidence that these cells may also use this site to undergo affinity maturation and perhaps isotype switching. Collectively, the 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 MALT Peyer's patches and isolated lymphoid aggregates are distinct and in the presence of antigens undergo clonal proliferation to become

predominantly IgA-secreting plasma cells (50, 51). In intestinal tissues, the prevalence of $HCAb^+$ cells was low; however, the presence of $IgG3_{1D1}{}^+$ cells within Peyer's patches may indicate that this isotype contribute significantly to mucosal immunity. In spleen, it was noted that only conventional $IgG1_{26G11}{}^+$ B cells populated marginal zone of white pulp follicles. Marginal zone B cells are a distinct lineage of cells that exhibit a pre-activated phenotype and provide the first line of defense against blood-borne antigens (52). These B cells respond to T-independent antigens to rapidly differentiate into IgM-secreting plasma cells (53). The presence of $IgG1_{26G11}{}^+$ B cells within marginal zones spleens implicates a role for this isotype in defense against blood-borne and T-independent antigens in alpacas. Finally, terminally differentiated B cells become plasma cells and reside within the bone marrow. Plasma cells are responsible for approximately 90 % of IgG and IgA present in serum. Our data indicate that HCAb-producing plasma cells also reside within bone marrow similar to conventional B cells.

In aggregate, the humoral branch of camelid immune system is comparable with that of ruminants and other GALT animals. We have shown that the HCAb-producing cells develop at the same time as conventional B cells within similar organs. In the adults, there is an overlap in distribution of $HCAb^+$ and conventional B cells, suggestive of an overlap of immune functions. Also, we have shown that conventional and heavy-chain IgGs demonstrate distinct catabolic rates in serum. We postulate that the HCAb isotypes may serve to augment the antigen binding repertoire of conventional IgG isotypes.

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CHAPTER 5

Final Discussion

The overall objective of this study was to contribute to the knowledge of camelid HCAbs by illuminating their role in immunity, and by describing the ontogeny of the B cells that produce them. The work described provides evidence that IgG2 and IgG3 HCAbs have distinct immunological functions. While IgG2 was induced during helminth infection, an anti-viral response produced potent neutralizing IgG3 antibodies. IgG2 antibodies produced by vaccination with virus demonstrated inferior neutralizing activities. Overall, the expression of conventional IgG1 appeared not to be influenced by the nature of the pathogen. In addition, the data revealed that all the IgG isotypes in serum are transferred to colostrum and subsequently to neonatal animals, including the heavy-chain isotypes, suggesting that HCAbs contribute to the passive transfer of maternal immunity. Finally, we showed that there is an overlap in anatomical distribution of conventional and HCAb-producing B cells during development and through life. These findings are compatible with the conclusions that the HCAb-producing B cells undergo development within conventional B cell compartments, and that they home to similar peripheral lymphoid tissues to execute traditional B cell functions.

The data presented here provide evidence that B cell development in the camelids is similar to that of the ruminants. Using reagents that demonstrated specificity for an uncharacterized B cell surface antigen (LH41 (1)), we demonstrated that fetal bone marrow likely served as the site for B cell lymphopoeisis. However, because these cells did not stain with any of the isotype-specific mAbs, we deduced that B cells were not expressing the BCR in bone marrow. Instead, the data implicate the spleen as being the primary organ within which both conventional and HCAb-producing B cells undergo VDJ rearrangement, BCR expression and class-switching. In sheep, B cells egress the spleen and relocate to the GALT to undergo repertoire diversification (2). These cells populate jejunal Peyer's patches by mid-gestation, then

ileal Peyer's patches during the late gestational period (Reviewed in (3)). We were fortunate to obtain lymphoid tissues from fetal alpacas at 5 m and 6 m of gestation. The distribution of B cells within the tissues was comparable in the two animals; however, the architecture of tissues was superior in the six month fetus. Follicles were observed within fetal alpaca ileal tissues; however, these cells did not stain with the isotype-specific reagents. Unfortunately, jejunal tissues had not been collected so we were not able to assess the prevalence of B cells within jejunal and ileal Peyer's patches. Because of the similarities of B cell distribution within lymphoid tissues at mid-gestation between sheep and alpaca, it seems likely that jejunal tissues would also stain positive for B cells.

This work provides the foundation for additional investigations into camelid B cell development. Assessment of lymphoid tissues collected from additional time points during gestation would provide a more comprehensive description of B cell development in camelids. In addition, as more reagents become available, other techniques can be applied, e.g. flow cytometry, to phenotype cells collected at different developmental stages. It is interesting that B cells within alpaca ileal follicles at mid-gestation were not identified using any of the isotype-specific reagents because in sheep, these cells express IgM receptors (4, 5). To date, the existence of a μ equivalent of HCAbs has not been ruled out. It is provocative to speculate whether B cells within ileal follicles at mid-gestation express BCR receptors that were undetected by the reagents used in the assays. Finally, it would be interesting to characterize the reactivity of the LH41 mAbs. Reactivity with markers expressed on B cell progenitors and throughout life suggests that LH41 may bind to B220 (CD45R), CD19 or CD21.

The distributions of B cells within adult tissues indicate that HCAb-cells populate conventional B cell areas. However, B cells within the marginal zones of adult, alpaca spleens expressed conventional $IgG1_{26G11}$ exclusively. Marginal zone B cells are a distinct lineage of cells that display a pre-activated phenotype, and are positioned at the blood-lymph interface to mount rapid responses to blood-borne bacteria (6-8). The data from our study suggest that we have identified an IgG isotype expressed by alpaca MZ B cells. An experiment that could address this would be to immunize alpacas with bacterial antigens to assess whether $IgG1_{26G11}$ is induced and compare the response with that of splenectomized animals. A predominance of bacteria-specific $IgG1_{26G11}$ and an absence of this response in splenectomized animals would support a MZ lineage. In addition, B cells present in adult, ileal follicles expressed on the $IgG3_{1D1}$ isotype. Assessments of IgG distribution in mucosal secretions by ELISA would a straightforward method to investigate whether this HCAb contributes to the protection of mucosal surfaces. These experiments would serve to further clarify specialized functions of camelid conventional and heavy-chain IgGs.

It would be of interest to investigate the cytokine milieu that regulates B cell expression of specific IgG isotypes. Recently, Odbileg ***et. al*** reported on the cloning, sequencing and analyses of cDNA encoding cytokines produced by camelids (9-12). These include the inflammatory cytokines, interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α; Th1 cytokines, interferon (IFN)γ, IL-2, IL-12p35 and IL-12p40; and Th2 cytokines, IL-4, and IL-13. In addition, cDNA sequences encoding the anti-inflammatory cytokine, IL-10, have also been reported. Phylogenetic analyses demonstrated high homology among the cytokines produced by camelids and those produced by other Artiodactyls. Future studies using recombinant forms of these cytokines would be useful in demonstrating how B cells are regulated to express the

different heavy-chain isotypes compared to conventional IgGs. To address this, an *in vitro* system could be designed in which B cells would be cultured in the presence of the various cytokines, and ask whether the different IgG isotypes are expressed in a cytokine-dependent manner. The technique described in Chapter 3 has been optimized to separate mononuclear cells from peripheral blood. An enriched primary, B cell population could be obtained and cultured in the presence of cytokines, and supernatant could be analyzed by ELISA using the isotype-specific mAbs. This information would help to further elucidate B cell regulation in camelids and will be useful for improving the design of vaccines.

Further, it would be informative to characterize additional effector functions of camelid IgG isotypes. Here, we illustrated that IgG3 antibodies were induced by a classical type 1 pathogen and that they were highly effective at neutralizing virus. This activity was shown to be C′-independent. However, we did not precisely address the mechanism by which neutralization was executed. Additional binding assays would have to be conducted to investigate whether neutralizing antibodies interfere with WNV attachment using Vero cells, a permissive cell line, or whether neutralization is effected at a stage downstream of attachment (13). Also, at low concentrations, virus-specific IgG1, IgG3 and IgG2 enhanced the ability of WNV to infect FcγR-bearing cells. This enhancement was diminished by complement. Experiments to ascertain that IgGs bind FcγRs could be conducted using the protocol described by Thrasher *et. al* (14). Immune complexes, prepared using IgG isotype-specific mAbs, or monomeric IgGs could be incubated with FcγR-bearing cells, e.g. macrophages or neutrophils. Differences in binding of monomeric IgGs or immune complexes observed by flow cytometric analysis would illustrate whether the different isotypes bind to low or high affinity receptors.

When Vero cells and alpaca macrophages were inoculated with WNV, C′ did not significantly alter the ability of the virus to infect and replicate compatible with the conclusion that C′ alone was not sufficient to neutralize. Of the three isotypes, neutralizing activities of IgG2 appeared to be most dependent on C′. In four of the five animals that produced anti-WNV IgG2, the presence of C′ improved the neutralizing effects as much as eight-fold. It would be interesting to conduct experiments comparing the ability of IgG1, IgG2 and IgG3 to activate the C′ cascade using the standard 50 % hemolytic complement assay (CH_{50}). Animals ($n > 1$) could be immunized to produce antibodies against sheep erythrocytes that could be affinity-purified using chromatography techniques described in Chapter 2, prior to use in CH_{50} assays.

Overall, the isotype specific reagents described here proved valuable in our investigations and will certainly be instrumental in future experiments. Presently, we have seventeen mAbs that are specific for camelid IgG1, IgG2 and IgG3 isotypes. Mono-specific clones, 27E10, 26G11 and 8E1 discern among two IgG1 sub-isotypes and one IgG3 isotypes, respectively, while 1D1 binds to both sub-isotypes of IgG3. Clone 19D8 binds all the IgG2 sub-isotypes. To precisely assess the immunological functions of distinct sub-isotypes, further characterization of the mAbs will have to be conducted. There are five additional anti-IgG2 mAbs that could be tested for sub-isotype specificity. IgGs purified over 1D1-columns from 8E1-depleted sera can be used to immunized mice in to produce hybridomas that express mAbs specific for the newly described IgG3. Finally, we have yet to determine which sub-isotypes of IgG1 and IgG3 are recognized by the mAbs, hence the temporary nomenclature $IgG1_{27E10}$, $IgG1_{26G11}$, $IgG3_{8E1}$ and $IgG3_{1D1}$. Collaborative efforts between Appleton and Muyldermans propose experiments to express characterized γ gene sequences and use these proteins in assays to determine isotype-specificity of the mAbs.

In summary, the work presented here has advanced our understanding of the functional significance of camelid HCAbs as well as fundamental knowledge of B cells in immunity. The clinical importance of this work is that we have set the foundation for evaluating IgG responses induced by infection and vaccination, which is applicable in designing vaccines and conducting trials to assess their efficacy in conferring protection. Also, simple, serologic, diagnostic assays can be designed to document exposure of animals to specific pathogens.

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