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Research paper

Structural analysis of effector functions related motifs, complement activation and hemagglutinating activities in *Lama glama* heavy chain antibodies

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ABSTRACT

Heavy chain antibodies (HCAbs), devoid of the light chains and the CH_1 domain, are present in the serum of camelids. IgG_2 and IgG_3 are HCAbs; whereas IgG_1 has the conventional structure. In order to study the immunological properties of llama HCAbs, from which to date little is known, llamas (*Lama glama*) HCAbs cDNA were cloned, sequenced and compared with other mammalian Igs. The sequence analysis showed that llama HCAbs cDNA organization is similar to other mammalian Igs and the presence of conserved binding motifs to Protein A, Protein G, FcγRI, FcγRIII and C1q in HCAbs were observed. In a previous work, different IgG isotypes purified by Protein A and Protein G chromatography, were assayed for their ability to fix complement. Both IgG_1 and the total serum were able to fix complement, whereas IgG_2 and IgG_3 fixed complement even in the absence of antigen (anti-complementary activity). Therefore, in this work we performed the complement activating activity of the different IgG isotypes purified under physiological conditions using Sephadex G-150 and their ability to induce hemagglutination. Llamas were immunized with sheep red blood cells (RBC) stroma and the different isotypes were purified from sera. Whole serum and IgG_1 could activate complement; however, HCAbs ($IgG_2 + IgG_3$) could not, despite the presence of the C1q binding motif in their primary sequence. Unlike IgG_1, the fraction corresponding to $IgG_2 + IgG_3$ did not display hemagglutinating activity. Our findings suggest that HCAbs cannot crosslink efficiently with different antigens and that the C1q binding site might be hindered by the proximity of the variable domains.

1. Introduction

Surprisingly, sera of camelids contain antibodies that lack the light chains and the first constant domain (CH_1) (Hamers-Casterman et al., 1993). These Abs are known as heavy chain antibodies (HCAbs) and have also been found in other species such as the nurse and wobbegong shark and ratfish (Roux et al., 1998; Dooley et al., 2003). However, HCAbs have never been documented in taxonomic suborders closely related to camelids such as ovine, bovine and swine. Achour et al. (2008) have shown that a single IgH locus contains all of the genetic elements required for the generation of conventional Abs and HCAbs in alpaca. This general organization resembles that of other typical mammalian V_n-D_n-J_n-C_n translocon lgH loci, unlike cartilaginous fishes (skates, sharks, rays) that have a *cluster-type* of heavy chain gene arrangement with hundreds of V-D-(D)-J-C_H loci (Stavnezer and Amemiya, 2004). The classification of HCAbs into different sub-isotypes is based

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upon the length and amino acids sequence of the hinge region as well as on their differential affinity for Protein A and Protein G. There are six sub-isotypes of IgG in llamas, namely IgG_1 (IgG_{1a} and IgG_{1b}), IgG_2 (IgG_{2a}, IgG_{2b} and IgG_{2c}) and IgG_3 of which, IgG_2 and IgG_3 are HCAbs. The antigen (Ag)-binding site of HCAbs is present as a single variable domain referred to as V_{HH} (Nguyen et al., 1998) and has remarkable sequence differences at the second framework (FR2) compared to conventional V_H domains. A number of substitutions involving conserved hydrophobic amino acids to hydrophilic, e.g. Leu11Ser, Val37Phe, Gly44Glu, Leu45Arg and Trp47Gly have been described (Kabat et al., 1991; Muyldermans et al., 1994; Vu et al., 1997). Although V_{HH} domains present some differences with conventional V_H domains, the Ag-binding repertoire of HCAbs is largely diversified by different mechanisms like the introduction of additional disulfide bridges within the CDR, the increased surface area of CDR3 and a high rate of paratope reshaping (Nguyen et al., 2000).

Over the last decades, the studies on the production of South American Camelids (SAC; llama, guanaco, vicugna and alpaca) have been intensified in order to promote the development of economy systems exploiting alternative regional products (wool, meat and leather) (Lichtenstein and Vila, 2003). Moreover, in some regions these animals are used for transport and as household pets. The development of the production of the SAC requires the knowledge of both their physiological parameters and their susceptibility and immune response against infectious agents. Studies performed to understand the immune response of SAC against infections agents are the basis for rational vaccine design. Even though a variety of vaccines are nowadays employed in SAC, they have been developed to be used in other livestock species.

Camelids, like cattle and swine, are susceptible to a wide range of infections and non-infections diseases. Nevertheless, studies performed on the immune response have not been as extensive in the former as in the latter species (Bastida-Corcuera et al., 1999; Crawley and Wilkie, 2003). The erradication of helminths and extracellular parasites is driven by type 2 cytokines (IL-4 and IL-5), and delayed type hypersensitivity responses and protection against intracellular pathogens and viruses are favored by type 1 cytokines (IFN-γ and IL-12). Besides, Immunoglobulin (Ig) isotypes are related to specific effector functions encoded in conserved motifs of the Fc region, such as complement activation, binding of Fc to phagocytic cells and delivering antibodies (Abs) to mucous secretions, tears, and milk. As a rule, class switch recombination takes place on specific particular Ig constant genes driven by signals generated from extracellular cytokines.

To date, little is known about the biological role of HCAbs. In a previous work, IgG_1, IgG_2 and IgG_3 were assayed separately (Hamers-Casterman et al., 1993; De Simone et al., 2006) for their ability to fix complement by indirect complement fixation tests. Both IgG_1 and the total serum were able to fix complement, whereas IgG_2 and IgG_3 fixed complement even in the absence of antigen (anti-complementary activity). Even though these antibodies have a considerably stable binding site; they are not stable enough as whole molecules and they present anti-complementary activity when subjected to the drastic conditions (very low pH elution) employed in their purification using Protein A and Protein G chromatography (De Simone et al., 2006). The production of specific polyclonal antibodies against the different IgG sub-isotypes (Saccodossi et al., 2006), was useful for the development of ELISAs for the measurement of IgM, total IgG and IgG isotypes. Sera from llamas have the following values of IgG isotypes and IgM: $IgG_1 = 6.2 \pm 1.6$ mg/ml, $IgG_2 = 0.7 \pm 0.3$ mg/ml, $IgG_3 = 1.2 \pm 0.4$ mg/ml and $IgM = 1.0 \pm 0.3$ mg/ml. These results indicate that HCAbs represent almost 25–30% of total IgG, being the IgG_3 sub-isotype the predominant HCAb in llamas (De Simone et al., 2008).

To develop rational vaccine schedules and to study the immunoprevalence of different IgG sub-isotypes against infectious diseases in the sera of camelids, it is necessary to study the biological activities of HCAbs. Besides, studies performed to understand the immune system of SAC will contribute to the development of projects of sustainable use and conservation of these species (De Lamo, 1997). Therefore, in this work we first cloned and analyzed the llamas' HCAbs cDNA organization by comparing their sequences to other mammalian Igs studying the presence of the amino acids involved in the interaction with Protein A, Protein G, FcγRI, FcγRIII and C1q. In order to study the ability of HCAbs to activate complement, we performed direct complement fixation tests with HCAbs purified under physiological conditions using Sephadex G-150 (Ferrari et al., 2007), to avoid subjecting the IgG fractions to drastic conditions as it was done previously using Protein A and Protein G chromatography (De Simone et al., 2006). Finally, to determine if HCAbs interact with Ags with both paratopes we analyzed their ability to induce hemagglutination.

2. Materials and methods

2.1. Animals

Llamas (*Lama glama*) were kept at the animals facilities at the School of Veterinary Sciences, University of Buenos Aires. Animals were handled according to local animal welfare regulations.

2.2. Cloning of the hinge regions, CH_2 and CH_3 domains of HCAbs cDNA

Lymphocytes were obtained from llamas' peripheral blood using Ficoll-Hypaque (GE Healthcare, Sweden). Total RNA from llama lymphocytes isolated with TRIzol Reagent (Invitrogen, USA), was reverse-transcribed using M-MLV reverse transcriptase (Promega, USA) with the oligo dT primer 5′-TCTAgACATATgAATTCTCgAgTTTTTTTTTTTTTTTT-3′.
The four HCAbs sub-isotypes cDNA fragments were first cloned using reverse transcription and heminested PCR. The first PCR was done using the following primers: oligoFR4 5′-gACCCAggTCACCgTCTCCTC-3′ as a sense primer and oligo$CH_{3.2}$ 5′-AgACTgggAgATggATTTCTgggT-3′ as an antisense primer, which were designed based

on the sequence homology of the previously published dromedary and llama IgG_{2b} and IgG_{2c} V regions (Harmsen et al., 2000; Conrath et al., 2001) and the llama IgG_{1b} (AF305955), dromedary IgG_{2a} (AJ131945) and IgG_3 (Atarhouch et al., 1997) third constant domain (CH_3). The second PCR was carried out employing the amplified fragment obtained from the first PCR as template, and using the following sense primers: oligoIgG_{2a}; 5′-CAAAACCTgAACCAgAATgCACgT-3′, oligoIgG_{2b}; 5′-ggATCCAgAACCCAAgACACCAAAACCACAACCA-3′, oligoIgG_{2c}; 5′-ggATCCAgCgCACCACAgCGAAgACCCCAgC-3′, and oligoIgG_3; 5′-gAACgAATgAAgTATgCAAgTgTC-3′ and oligo$CH_{3.2}$ as antisense primer. The sense primers were designed based on the previously published sequences of the hinge regions specific of each Ig sub-isotype: oligoIgG_{2a} (Nguyen et al., 1999), oligoIgG_{2b} and oligoIgG_{2c} (known as Lam08 and Lam07, respectively; Harmsen et al., 2000), and oligoIgG_3 (Atarhouch et al., 1997). The amplified cDNA segments were analyzed by gel electrophoresis and cloned into the TOPO vector (Invitrogen, USA). *Escherichia coli* JM109 was transformed with the recombinant constructs by electroporation (*E. coli* pulser apparatus, Bio-Rad) and sequencing was carried out using fluorescent dye-labeled (BigDye) terminators employing an automated fluorescent sequencer (ABI 377 Automated Sequencer, Macrogen service, Korea).

2.3. Sequence analysis

Nucleotide and amino acid sequences were analyzed using the Expasy Protein Translate software (SIB, Lausanne, Switzerland). Alignments were performed with the BLAST-NCBI and ClustalW software. Sequence identity (%) between the llama HCAbs CH_2 and CH_3 domains and mammalian domains were calculated with the BLAST-NCBI software. The Ig Cγ protein sequences used for sequence comparison were retrieved from the NCBI protein database. In the present study, the following mammalian Ig Cγ protein sequences were used: *Lama glama* IgG_{2a} (DQ058713), *Lama glama* IgG_{2b} (AY874455), *Lama glama* IgG_{2c} (AY874456), *Lama glama* IgG_3 (HM209028), which were originated in this work and *Lama glama* IgG_{1b} (AAG42243), *Equus caballus* IgG_1 (CAC44760), *Tursiops truncatus* IgG_2 (AAT65197), *Bos taurus* IgG_3 (AAC48761), *Bos taurus* IgG_{2a} (AAB37380), *Bos taurus* IgG_1 (AAB37381), *Ovis aries* IgG_1 (S31459), *Sus scrofa* IgG_{2a} (I47159), *Homo sapiens* IgG_1 (AAD38158). Finally, the amplified cDNA sequences were modeled using the crystallographic structure PDB 1hZhH (human IgG_1) as template with the Expasy-Swiss Model software. The identity between the cDNA sequences and the template is 72%. Figures were designed with the Pymol molecular graphic system (Schrodinger, Portland, USA).

2.4. Obtention of ovine red blood cell stroma

The red blood cells (RBC) stroma was obtained as described by Margni (1996). Briefly, 250 ml of peripheral blood was collected from four animals and diluted in 3.8% (w/v) sodium citrate at a ratio of 1:9 (1 part of sodium citrate to 9 parts of blood). Samples were centrifuged at 1700 × *g* for 15 min. After two washes with phosphate buffered saline (PBS), cells were resuspended in 0.001 N acetic acid and incubated overnight. The resulting stroma was washed four times with 0.001 M sodium acetate pH 5.0 and centrifuged at 12,000 × *g* for 30 min at 4 °C. The pellet was resuspended in 0.15 M NaCl and centrifuged at 12,000 × *g* for 30 min at 4 °C. This procedure was repeated three times and stroma was finally resuspended in 75 ml of 0.15 M NaCl. The stroma supernatant was sonicated with 3 pulses of 30 s each, amplitude 60 (Transsonic 540 Sonicator, Germany) and protein concentration was measured by the BCA method (Pierce, USA).

2.5. Immunization of llamas with ovine RBC stroma and sera titration

Two llamas were immunized with 1 mg of ovine RBC stroma on days 0, 14, 28 and 35. Briefly, 0.5 ml of the antigen (2 mg/ml) were emulsified with an equal volume of Complete Freund's Adjuvant (CFA, Sigma Chemicals Co., USA) and injected i.m. in the biceps femoral muscle. This process was repeated on days 14, 28 and 35 employing Incomplete Freund's Adjuvant (IFA, Sigma Chemicals Co., USA). Llamas were bled by the jugular vein on day 60 (De Simone et al., 2008). Serum antibodies were titrated by ELISA. Briefly, 96 well microplates (Maxisorp, NUNC, Denmark) were coated with 100 μl of 10 μg/ml stroma supernatant during 1 h at 37 °C. Plates were then washed and blocked with 200 μl of 3% dry non-fat milk in PBS (PBS-M) and incubated overnight at 4 °C. After incubation, plates were washed and incubated with 100 μl of twofold serial dilutions ranging from 1/100 to 1/25,600 of each llama serum in 1% PBS-M for 1 h at 37 °C. After incubation, plates were washed and 100 μl of a rabbit anti-llama IgG serum diluted 1/2000 in 1% PBS-M was added and incubated for 1 h at 37 °C. Finally, plates were washed and 50 μl of the substrate-chromogen solution containing hydrogen peroxide (0.004%) and 2 mg/ml *o*-phenylendiamine dihydrochloride (OPD, Sigma Chemicals Co., USA) in citrate buffer pH 5.0 were added. The color reaction was stopped by the addition of 50 μl of 4 N sulfuric acid. Absorbance was measured at 490 nm in an ELISA microplate reader (Metertech Σ960, Taiwan). Abs titers were determined as the highest positive dilution over twice the OD obtained with pre-immune sera.

2.6. Purification of llama sera

Samples were obtained from two immunized llamas and precipitated with 50% (w/v) ammonium sulfate. Subsequently, 0.5 ml (20 mg/ml) of the precipitated Igs were loaded on a Sephadex G150 (GE Healthcare, Sweden) column (15 mm × 850 mm) equilibrated in PBS. Fractions of 1 ml were collected and absorbances were measured at 280 nm employing an U.V. detector (Milton Roy, USA). As the fractions collected had different protein concentrations, each isotype fraction (IgG_1 and $IgG_2 + IgG_3$) was adjusted to 3 mg/ml (a concentration value that is physiologically found in llamas sera) (De Simone et al., 2008).

```
                                                    234          248
IgG2a          EPKIPQPQPKPQPQPQPQPKPQPKPEPECTCPKCPAPELLGGPSVFIFPPKPKDVLSISG
IgG2b               EPKTPKPQPQPQPQPQPNPTTSESKCTCPKCPAPELLGGPSVFIFPPKPKDVLSISG
IgG2c                          AHHSEDPSSKCPKCPGPELLGGPTVFIFPPKPKDVLSITR
IgG3                              GTNEVCKCPKCPAPELLGGPTVFIFPPKPKDVLSISG
                                    :  ..*****.*******:**************:
                        265                          297         309
IgG2a          RPEVTCVVVDVGQEDPEVSFNWYIAGAEVRTANTRPKEEQFNSTYRVVSVLPIQHQDWLT
IgG2b          RPEVTCVVVDVGQEDPEVSFNWYIDGAEVRTANTRPKEEQFNSTYRVVSVLPIQHQDWLT
IgG2c          KPEVTCVVVDVGKEDPEIEFSWSVDDTEVHTAETKPKEEQFNSTYRVVSVLPIQHQDWLT
IgG3           RPEVTCVVVDVGQEDPEVSFNWYIDGAEVRTAKTRPKEEQFNSTYRVVSVLPIQHQDWLT
               :***********:****:.*.* : .:**:**:*:*************************
                        327
IgG2a          GKEFKCKVNNKALPAPIERTISKAKGQTREPQVYTLAPHREELAKDTVSVTCLVKDFYPV
IgG2b          GKEFKCKVNNKALPAPIEKTISKAKGQTREPQVYALAPHREELAKDTVSVTCLVKGFYPP
IgG2c          GKEFKCKVNNKALPAPIERTISKAKGQTREPQVYALAPHREELAKDTVSVTCLVKGFFPA
IgG3           GKEFKCKVNNKALPAPIEKTISKAKGQTREPQVYALAPHREELAKDTVSVTCLVKGFYPP
               ******************:***************:*******************.*:*
                  380                                                 432
IgG2a          DINIEWQRNGQPESEGTYATTPPQLDNDGTYFLYSKLSVGKNTWQRGETFTCVVMHEAL
IgG2b          DINVEWQRNRQPEPEGTYATTPPQLDNDGTYFLYSKLSVGKNTWQRGETFTCVVMHETL
IgG2c          DINVEWQRNGQPESEGTYATTLPQLDNDGTYFLYSKLSVGKNTWQQGEVFTCVVMHEAL
IgG3           DINVEWQRNGQPESEGTYANTPPQLDNDGPYFLYSKLSVGKNTWQRGETLTCVVMHEAL
               ***:***** ***.*****.* *******.***************:**.:*******:*
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Fig. 1. Alignment of the llama HCAbs deduced amino acid sequences obtained in this work. The N-glycosilation site at Asn297 is underlined. Cys residues are highlighted. NCBI protein database accession numbers are IgG_{2a} (DQ058713), IgG_{2b} (AY874455) IgG_{2c} (AY874456) and IgG_3 (HM209028). Symbols at the end of the sequence alignment represent: (*) identity, (:) conservative substitution and (.) semi-conservative substitution.

2.7. Direct complement activation test

The complement activation test was performed with llama sera and fractions collected after the gel filtration chromatography. The reaction was carried out in 96 well U-shaped bottom microplates. Fifty microliters of fresh Guinea pig or llama serum diluted 1/15 in buffer Mayer (veronal buffer saline containing 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 7.5) and 50 μl of a 10% RBC suspension in buffer Mayer were placed in each well together with 100 μl of total serum at twofold serial dilutions (ranging from 1:2 to 1:256) or 100 μl of each isotype fraction (IgG_1 and $IgG_2 \pm IgG_3$) at twofold serial dilutions (ranging from 3 to 0.024 mg/ml). Reactions were then incubated for 45 min at 37 °C and microplates were centrifuged at 500 rpm for 10 min. Absorbances of supernatants were read in a microplate reader at 540 nm (Metertech Σ960, Taiwan). The 100% of hemolysis was considered as the absorbance obtained after the addition of 150 μl distilled water to 50 μl of a 10% RBC suspension.

2.8. Hemagglutination assay

The hemagglutination test was performed with pre-immune and immune sera against ovine RBC and with the purified Igs (IgG_1 and $IgG_2 + IgG_3$). Briefly, 0.5 ml of total sera at twofold serial dilutions (ranging from 1:2 to 1:256) or 0.5 ml of each isotype fraction at twofold serial dilutions (ranging from 3 to 0.024 mg/ml) were incubated with 0.5 ml of a 0.5% RBC suspension in PBS. Reaction mixtures were incubated at room temperature for 90 min and then the presence of hemagglutination was evaluated.

3. Results

3.1. Llama HCAbs cDNA organization is similar to other mammalian Igs

Four llama HCAbs constant regions were cloned and sequenced which corresponded to IgG_{2a}, IgG_{2b}, IgG_{2c} and IgG_3 according to the hinge region classification (Nguyen et al., 1998). The presence of a CH_2 glycosylation site at Asn297 was found and the Cys involved in the formation of intradomain disulfide bonds Cys260, Cys321, Cys369 and Cys425 were conserved (Atarhouch et al., 1997) (Fig. 1).

The translated amino acid sequences (CH_2 and CH_3) from llama HCAbs were compared to each other, obtaining sequence identities that ranged from 88% to 96%. The lower value (88%) was always obtained when the comparison was made between IgG_{2c} and the other sub-isotypes. As a rule, a higher degree of identity was observed between the CH_2 domains than between the CH_3 domains of llama HCAbs. In parallel, translated amino acid sequences (CH_2 and CH_3) from llama HCAbs were compared to other mammalian Fc domains resulting in 66–76% with a mean value of 72%. When the CH_2 domain was compared, sequence identity varied between 71% and 82% with a mean value of 78%, and the comparison between CH_3 domains sequence identity varied between 65% and 74% with a mean value of 69%. This result, in agreement with those of Atarhouch et al. (1997), would suggest that a different evolutionary process might be acting upon the different constant domains of Igs.

3.2. Conserved binding motifs are present in HCAbs

Effector functions are related to different sequence motifs present in the Fc domain such as the C1q and the Fc receptor (FcR) binding site. The motifs involved in the

C1q binding	269-271…318-331	C´ activation
IgG2b mouse	D**D**P…**E**F**K**C**K**VNNKDL**P**S**P**	+
IgG1 human	E**D**P…**E**Y**K**C**K**VSNKAL**P**A**P**	+
IgG2a llama	E**D**P…**E**F**K**C**K**VNNKAL**P**A**P**	-
IgG2b llama	E**D**P…**E**F**K**C**K**VNNKAL**P**A**P**	-
IgG2c llama	E**D**P…**E**F**K**C**K**VNNKAL**P**A**P**	-
IgG3 llama	E**D**P…**E**F**K**C**K**VNNKAL**P**A**P**	-
IgG1b llama	E**D**P…**E**F**K**C**K**VNNKAL**P**A**P**	+

FcγRc (#)	234-239…265-269…327-332
IgG1 human	**LLGGPS**…**DVSHE**…**NST**…**ALPAPI**
IgG2a llama	**LLGGPS**…**DVGQE**…**NST**…**ALPAPI**
IgG2b llama	**LLGGPS**…**DVGQE**…**NST**…**ALPAPI**
IgG2c llama	**LLGGPT**…**DVGKE**…**NST**…**ALPAPI**
IgG3 llama	**LPGGPS**…**DVGKE**…**NST**…**ALPAPI**
IgG1b llama	**LPGGPS**…**DVGKE**…**NST**…**ALPAPI**
	* ***: **.:* *** ******

Protein A	251-254…310-314…432-436	PA Binding
IgG1 human	**LMIS** … **HQ**NW**L** … **LHNHY**T	+
IgG2a mouse	**LMIS** … **HQ**DW**M** … **LHNHH**T	+
IgG2a llama	**LSIS** … **HQ**DW**L** … **L-----**	+
IgG2b llama	**LSIS** … **HQ**DW**L** … **LHNHY**T	+
IgG2c llama	**LSIT** … **HQ**DW**L** … **LHNHS**T	+
IgG3 llama	**LSIS** … **HQ**DW**L** … **L-----**	+
IgG1b llama	**LSIS** … **HQ**DW**L** … **LHNHY**T	+
	* *: **:*: **** *	

Protein G	248-254…309-311…380-382…424-436	PG Binding
IgG1 human	**K**DTL**MIS**…H**Q**D…**E**W**E**…**S**V**M**HEAL**HNHYTQ**	+
IgG2a mouse	**K**DVL**MIS**…H**Q**D…**E**W**T**…**S**V**V**HEGLHNHHTT	+
IgG2a llama	**K**DVL**SIS**…H**Q**D…**E**W**Q**…**V**V**M**HEAL------	–
IgG2b llama	**K**DVL**SIS**…H**Q**D…**E**W**Q**…**V**V**M**HETLHNHYTQ	–
IgG2c llama	**K**DVL**SIT**…H**Q**D…**E**W**Q**…**V**V**M**HEALHNHSTQ	–
IgG3 llama	**K**DVL**SIS**…H**Q**D…**E**W**Q**…**V**V**M**HEAL------	+
IgG1b llama	**K**DVL**SIS**…H**Q**D…**E**W**Q**…**V**V**M**HEALHNHYTQ	+
	.* *: * ** *:**.**** *	

Fig. 2. Residues known to be involved in effector functions such as binding to C1q or FcγR and residues that interact with Protein A and Protein G are in black fonts. Top sequences from species other than llama are the reference sequences from which the original binding motif was described. Identical amino acids are indicated by (*), conservative substitutions by (:) and semi-conservative substitutions by (.). Numbering is according to Kabat et al. (1991). Experimental related assays are shown on the right side of the sequences. (#) At present, no data is available regarding the interaction of the different IgG fractions with the FcγR. C′: complement activation, PA: Protein A, PG: Protein G binding motifs.

human C1q binding Glu318-X-Lys320-X-Lys322, Pro331 (Duncan and Winter, 1988) and in the mouse C1q binding Asp270, Lys322, Pro329 and Pro331 (Idusogie et al., 2000) were found in llama HCAbs. Besides, the motifs that interact with human FcγRI and FcγRIII are highly conserved. The residues involved in the interaction with the FcγRI and FcγRIII are mainly defined by Leu234-Leu-Gly-Gly-Pro-Ser239 (Duncan et al., 1988), and Asp265-Val-X-X-Glu269, Asn297-Ser-Thr299, Ala327-Leu-Pro-Ile332 respectively (Sondermann et al., 2000). However, some changes were observed, i.e. Gly267Ser and Gln/Lys268His in all HCAbs and Thr239Ser in llama IgG_{2c} and Pro235Leu in llama IgG_{1b} and IgG_3 (Fig. 2). The IgG_{2c} sub-isotype presents a conservative substitution, whereas IgG_3 and IgG_{1b} present a non-conservative substitution at amino acid position 235 which is crucial for the FcγR-Fc interaction (Duncan et al., 1988).

Since all camelid IgG isotypes interact with Protein A, but only IgG_1 and IgG_3 interact with Protein G (Hamers-Casterman et al., 1993; De Simone et al., 2006), the presence of the amino acid residues involved in these interactions was analyzed. Protein A and Protein G interact with the same Fc region: 252–254, 311–313 and 426–438 (Burton, 1985; Stone et al., 1989). The amino acid residues that are involved in Protein A interaction are Leu251-X-Ile-Ser254, His310-Gln311-X-X-Leu314 and Leu432-His-Asn-His-His436 in mice and Tyr436 in humans (Deisenhofer, 1981; Kato et al., 1993); most of them are highly conserved in llama HCAbs and IgG_{1b}. However, some changes were observed in all IgG sub-isotypes such as Ser254Met and Gln309Leu, and Thr254Ser only occurred in llama IgG_{2c} (Fig. 2). Nevertheless, these substitutions do not affect the Protein A-Fc interaction.

As mentioned above, only camelid IgG_1 and IgG_3 interact with Protein G. However, in all HCAbs sequences, the residues involved in this interaction are highly preserved. These residues are: Lys248, Ile253-Ser254, Gln311, Glu380, Met428 and all the residues flanked by His433-Gln438 (Kato et al., 1995; Sauer-Ericsson et al., 1995). Although some substitutions can be observed in all IgG sub-isotypes

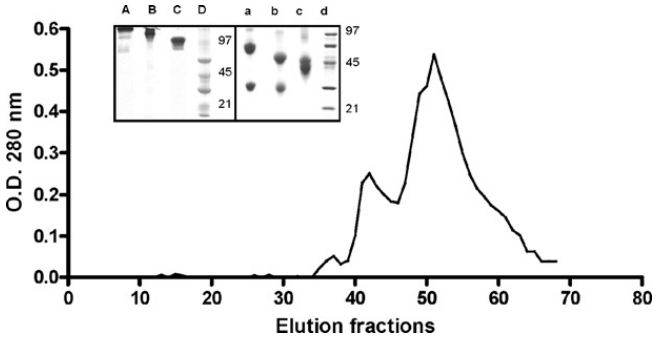

Fig. 3. Gel filtration of llama serum on Sephadex G150. Insert: 12% SDS-PAGE stained with Coomassie blue of eluted fractions with (right, lower case letters) and without (left, upper case letters) DTT. Lanes: A and a correspond to IgM from elution fraction 42, B and b correspond to IgG_1 from elution fraction 49 and C and c correspond to $IgG_2 + IgG_3$ from elution fraction 57. MW (kDa): 97, 66, 45, 31 and 21.

and particularly in IgG_{2c}, no differences were found in the residues responsible for this interaction between the IgG_2 sub-isotypes and IgG_{1b} and IgG_3 (Fig. 2).

3.3. The presence of the C1q binding site is not enough to activate complement

In a previous study, we had failed to demonstrate the ability of HCAbs to fix complement. These negative results were probably due to the anti-complementary activity of HCAbs caused by the drastic conditions employed in their purification by Protein A and Protein G chromatography (De Simone et al., 2006). For that reason, in this work we decided to use Igs purified by gel filtration under physiological conditions to perform the direct complement activation test. Fractions corresponding to IgG_1 and $IgG_2 + IgG_3$ could be purified by Sephadex G-150 gel filtration. HCAbs were obtained in one fraction because of their close molecular size (Fig. 3). Although the C1q binding motifs of llama HCAbs are preserved, it was interesting to assess whether the atypical structure of these Abs could interfere in the C1q binding to the Fc and/or subsequent complement activation. For this reason functional tests were performed.

By ELISA, anti-RBC stroma sera showed titers of 800 and 1600 for each llama. Pooled sera from both llamas were used in the complement activation and hemagglutination tests. When the direct complement activation test was performed with different serum dilutions from immunized llamas, hemolysin activity was detected (Fig. 4A). The same result was obtained with IgG_1. Unlike conventional Igs, HCAbs ($IgG_2 + IgG_3$) were unable to lyse sheep RBC (Fig. 4B) neither with Guinea pig serum nor with llama serum. Controls were done by adding IgG_1 in those wells where no complement activity was detected. In the latter control, it was observed that the addition of IgG_1 resulted in hemolysis of RBC (data not shown).

To study the valence with which HCAbs bind RBC we performed hemagglutination tests. In these tests, it was observed that all dilutions of sera and IgG_1 fractions could agglutinate RBC, whereas dilutions of HCAbs could not. However, when anti-llama IgG was added as a secondary Ab to those RBC incubated with the HCAbs fraction, hemagglutination was observed (Table 1).

Table 1
Direct hemagglutination assay.

	Hemagglutination
Serum	1:64
IgG_1	1:32 (0.095 mg/ml)
IgG_2–IgG_3	–
IgG_2–IgG_3 + anti-IgG[a]	+
Anti-IgG	–

Titers correspond to maximum hemagglutination positive dilution. The dilutions tested for IgG_2–IgG_3 were all negative.

[a] This result was obtained by indirect hemagglutination assay using a second polyclonal antibody raised against llama IgG. All reactions were analyzed in duplicate.

4. Discussion

To date, most of the works published related to HCAbs have described the biotechnological uses of the V_{HH} domains (Wesolowski et al., 2009), but little is known about the immunological properties of HCAbs.

In this work we first cloned and analyzed the CH_2–CH_3 domains of llama HCAbs and compared them to other mammalian IgGs. The high degree of identity between llama and dromedary HCAbs reflects the close phylogenetic relationship between them. Upon comparing llama HCAbs sequences with those of other mammalian IgGs it can be observed that HCAbs and conventional IgG molecules are structurally similar. The high degree of identity between them supports the idea that HCAbs were generated as a mechanism of evolution from conventional Igs genes (Nguyen et al., 2002; Conrath et al., 2003). In this sense, the main difference between the HCAbs sub-isotypes lies on the hinge region.

Regarding the interaction between IgG isotypes with Protein A and Protein G, all llama IgG isotypes (IgG_1, IgG_2 and IgG_3) bind to Protein A, however only IgG_1 and IgG_3 bind to Protein G. The contact analysis of the residues involved in the interaction revealed that all γ sub-isotypes present almost the same amino acid composition in the Protein G binding site. The failure of IgG_2 to bind to Protein G is due either to long-distance conformational changes between the different sub-isotypes or to the presence of distant interacting residues of the described binding site with Protein G.

To develop rational vaccination schedules it is necessary to study the characteristics of the immune response against pathogens. Protection against intracellular pathogens and viruses are favored by antibody-dependent cell cytotoxicity (ADCC), opsonization and phagocytosis. Regarding the humoral effector response, antigen–antibody complexes are recognized through the Fc region by effector molecules such as cellular receptors and complement. For these events to occur, the interaction between FcγR and the Fc region of IgG is necessary. Although functional tests to study the binding of llama IgG to FcγRc have not been described; the study of the presence and characteristics of the binding motifs to the FcγRc described in other species allow speculating about the possible effector functions of

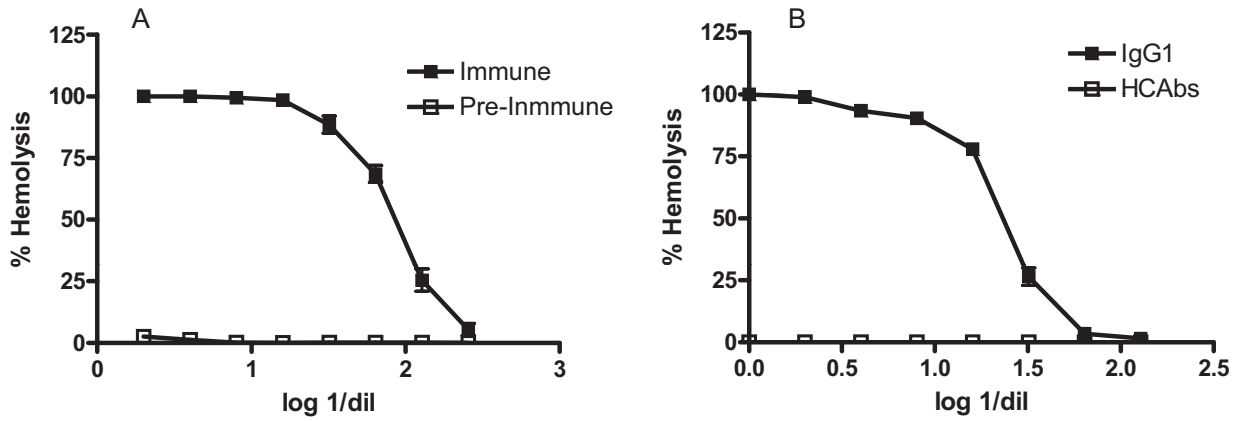

Fig. 4. Complement activation assay. (A) The results shown here correspond to pre-immune and immune serum at twofold serial dilutions (from 1:2 to 1:256). (B) Analysis of each isotype fraction at twofold serial dilutions (3–0.024 mg/ml) were dilution 1 corresponds to 3 mg/ml. 100% hemolysis was determined using sheep RBC treated with distilled water. All reactions were analyzed in duplicate. Error bars represent SE.

llama HCAbs. The main FcγR binding motif is preserved in llama IgG_{2a} and IgG_{2b}, whereas, IgG_{2c}, IgG_3 and IgG_1 present some substitutions. These amino acid substitutions may be important to establish the affinity and specificity with which HCAbs interact with the different FcγRs. Furthermore, Daley et al. (2010) determined that conventional Igs and HCAbs bind receptors displayed on the surfaces of mononuclear cells. Regarding complement binding and

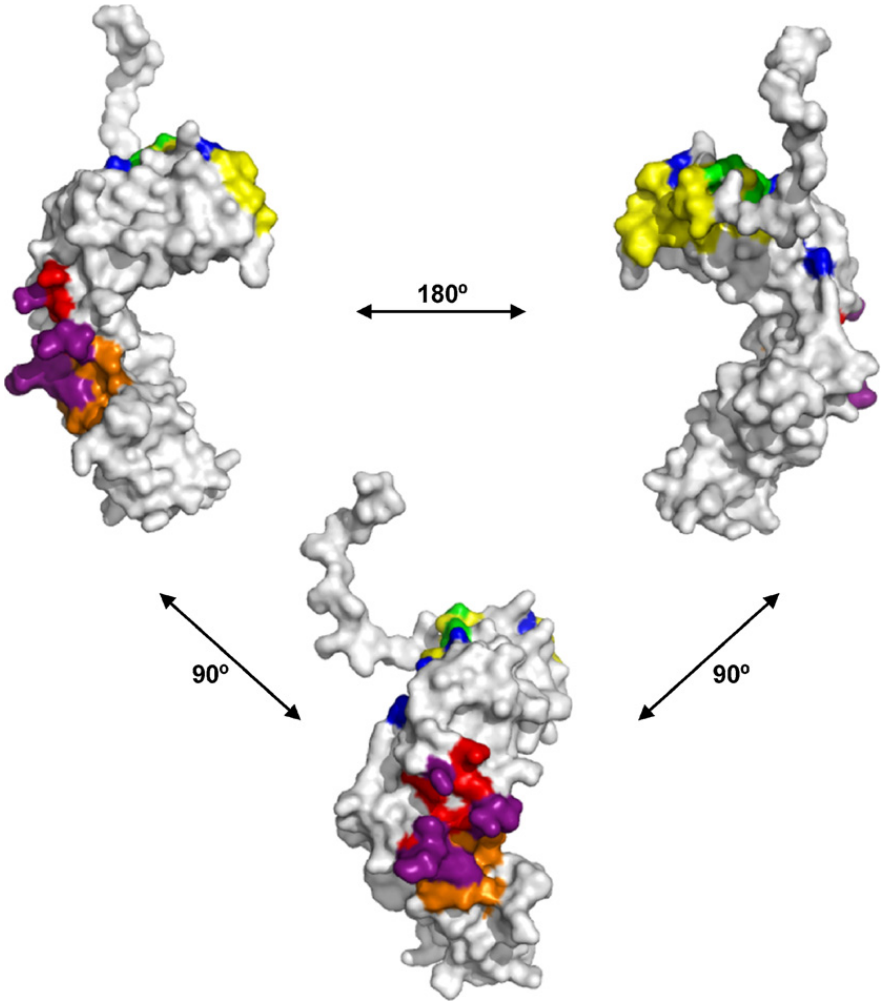

Fig. 5. Three-dimensional model of llama IgG_{2b} constant region. Protein interaction sites are colored. The residues that interact with FcγR are in yellow, the residues that interact with C1q are in blue and the residues involved in both interactions are in green. The residues that interact with Protein G are in orange, the residues that interact with Protein A are in red and the residues involved in both interactions are in purple. Molecular modeling was done using as template PDB: 1hzhK (human IgG_1). The identity between both sequences is 72%. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activation, the presence of the highly conserved C1q binding site in llamas HCAbs and the absence of the light chain would allow a better interaction between C1q and the C1q binding motif. Nevertheless, in this work it was demonstrated that HCAbs were not able to activate complement. In one study, West Nile virus neutralization by HCAbs was enhanced in the presence of complement; however, complement activation was not evaluated by these authors (Daley et al., 2010). Moreover, hemagglutination assays revealed that HCAbs failed to agglutinate RBC and this was reverted when a secondary anti-llama IgG antibody was added. Although HCAbs are not able to crosslink antigens efficiently with both paratopes, it has been described that the binding of one paratope to the Ag is sufficient to activate C1q (Couderc et al., 1985). Certainly, this is not the case of asymmetric antibodies which also present a conserved C1q binding site, but as a result of their univalence, which is due to steric hindrance present in one of the paratopes by the carbohydrate moiety, they cannot trigger complement fixation, phagocytic activity or antigen clearance (Labeta et al., 1986; Margni and Borel, 1998). In terms of length and flexibility, HCAbs sub-isotypes possess different properties in their hinge regions, however, it has been demonstrated that the most important requirements for the Fc to bind and activate C1q are the presence of a preserved C1q binding site, the formation of disulfide bonds that stabilize the structure at the middle hinge region and that the binding of C1q to the Fc is not hampered by steric hindrance (Brekke et al., 1995). On the other hand, the CH_1 domain allows the interaction between the C1q binding site and the C1q, acting as a spacer between the V regions that are complexed with the Ag. Additionally, the CH_1 domain stabilizes the hinge region structure through the interaction with the upper site of the hinge region by van der Waals bonds. For these reasons, the interaction of C1q with the HCAbs CH_2 domain is probably sterically hindered by the proximity of the V domains with the CH_2. The llama IgG_{2b} molecular modeling showed that C1q and the FcγR binding sites are nearby the hinge region, suggesting that the accessibility of these sites to their ligands would depend on the steric hindrance generated by the interaction of the V_{HH} with the Ag (Fig. 5).

Hemagglutination tests are widely used to evaluate the humoral response in different animal species, because it is an unexpensive and easy methodology to perform. Since HCAbs are present at a high proportion within the IgG isotype fraction in camelids, during the study of their humoral response against a pathogen it is important to bear in mind that HCAbs might be present in such humoral response and which might go undetected in hemagglutination tests.

This study demonstrates that HCAbs present effector functions motifs highly preserved such as the C1q, FcγRI and FcγRIII binding sites. Although functional tests to study the binding of llama IgG to FcγRc have not been described, the fact that HCAbs are not able to activate complement might indicate that their main effector function lie on the neutralization activities (Daley et al., 2010). The data presented represent a mayor advance in the HCAbs characterization by providing new insights into the HCAbs effector functions; however, further studies should be conducted to understand the importance of HCAbs in the immunobiology of camelids.

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