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Bovine (*Bos taurus*) Humoral Immune Response Against *Echinococcus granulosus* and Hydatid Cyst Infertility

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ABSTRACT

Echinococcus granulosus, the agent of hydatid disease, presents an indirect life cycle, with canines (mainly dogs) as definitive hosts, and herbivores and human as intermediary ones. In intermediary hosts fertile and infertile cysts develop, but only the first ones develop protoscoleces, the parasite form infective to definitive hosts. We report the presence of bovine IgGs in the germinal layer from infertile cysts (GLIC), in an order of magnitude greater than in the germinal layer from fertile cysts (GLFC). When extracted with salt solutions, bovine IgGs from GLIC are associated with low or with high affinity (most likely corresponding to non specific and antigen specific antibodies, respectively). Specific IgGs penetrate both the cells of the germinal layer and HeLa cultured cells and recognize parasitic proteins. These results, taken together with previous ones from our laboratory, showing induction of apoptosis in the germinal layer of infertile hydatid cysts, provide the first coherent explanation of the infertility process. They also offer the possibility of identifying the parasite antigens recognized, as possible targets for immune modulation. J. Cell. Biochem. 112: 189–199, 2011.

KEY WORDS: CYST FERTILITY; CYSTIC ECHINOCOCCOSIS; ECHINOCOCCUS GRANULOSUS; HYDATID CYSTS; PROTOSCOLEX

Cystic echinococcosis (CE), caused by infection with the metacestode stage of the flatworm *Echinococcus granulosus*, is a major zoonotic disease of world-wide distribution, that generates an important impact in public health and serious economic costs [Eckert et al., 2000; Battelli, 2009]. This disease is present in all continents and it has been described in 100 countries at least. Higher prevalence in humans has been reported in Eurasia (in the Mediterranean region, the Russian Federation, and its adjacent independent states), in China, in Africa (east and north regions), in Australia, and in South America [Eckert and Deplazes, 2004; Magambo et al., 2006; Moro and Schantz, 2006; Sadjjadi, 2006; Shaikenov, 2006].

In intermediary hosts two types of hydatid cysts can be observed: fertile, which produce protoscoleces, the infective form of the parasite to dogs, attached to germinal layer and free in the hydatid fluid; and infertile, that do not present protoscoleces and therefore

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are not able to continue with the parasite life cycle. The biological and molecular backgrounds underlying the generation of both types of cysts have not been elucidated. The World Health Organization indicates the importance of developing effective serologic methods that allow the accurate and early diagnosis of CE [Eckert et al., 1995a; Zhang et al., 2007], particularly of cyst fertility, previous to any medical treatment or surgical removal of the hydatid cyst [Eckert et al., 1995b]. This indication is based on the existence of humoral immune response in this type of parasitic diseases. In fact, most of the patients produce several antibodies against parasite antigens, but they have not been associated to protection, although they would be relevant in diagnoses [Eckert and Deplazes, 2004; Gatti et al., 2007; Zhang et al., 2007].

Bovines are very susceptible to *E. granulosus* infection nonetheless the cysts are mainly infertile. In contrast, ovine usually develop a high percentage of fertile hydatid cysts. In different geographic locations, where a single *E. granulosus* strain prevails, cyst fertility is more related to the intermediary host species [Zhang et al., 2003; Daryani et al., 2009]. Molecular studies using mitochondrial DNA sequences have identified 10 distinct genetic types (G1–10) within *E. granulosus* [Moro and Schantz, 2009]. Thus, several groups have reported the presence of fertile and infertile cysts produced by the same *E. granulosus* strain, as is the case with the G1 strain in bovine, ovine and human, and the G7 strain in pigs [Rosenzvit et al., 1999; Kamenetzky et al., 2000; Kamenetzky et al., 2002; Guarnera et al., 2004].

The fact that a single strain generates fertile or infertile cysts in different hosts species would indicate natural immunity in some hosts, thus preventing the development and growth protoscoleces and maintaining the cyst in an infertile state [Zhang et al., 2003]. IgGs are present in hydatid fluid and some reports suggest the presence of IgGs at the internal face of the cysts (including both germinal and laminar layers) [Coltorti and Varela-Diaz, 1972, 1975; Varela-Diaz and Coltorti, 1972, 1973; Shapiro et al., 1992], although this fact was not studied further. Recently, Taherkhani et al. [2007] described that in the laminar layer of ovine hydatid cysts proteins with molecular masses of approximately 55 and 25–29 kDa are present that are recognized by antibodies against ovine IgGs. The same proteins were also recognized by a significant proportion of sera from human hydatid patients, particularly by IgG4 antibodies and not by sera from negative control individuals. These proteins could correspond to glycoproteins similar to mucins with molecular masses near to those expected for light and heavy chains of IgG. A possible explanation to these results is the coexistence of Igs and mucins in the laminar layer of the cyst or that parasitic glycoproteins share epitopes with Igs making possible a cross-reaction between them.

In this work, we describe the presence of host specific IgG in the germinal layer from bovine hydatid cysts. In fertile cysts, we detected only an unspecific IgG that is able to penetrate the cyst wall, accumulating in the hydatid fluid. In contrast, in infertile cysts a specific fraction of IgG was found tightly bound to the inner surface of the cyst wall. We propose that intermediary hosts could establish different immunological responses against *E. granulosus*, independent of the parasite strain, thus generating fertile or infertile cysts according to the host immunological capacity.

MATERIALS AND METHODS

HYDATID CYSTS

Fertile and infertile *E. granulosus* hydatid cysts were obtained from fresh bovine livers or lungs at abattoirs in Santiago, Chile. Cysts were processed as described before [Galindo et al., 2003; Martinez et al., 2005; Paredes et al., 2007]. Macroscopic observation was used to define as fertile a cyst presenting free protoscoleces in the hydatid fluid, a whitish color and a thick laminar layer [Bortoletti and Ferretti, 1978]. They were confirmed under light microscopy by the presence of buds and both grown protoscoleces attached to the germinal layer and free protoscoleces in the hydatid fluid. Infertile cysts showed a clear hydatid fluid, a yellow-brown color of the internal surface and no protoscoleces, either by macroscopic or microscopic observations. All cysts used, either fertile or infertile, were at least of 3 cm diameter.

INNER SURFACE OF HYDATID CYSTS EXTRACTION AND PROTOSCOLECES COLLECTION

The inner layer of fertile and infertile cysts containing the germinal layer and part of the cyst wall were aseptically obtained by gentle scraping of the inner surface of the cysts (previously washed with 10 ml of sterile phosphate buffered saline (PBS) pH 7.4) in a laminar flow chamber. Protoscoleces were decanted by gravity from the hydatid fluid, washed in PBS pH 7.4 at 38.5°C, and treated with pepsin 0.1% in Hanks' salt solution pH 2.0 at 38.5°C for 15 min to eliminate remnants of the germinal layer and death protoscoleces [Thompson et al., 1990; Galindo et al., 2003; Hou et al., 2007].

IMMUNOGLOBULIN IDENTIFICATION ON THE INNER SURFACE OF HYDATID CYSTS

The germinal fertile and infertile layers obtained by gentle scraping of the inner surface of the cysts and protoscoleces were homogenated in 30 mM Tris pH 7.6, 150 mM NaCl, 5 μg/ml aprotinin, 2 mM EDTA, 5 mM PMSF, and 2.5 mM *N*-α-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK). Proteins from these homogenates were separated on SDS–12% polyacrylamide gels in a Mini Protean 3 Electrophoresis chamber (Bio-Rad) under reducing conditions. Electrophoresis was performed at 100 V/gel and either stained with Coomassie blue or blotted on to nitrocellulose paper at 200 V for 1 h. Silver stain was used when low concentration of proteins was obtained following salt extractions. The electrophoretic transfer was performed in a mini Trans-Blot Cell (Bio-Rad). The nitrocellulose paper was blocked overnight using 0.5% soybean proteins in PBS and washed in PBS/0.05% Tween 20. Blots were incubated with HRP conjugated anti bovine IgG whole molecule (Sigma #A-5295) at 1/10,000 v/v dilution and developed with ECL Western blotting (GE Healthcare) in a Bio-Max film (Kodak).

Antibodies present in the samples were isolated using the Seize X Protein G Immunoprecipitation Kit (Promega). Briefly, germinal layer homogenates from fertile and infertile cysts were centrifuged twice at 31,870*g* for 15 min; 400 μl of protein G affinity column was equilibrated in binding/wash buffer (0.14 M NaCl pH 7.4, 0.008 M sodium phosphate, 0.002 M potassium phosphate, and 0.01 M KCl) and 800 μl of sample was applied to the column. After 15 min incubation the column was washed with binding/wash buffer and

the elution was performed with 100 mM glycine pH 2.5. Proteins were quantified by the Bradford assay [Bradford, 1976] for all samples and Western blot analysis was performed against bovine IgG as a control of the Ig isolation efficiency.

IMMUNOFLUORESCENCE MICROSCOPY

Histological sections of cysts wall pieces obtained from fertile and infertile cysts were fixed in 4% (w/v) paraformaldehyde in PBS pH 7.2 at 4°C for 24 h and embedded in paraffin. Five micrometers sections were blocked overnight with Cas-Block (Zymed) and incubated for 2 h at room temperature with polyclonal antibodies against bovine IgG whole molecule (Sigma #A-5295) at 1/50 v/v dilution and with a polyclonal anti-rabbit IgG conjugated with FITC (Dako N8 F0205) diluted 1/100 v/v. Finally, the samples were mounted in VECTASHIELD and visualized in a Nikon Eclipse E400 epifluorescence microscope. Five micrometers sections of bovine spleen were used as positive control for bovine IgGs using the same dilution of antibodies as described above.

MICROSEQUENCING OF THE IMMUNOGLOBULIN PEPTIDES FROM THE GERMINAL LAYER

After separation in SDS–PAGE (12.5% polyacrylamide) in a Protean II XL cell chamber (Bio-Rad), the gel was fixed for 1 h in 45% methanol, 15% trichloroacetic acid; stained overnight with Amido Black reagent (0.58% Amido Black, Sigma, in 40% methanol, 10% acetic acid) and destained with 25% methanol and 5% acetic acid. Bands between 24 and 27 kDa were excised from the gel and digested with endoproteinase Lys-C (*Achromobacter* lyticus, WAKO Chemicals), which cleaves peptide C-terminal to lysine residues. The peptides were purified by HPLC as described [Toro et al., 1993] and analyzed by Edman degradation in a Procise micro sequencing instrument (Applied Biosystem), following the manufacture's instruction.

HIGH AFFINITY SPECIFIC IgGs FROM THE GERMINAL LAYER OF HYDATID CYSTS

Proteins from germinal layers of fertile and infertile cysts as well as from protoscoleces were extracted with increasing ionic strength salt solutions (0.154–3.0 M NaCl). One gram of germinal layer obtained from at least 20 different fertile or infertile cysts were homogenized in a Potter Elvehjem with 50 strokes at 1,400 rpm in PBS pH 7.2 containing 5 mM PMSF, 2.5 mM *N*-α-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), and 1 mM EDTA as protease inhibitors. The homogenate was centrifuged at 7,000*g* for 10 min at 4°C and the supernatant, containing proteins extracted with 0.154 M NaCl, was collected. To assure the efficiency in the protein extraction, the sediment was resuspended in PBS, homogenized with 20 strokes and centrifuged at 7,000*g* for 10 min at 4°C. This process was repeated until the protein concentration in the supernatant was negligible. All supernatants from the PBS extraction were mixed. Proteins from the sediment were then sequentially extracted with 0.5, 1.0, 2.0, and 3.0 M NaCl solutions, as described previously. Then, each extract was dialyzed against distilled water, lyophylized, and suspended in PBS in the presence of protease inhibitors. In each extract and in the 3.0 M NaCl sediment, protein concentration was measured by the Bradford assay and separated by SDS–PAGE. Proteins were transferred to a nitrocellulose membrane (Bio-Rad), blocked overnight with 0.5% soy bean proteins in PBS, incubated for 2 h with specific antibodies against bovine immunoglobulin (whole molecule, Sigma) conjugated with horseradish peroxidase and visualized by ECL Western blotting (GE Healthcare) in Bio-Max film (Kodak). Densitometric analysis in this and the following experiments were performed using UN-SCAN-IT gel version 4.1 for Windows.

PROTEIN EXTRACTION FROM THE INNER SURFACE OF HYDATID CYSTS WITH CHAOTROPIC AGENTS

Antibodies with high affinity to the germinal layer were extracted with chaotropic agents. The same protocol as described above for NaCl was used, except sequentially extracting with 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 M KI or NaSCN, this last extending up to 6.0 M. In each extract protein concentration was measured by the Bradford assay. Proteins were separated by SDS–PAGE and the immunoglobulins were visualized by Western blot using an anti-bovine IgG antibody (whole molecule, Sigma and Fc fraction, Jackson).

SUBCELLULAR FRACTIONS OF THE GERMINAL LAYER FROM HYDATID CYST

Subcellular fractions from the germinal layer were obtained by differential centrifugation, according to the method described by Cabezón [2002]. Samples of the inner surface from hydatid cysts were homogenized in buffer A (0.25 M KCl, 0.003 M $MgCl_2$, 0.002 M $CaCl_2$, 0.32 M sucrose, 0.05 M Tris pH 7.4) and centrifuged twice at 650*g* for 15 min at 4°C. The sediment was identified as Crude Nuclear Fraction (CNF). The supernatant was centrifuged at 15,000*g* for 10 min at 4°C; the sediment obtained (Mitochondrial Fraction or MitF) was washed twice in the same conditions. The resulting supernatant was centrifuged at 100,000*g* 1 h at 4°C obtaining the Microsomal Fraction (MF) (sediment) and the Supernatant (S). Aliquots from CNF were resuspended in 1.6 M sucrose and placed over a 2.2 M sucrose solution. After centrifugation at 70,000*g* for 1 h, the Enriched Nuclei Fraction (ENF) was recovered from the interface of the sucrose gradient. The quality of each fraction was determined according to Cabezón [2002]. DNA, protein, and oxygen consumption were measured in each fraction as previously described by Cabezón [2002]. The proteins from each fraction, previously quantified, were separated by SDS–PAGE, transferred to nitrocellulose membranes and incubated with anti bovine IgG (whole molecule).

PENETRATION OF IgGs FROM HYDATID CYSTS IN HELA CELLS

HeLa cells were cultured in RPMI 1640 medium (Gibco) with 5% heat-inactivated FCS (Gibco) and penicillin–streptomycin medium (Gibco) at 37°C in 5% CO_2. IgGs were extracted with NaCl solutions from the inner surface of hydatid cysts as described above and purified using a Protein G-agarose (Pierce) column.

Cells in 2.5 ml of medium were placed in the wells of a six-well plate containing a 12 mm glass coverslip each and grown until 50% confluence. Afterwards, following Seddiki et al. [2001], 10 μg/ml of purified IgGs extracted with PBS, 1 or 3 M NaCl from the inner surface of infertile cysts were added and further incubated for 2 h at 37°C in 5% CO_2. IgGs extracted with PBS or 1 M NaCl from the inner

surface of fertile cysts and IgGs purified from normal bovine serum were used as controls. Cells were then washed with sterile PBS to remove unbound IgGs and fixed in ice-cold 90% ethanol. Samples were blocked with Cas-Bloc (Zymed) overnight, rabbit Ab anti bovine IgG Fc fraction (Jackson #301-035-008) was added and incubated for 2 h at 37°C. Finally, Ab anti rabbit IgG conjugated with Alexa 488 was used as secondary antibody and further incubated for 1.5 h at 37°C. The coverslips were mounted in 70% glycerol with 1 μg/ml Hoescht. Cells were analyzed using a Nikon Eclipse E400 epifluorescence microscope. Images were obtained using a Coolpix 4500 Nikon camera.

PROTEINS FROM PROTOSCOLECES ARE RECOGNIZED BY IgGs EXTRACTED FROM INFERTILE CYSTS WITH 3 M NaCl

Total proteins from protoscoleces were separated by SDS–PAGE, transferred to nitrocellulose membranes and incubated with IgGs purified from proteins extracted with increasing salt solutions, as described above. Briefly, each extract was passed through a Protein G affinity column, eluted with 0.1 M Glycine pH 2.0 and concentrated at 1 μg/μl in Microcon 3 kDa (Millipore). Purity of IgGs was confirmed by SDS–PAGE and silver staining; additionally IgGs were assayed by Western blot using Ab anti bovine IgG whole molecule. IgGs extracted from infertile cysts with 0.1, 1.0, and 3.0 M NaCl and purified as indicated above were used. For fertile cyst all proteins were extracted in 1.0 M NaCl; then, only 0.1 and 1 M NaCl extracts were used. Bound IgGs were recognized using Ab anti bovine IgG whole molecule HRP conjugated as secondary antibody. A negative control using only the secondary Ab was performed.

RESULTS

BOVINE IMMUNOGLOBULINS ARE PRESENT IN THE GERMINAL LAYER OF HYDATID CYSTS

Figure 1A,a shows the electrophoretic protein pattern obtained by SDS–PAGE from infertile (lane 3) and fertile (lane 4) hydatid cyst inner surfaces. Bands of approximately 50 kDa (thick arrow) and 25 kDa (thin arrow) are present in both preparations, showing similar electrophoretic mobility than heavy and light bovine IgGs chains (Fig. 1A,a lane 2). To confirm the identity of these proteins, a Western blot using specific antibodies against whole molecule bovine IgGs was performed. Figure 1A,b lanes 7 and 8, shows recognition of both heavy (thick arrow) and light (thin arrow) IgGs chains by this antibody, confirming the presence of bovine IgGs in the inner surface of both infertile and fertile hydatid cysts. The specificity of the antibody is evident when chased against rabbit serum (lane 5) compared to IgGs purified from bovine serum (lane 6). Although IgGs are present in the germinal layer of both infertile (GLIC) and fertile (GLFC) cysts (Fig. 1A,b lanes 7 and 8), the relative proportion of these proteins seems to be higher in GLIC (Fig. 1A lane 7).

To verify the identity of the proteins recognized by immunological methods, the bands from GLIC located around 25 kDa on the SDS–PAGE gel were analyzed by Edman microsequencing. Three peptides matched with the expected sequence for bovine IgGs, thus confirming the results obtained by Western blot (DEVTQSSSNFQNSFT = light chain fragment from bovine IgG; PVTWIRQAPGK = heavy chain fragment from bovine IgG, and NVDWIRQAPGK = heavy chain fragment from bovine IgG). Interestingly, the first fragment that corresponds to a 27 kDa protein was only found in the germinal layer from infertile cysts, suggesting that a particular IgG subtype is present in this type of cysts (Fig. 1B).

BOVINE IgGs ARE PRESENT IN LARGER AMOUNTS IN THE INNER SURFACE OF INFERTILE CYSTS AS COMPARED TO FERTILE ONES

In order to estimate the relative proportion of IgGs in the germinal layer of fertile and infertile cysts and protoscoleces (PSc), a Western blot with the proteins obtained from these three structures was performed, using antibodies against bovine IgG whole molecule and α-actin as charge control. Densitometric analysis shows that in the GLIC (Fig. 1C, lane 3) the proportion of IgG heavy chain is around two orders of magnitude higher than in GLFC (Fig. 1C, lane 4) while for the IgG light chain the difference was one order of magnitude higher. IgG was not detected in protoscoleces (Fig. 1C, lane 5).

Using a recombinant G protein affinity column, purified IgGs obtained from the inner surface of infertile cysts was fourfold the amount of purified IgGs estimated from inner surface of fertile cysts. The presence of bovine IgGs in the germinal layer of hydatid cysts was confirmed by in situ immunofluorescence (Fig. 2). Bovine spleen was used as positive control of IgGs presence (Fig. 2A). The germinal layer of infertile cysts (GLIC) presents an intense immunochemical reaction indicative of high levels of IgGs (Fig. 2B). This reactivity is distributed in patches (not shown). The germinal layer of fertile cysts (GLFC) (Fig. 2C) shows a diffuse distribution of immunochemical reactivity. Interestingly, only a light positive signal was observed in the laminar layer (LL) in both types of cysts (Fig. 2B,C), indicating low antibody presence in this cyst structure. Controls using only the secondary antibody were negative (not shown).

BOVINE IMMUNOGLOBULINS HAVE HIGH AFFINITY FOR THE INNER SURFACE OF INFERTILE HYDATID CYSTS

Cucnik et al. [2004, 2005] have described that a 0.5 M NaCl solution decrease to 25% the initial binding of an antigen to its antibody. Then, it is possible to discriminate between low- and high-affinity Ab-antigen interactions using increasing ionic strength salt solutions. Proteins from the whole homogenate of the inner surface of fertile cysts (Fig. 3A, lane 1) and from repeated extractions with 0.154 M (lane 2) or 1 M (lane 3) NaCl are shown in Figure 3A. The right panel shows the same protein extracts detected with anti bovine IgG whole molecule (Fig. 3A, lanes 4–6). Rabbit serum and bovine purified IgGs were used as negative and positive controls, respectively (Fig. 3A, lanes 7 and 8). Proteins remaining after 0.5 M NaCl extraction were completely soluble in 1 M NaCl, so no further extractions with higher NaCl concentrations were performed. Most IgGs were extracted with PBS (Fig. 3A, right panel, lane 4) indicative of antibodies showing low affinity to the germinal layer from fertile cysts; only faint bands were observed in 0.5 and 1.0 M NaCl extracts (Fig. 3A, right panel, lanes 5 and 6, respectively). In contrast, in order to extract IgGs from GLIC, higher concentrations of NaCl were needed. Thus, IgGs were found in all extracts, from PBS up to 3.0 M NaCl (Fig. 3B, left panel, lanes 2–5, silver stain and Fig. 3B, right panel, lanes 7–11, Western blot). Moreover, a high proportion of

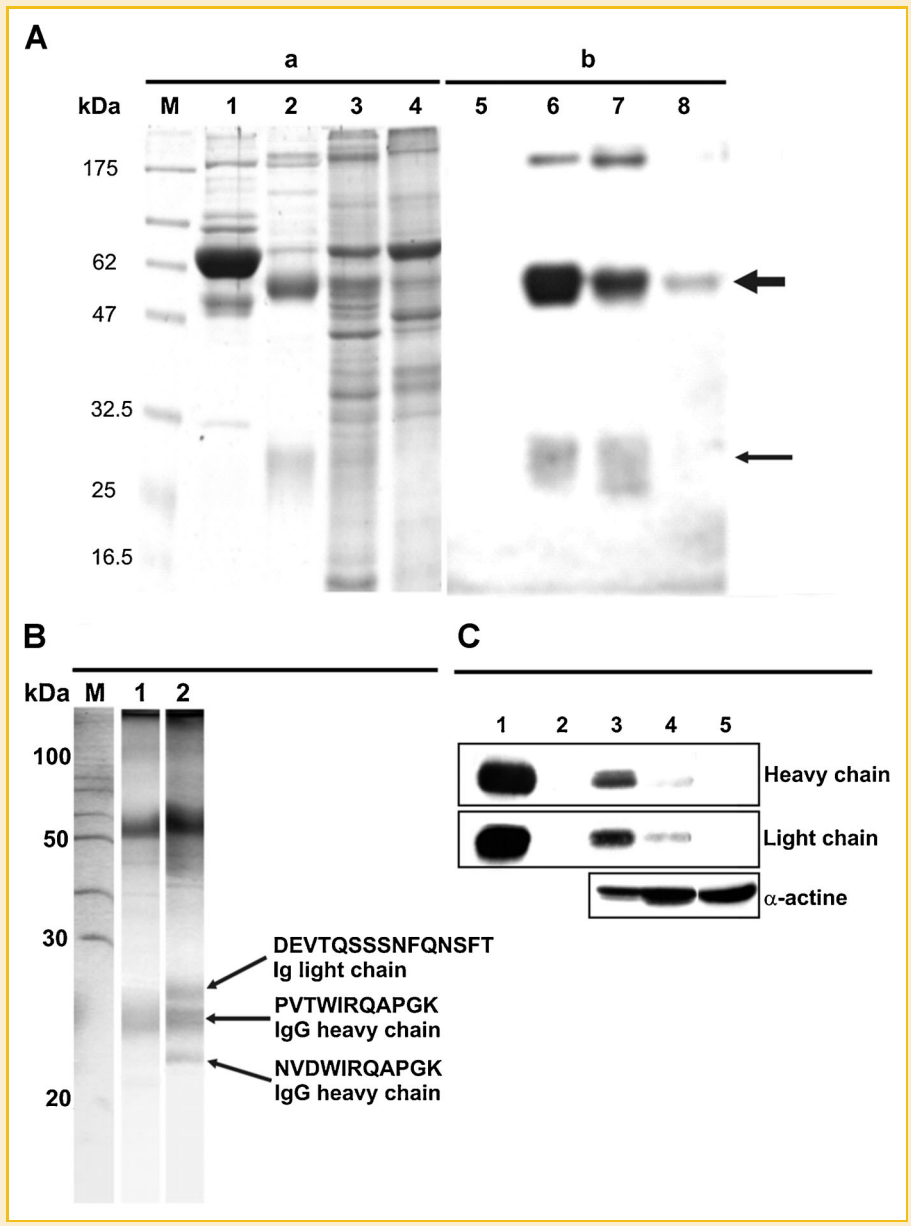

Fig. 1. A: Bovine IgGs are present on the inner surface of fertile and infertile hydatid cysts. a: Proteins (20 μg) from infertile (lane 3) and fertile (lane 4) inner surfaces of hydatid cysts were separated by SDS–PAGE as indicated under Materials and Methods Section and stained with Coomassie blue. b: Western blot of gel shown in part (a) using anti bovine IgG whole molecule that recognizes both heavy (thick arrow) and light (thin arrow) chains. Lanes 1 and 5: rabbit IgGs; lanes 2 and 6: bovine IgGs; lanes 3 and 7: proteins from the inner surface of infertile cysts; lanes 4 and 8: proteins from the inner surface of fertile hydatid cysts. B: Three bands from the inner surface of infertile cysts (22–27 kDa) were sliced out of the gel and digested in situ with lysine-C. Selected peptides, separated by HPLC, were microsequenced by Edman degradation. Three sequences are shown. Lane 1: proteins from fertile cysts; lane 2: proteins from infertile cysts. C: Estimation of IgGs relative proportion in the inner surface of fertile and infertile cysts and protoscoleces. Western blot using antibodies against bovine IgG whole molecule-HRP conjugated. Lane 1: IgG from normal bovine serum; lane 2: rabbit normal serum; lane 3: IgGs from the inner surface of infertile cysts; lane 4: IgGs from the inner surface of fertile cysts; lane 5: protoscoleces. α-Actin was used as charge control. M: molecular weight standards in kDa.

IgGs was found in the sediment after 3.0 M NaCl extraction (Fig. 3B, right panel, lane 6 and left panel, lane 12).

The presence of immunoglobulins in the inner surface of infertile hydatid cysts was also studied with KI extraction. After exhaustive extraction with PBS an important fraction of IgGs is evident (Fig. 4A, lane 2) as compared with IgGs present in the homogenate (Fig. 4A, lane 1). On the other hand, the chaotropic agent extracts a maximum of IgGs at 2.0 M (lane 5) in spite of the fact that these antibodies are present up to the 5.0 M extract (lane 8), indicating a very strong binding of IgGs to the germinal layer. Interestingly, at 5.0 M KI all proteins are solubilized. A similar result was obtained using NaSCN in a concentration ranging from 0.5 to 6.0 M (data not show). Densitometric analysis of heavy (Fig. 4B, left panel) and light chains (Fig. 4B, right panel) of each band in PBS extract and in each

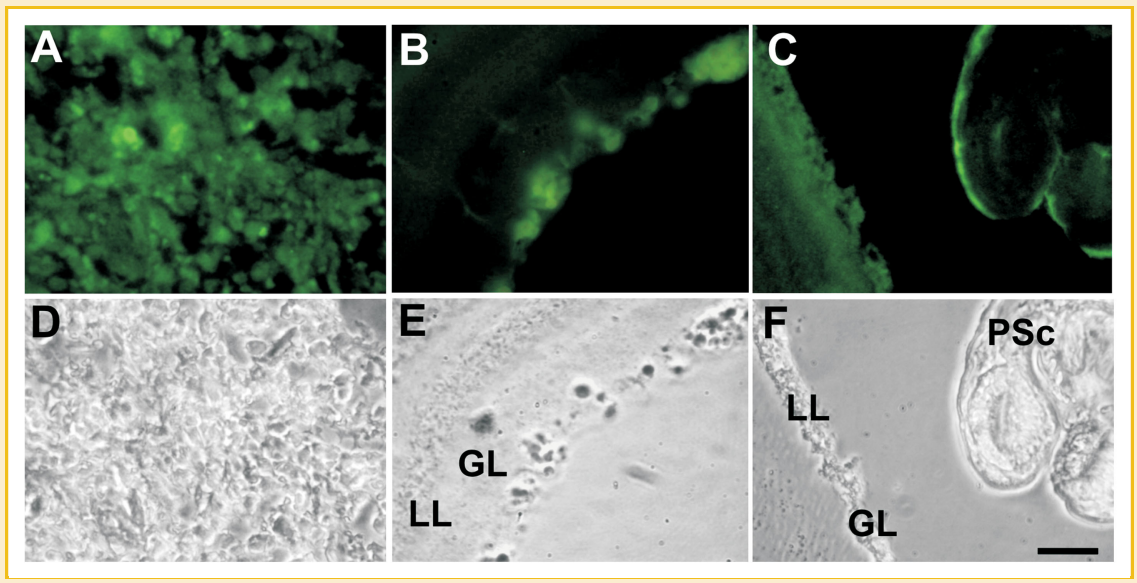

Fig. 2. Bovine IgGs are present in the germinal layer of fertile and infertile cysts. Rabbit antibodies against bovine IgG whole molecule and anti-rabbit IgG conjugated with FITC as secondary antibody were used. Panels A–C show presence of IgGs (green). D–F: Phase contrast. A,D: Bovine spleen used as positive control of IgGs presence; B,E: infertile cyst; C,F: fertile cyst. GL: germinal layer; LL: laminated layer; PSc: protoscolex. Bar: 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

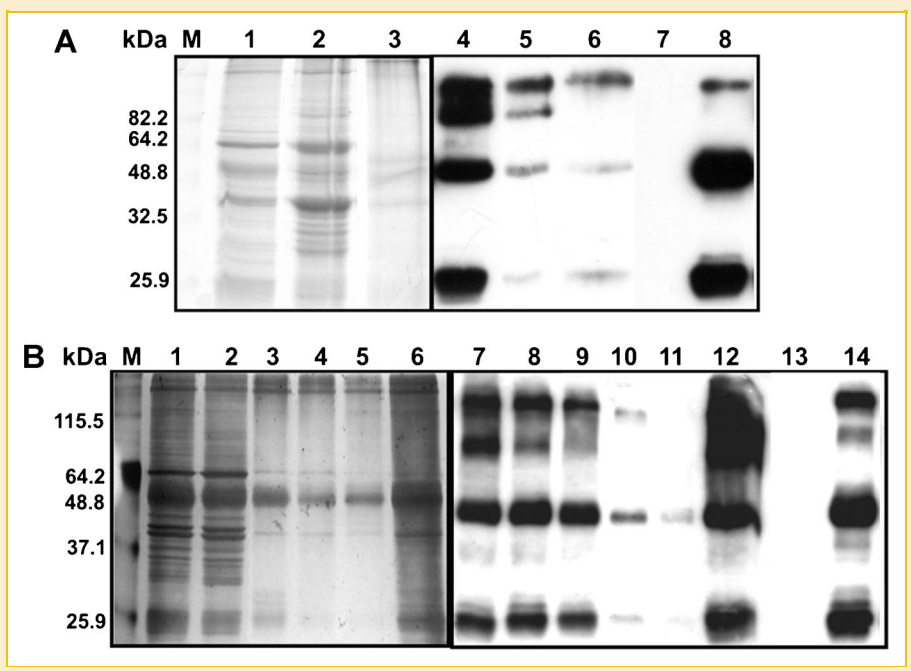

Fig. 3. Bovine IgGs are released from the inner surface of fertile and infertile cysts with increasing NaCl concentrations. A: Protein extraction from the inner surface of fertile cysts (GLFC). Lanes 1 and 4: total proteins from GLFC; lanes 2 and 5: proteins extracted with 0.154 M NaCl; lanes 3 and 6: proteins extracted with 1 M NaCl; lane 7: rabbit IgGs; lane 8: bovine IgGs. Left panel: SDS–PAGE, Coomassie blue stain. Right panel: Western blot using an Ab anti-bovine IgG, whole molecule, HRP conjugated. B: Protein extraction from the inner surface of infertile cysts (GLIC). Lanes 1 and 7: total proteins from GLIC; lanes 2 and 8: proteins extracted with 0.154 M NaCl; lanes 3 and 9: proteins extracted in the 0.5 M NaCl; lanes 4 and 10: proteins extracted in 1 M NaCl; lanes 5 and 11: proteins extracted in 3 M NaCl; lanes 6 and 12: proteins present in the sediment remaining after exhaustive extraction with 3 M NaCl; lane 13: rabbit IgGs; lane 14: bovine IgGs. Left panel: SDS–PAGE, silver stain. Right panel: Western blot using an Ab anti-bovine IgG, whole molecule, HRP conjugated.

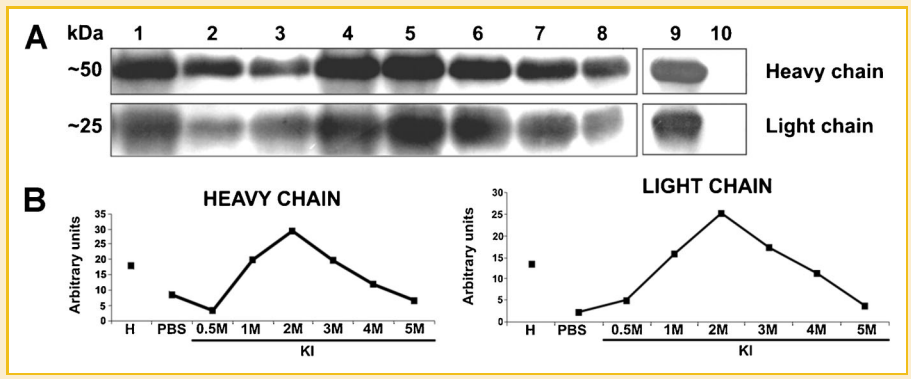

Fig. 4. Bovine IgGs are released from the inner surface of infertile hydatid cysts with increasing concentrations of the chaotropic agent KI. A: Identification of bovine IgGs by Western blot, using an Ab anti-bovine IgG HRP conjugated and developed by chemiluminescence. Lane 1: total proteins from inner surface of infertile cysts (GLIC); lane 2: proteins extracted with PBS. Proteins extracted with 0.5 M KI (lane 3), 1.0 M KI (lane 4), 2.0 M KI (lane 5), 3.0 M KI (lane 6), 4.0 M KI (lane 7), 5.0 M KI (lane 8). Lane 9: IgGs purified from normal bovine serum, lane 10: rabbit normal serum. B: Densitometric analysis of heavy (left panel) and light (right panel) IgG chains present in each KI protein extract.

KI extraction is shown. These results confirm that in infertile cysts an IgG fraction is present showing high affinity to moieties present in the germinal layer.

IgGs STRONGLY BOUND TO THE INNER SURFACE OF INFERTILE HYDATID CYSTS PENETRATE LIVE MAMMALIAN CELLS

Figure 5A shows a light micrograph of the enriched nuclear fraction, showing that these subcellular structures are concentrated in this sample (arrows). In Figure 5B, an analysis of DNA concentration and respiratory activity in each fraction is shown. DNA is clearly concentrated in the nuclear fractions while most respiratory activity is found in the mitochondrial fraction, although mitochondria are also evident in the nuclear fractions, as expected. Figure 5C,a, shows the electrophoretic pattern of the homogenate (lane 3), crude nuclear (lane 4), enriched nuclear (lane 5), mitochondrial (lane 6), microsomal (lane 7), and supernatant (lane 8) fractions of the inner surface of infertile cysts. In Figure 5C,b, the corresponding Western blots are shown after chasing with bovine IgG antibodies. Lanes 1 and 2 of Figure 5C correspond to rabbit serum and purified bovine IgGs, respectively, used as controls. Figure 5C,b, shows that IgGs are present in all fractions studied, though more intense reactivity was found in the crude (lane 12) and enriched (lane 13) nuclear fractions as well as in the supernatant (lane 16). Light chain densitometric analysis indicates that the crude (Fig. 5C,b, lane 12) and enriched (Fig. 5C,b, lane 13) nuclear fractions present 10- and 9-fold more IgGs, respectively, than the homogenate (Fig. 5A,b, lane 11). These results suggest that bovine IgGs found strongly bound to the inner surface of infertile hydatid cysts are those that pass across the adventitious layer, the laminar layer, and the plasma membrane, finally locating in subcellular structures of the germinal layer.

To further assess the ability of IgGs present in the inner surface of infertile hydatid cysts to penetrate mammalian cells, IgGs were purified from bovine serum or from 0.154 M NaCl extracts of fertile and infertile inner cyst surfaces as well as from the supernatants after extraction with 1.0 and 3.0 M NaCl and the remaining sediment after 3.0 M extraction of infertile cysts. The purified IgGs were incubated with HeLa cells and followed inside the cells using a fluorescent specific antibody. Figure 6B shows that serum bovine IgGs cannot penetrate these cells. The same results were observed when IgGs purified from extracts obtained with 0.154 M NaCl from fertile (Fig. 6E) or infertile (Fig. 6H) cysts were incubated with HeLa cells. However, IgGs purified from the 1.0 M NaCl extract from the inner surface of infertile hydatid cysts (Fig. 6K) or with IgGs purified from the supernatant of the 3.0 M extract of the infertile cysts (Fig. 6N) did penetrate HeLa cells. Same results were obtained using Vero cells (not shown).

IgGs STRONGLY BOUND TO THE INNER SURFACE OF INFERTILE HYDATID CYSTS RECOGNIZE PARASITIC PROTEINS

To test whether IgGs extracted from the germinal layer of fertile and infertile hydatid cysts recognize parasitic proteins, these IgGs were challenged against protoscolex homogenates. IgGs extracted with PBS (Fig. 7, lane 3) or 1.0 M NaCl (Fig. 7, lane 4) from fertile cysts do not recognize parasitic proteins. Contrarily IgGs extracted with 1.0 M (Fig. 7, lane 6) or 3.0 M (Fig. 7, lane 7) NaCl from infertile cysts recognized up to 5 parasitic proteins (69, 62, 57, 47, and 28 kDa, arrows). IgGs extracted with PBS from infertile cysts showed only a weak signal (Fig. 7, lane 5). A negative control using only the secondary Ab is shown in lane 2.

DISCUSSION

Despite the fact that host immunoglobulins have been previously found in the hydatid fluid present in the lumen of the cyst [Coltorti and Varela-Diaz, 1972, 1975; Varela-Diaz and Coltorti, 1972; Shapiro et al., 1992], this observation was not associated with the ability of these proteins to pass across the plasma membrane located between the laminar and the germinal layers of the cyst. Indeed, to be present in the hydatid fluid the IgGs should be able to first penetrate the germinal layer. On the other hand, IgGs were reported to be present in a "cyst membrane" preparation, corresponding to the whole wall of the cyst [Varela-Diaz and Coltorti, 1973] and in the laminar layer of the cyst as well. These reports points to a humoral

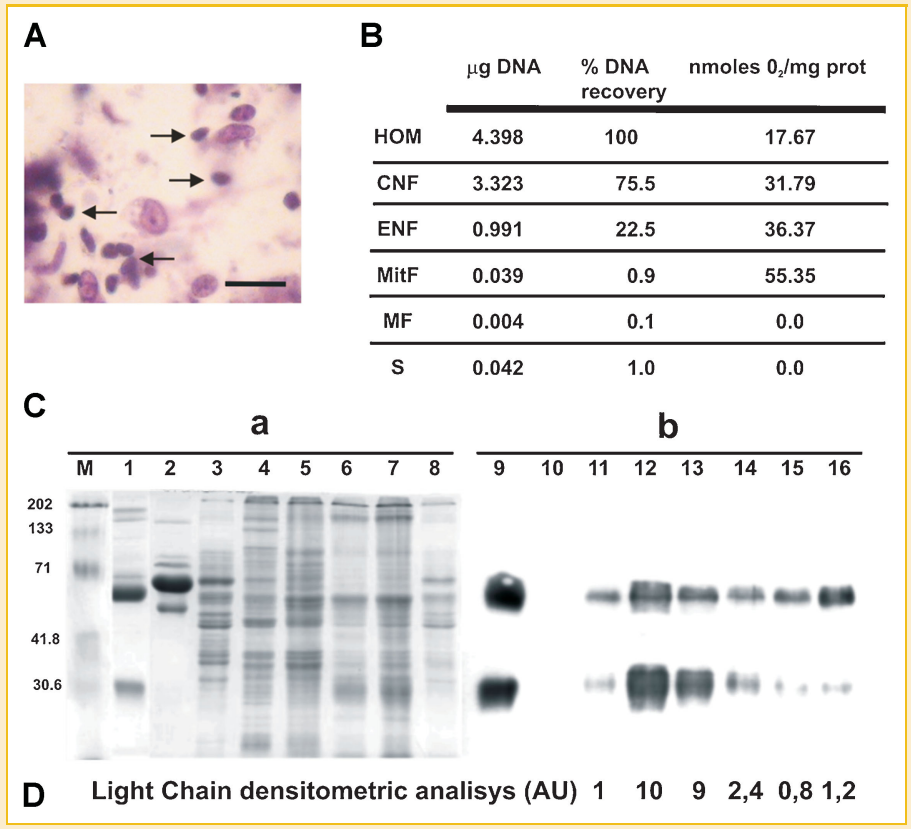

Fig. 5. Bovine IgGs are present in sub-cellular fractions of cells from the inner surface of infertile cysts (GLIC). A: Light microscopy of an enriched purified nuclear fraction obtained from the inner surface of infertile cysts. B: Amount of DNA, DNA recovery, and oxygen consumption from the crude nuclear (CNF), enriched nuclear (ENF), mitochondrial (MitF), microsomal, and supernatant fractions. C: Proteins separated in SDS–12% PAGE, 20 μg per line. Lanes 1 and 9: purified bovine IgGs; lanes 2 and 10: rabbit serum; lanes 3 and 11: homogenate from the inner surface of infertile cysts; lanes 4 and 12: Crude Nuclear Fraction; lanes 5 and 13: Enriched Nuclei Fraction; lanes 6 and 14: Mitochondrial Fraction; lanes 7 and 15: Microsomal Fraction; lanes 8 and 16: Supernatant. a: Coomassie blue stain. b: Identification of bovine IgGs by Western blot, using an Ab anti-bovine IgG HRP conjugated and developed by chemiluminescence. D: Densitometric analysis of the IgG light chain. Light IgG chain quantity present in the homogenate (lane 11) was used as a reference (1 AU) in arbitrary units. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

immune response of the host against the parasite larval form, the hydatid cyst.

Regarding the cellular immune response, in established cysts eosinophils, neutrophils, macrophages, and fibrocytes are the main cellular types found in the outer wall, without a noticeable inflammatory response [Zhang et al., 2003].

As other helminths, *E. granulosus* induces a balance between the immune T_H1 and T_H2 responses [Cardozo et al., 2002]. Nevertheless, in those cysts that continue their growth, $CD8^+$ lymphocytes predominate in the pericystic region, whereas in cysts of regressive type lymphocytes $CD4^+$ predominate, associated with abundant eosinophilic infiltration, probably related with the destruction of the laminar layer [Sakamoto and Cabrera, 2003]. These responses of the immune cellular system mediate the growth or regression of hydatid cysts, showing that the cellular immune system, with the innate immune system, play a role in the formation of progressive or regressive cysts. Thus, type T_H2 responses are directly related to susceptibility to the infection, while T_H1 answers are correlated with protective immunity [Ortona et al., 2003; Amri et al., 2009].

Additionally, a protein from the tegument of the cyst has been described (EgTeg) that would participate in creating a favorable anti-inflammatory environment for the parasite; thus, EgTeg significantly inhibits chemiotaxis of polymorphonuclear cells to the pericystic region [Ortona et al., 2005]. On the other hand, in natural infections antigen B of *E. granulosus* takes part in early events, inhibiting the recruitment of neutrophils and directing the immune response to a non-protective T_H2 type [Rigano et al., 2007].

In this work, we have confirmed the presence of a humoral response to the cyst. Specifically, we have determined the presence of IgGs in germinal layer of both fertile and infertile cysts. These results are of importance considering that the germinal layer is the structure in which protoscoleces are formed [Thompson, 1976; Galindo et al., 2002; Martinez et al., 2005; Galindo et al., 2008]. Interestingly, the inner surface of infertile hydatid cysts presents a higher concentration of IgGs in their germinal layer than fertile cysts and the affinity of these IgGs to this structure is compatible with an antigen–antibody specific binding.

Considering that cyst viability depends on the capacity of the germinal layer to produce protoscoleces, we propose that the higher concentration of IgGs in the germinal layer of infertile cysts as well as their high affinity to this parasitic structure is associated with infertility. If this is the case and taking into account that protoscolex

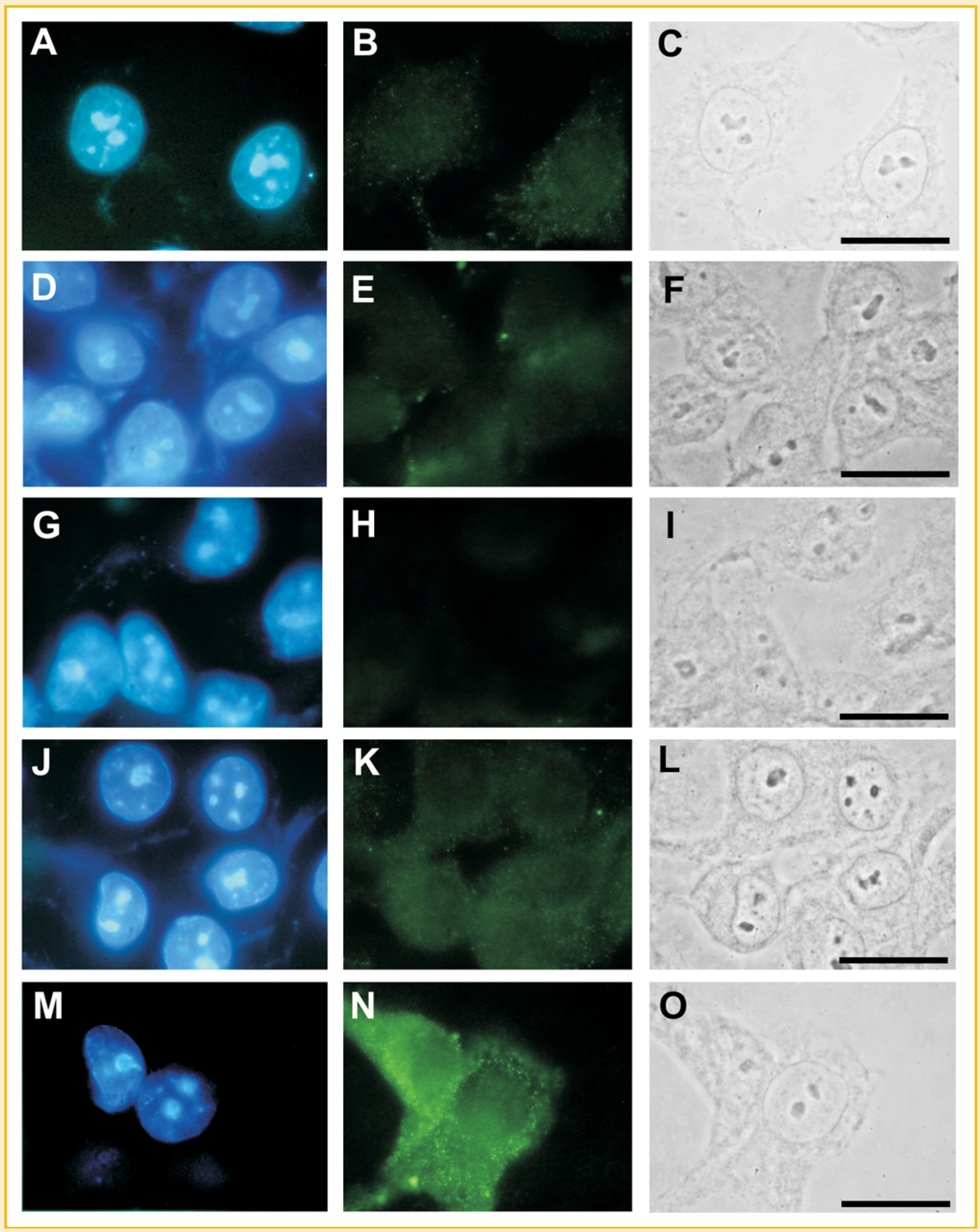

Fig. 6. Bovine IgGs with high affinity to the germinal layer of infertile cysts (GLIC) penetrate HeLa cells. 100 μg/ml IgGs purified with Protein G from proteins extracted with different NaCl concentrations was incubated with HeLa cells for 2 h at 37°C in 5% CO_2. A, D, G, J, and M: Hoescht nuclei stain. B, E, H, K, and N: IgGs identified by indirect immunofluorescence (green). C, F, I, L, and O: Phase contrast images. Bar: 10 μm. A–C: IgGs purified from normal bovine serum; D–F: IgGs purified from proteins extracted with 0.154 M NaCl from the inner surface of fertile cysts; G–I: IgGs purified from proteins extracted with 0.154 M NaCl from the inner surface of infertile cysts; J–L: IgGs purified from proteins extracted with 1 M NaCl from the inner surface of infertile cysts; M–O: IgGs purified from proteins remaining in the sediment after extraction with 3 M NaCl from the inner surface of infertile cysts. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

formation is based on cell proliferation at the germinal layer [Galindo et al., 2002, 2003; Martinez et al., 2005], specific IgGs may be inhibiting this process. For example, anti-U1snRNP antibodies of the IgG class penetrate into a subset of human T lymphocytes, induce arrest of the cycle in the G0/G1 phases, and ultimately trigger active cell death [Ruiz-Arguelles and Alarcon-Segovia, 2001]. Penetration of antibodies modify cell functions, arrest the progression of cell cycle, abrogate the expression of some genes,

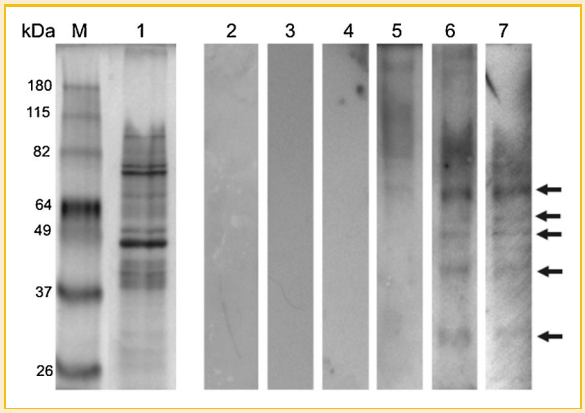

Fig. 7. IgGs extracted from the germinal layer of infertile hydatid cysts recognize parasitic proteins. Protoscolex proteins were challenged against IgGs extracted from the germinal layer of fertile and infertile cysts. The antigen–Ab complexes were recognized by Western blot using as primary antibody IgGs (dilution 1:1,000 v/v) purified with Protein G from each germinal layer NaCl extract. As a secondary Ab bovine IgG HRP conjugated (dilution 1:10,000 v/v) was used and the antigen–Ab complexes were developed by chemiluminescence. M: molecular weight standards. Lane 1: protoscolex proteins in SDS–PAGE silver stained. Protoscolex proteins recognized by IgGs extracted in PBS (lane 3) or in 1.0 M NaCl (lane 4) from the germinal layer of fertile cysts. Protoscolex proteins recognized by IgGs extracted in PBS (lane 5), in 1.0 M NaCl (lane 6) and in 3.0 M NaCl (lane 7) from the germinal layer of infertile cysts. Arrows: molecular weight of parasitic proteins recognized by IgGs from infertile cysts. A negative control using only the secondary Ab is shown in lane 2.

and cause cell death through apoptosis [Alarcon-Segovia et al., 1996a].

On the other hand, proliferating cells at the germinal layer are distributed in patches and from these aggregates of dividing cells protoscoleces are formed [Galindo et al., 2003; Martinez et al., 2005]. Interestingly, hydatid cyst infertility was related with the presence of programmed cell death at the germinal layer and apoptotic cells were also distributed in patches [Paredes et al., 2007]. Specific IgGs may be thus directed towards proliferating cells inducing an apoptotic process leading to cyst infertility. It is also possible that IgGs alter and block the cellular differentiation process leading to protoscolex formation, as was previously described [Galindo et al., 2002; Martinez et al., 2005].

To interfere in these processes, host IgGs should be able to penetrate inside cells. A subfamily of IgGs do penetrate living cells, recognizing specific antigen epitopes and inducing cell death [Alarcon-Segovia et al., 1978, 1996b; Seddiki et al., 2001]. The entrance of those IgGs in the cells involves, among others, presence of calreticulin at the cell surface and the translocation of the calreticulin–IgG complex to a nuclear or perinuclear area [Seddiki et al., 2001]. Indeed, we have previously found this chaperone protein in *E. granulosus* in a perinuclear location [Cabezon et al., 2008]. Bovine IgGs showing high affinity for the inner surface of infertile hydatid cysts penetrate HeLa cells, showing a perinuclear location and these IgGs are found in subcellular fractions of the inner surface of infertile hydatid cysts, mostly in a nuclear enriched fraction. Furthermore, this subfamily of IgGs recognizes specific antigens in protoscoleces (see Fig. 7).

In synthesis, we propose that bovine infected with *E. granulosus* that develop to the larval, cystic form induce a humoral immune response represented by IgGs that cross the tegument and plasma membrane present between the laminar and the germinal layers of the cyst. In this last structure, these IgGs recognize specific antigens involved in the cell proliferation process and/or in the differentiation mechanisms leading to protoscolex formation. This antigen–antibody interaction may inhibit cell proliferation and/or cell differentiation involved in the formation of buds and protoscoleces and may induce apoptosis leading to cyst infertility. This proposal is a matter of further studies.

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