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Fc-binding molecules specific for human IgG1 and IgG3 are present in *Echinococcus granulosus* protoscoleces

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SUMMARY

In this work we describe the presence of Fc-binding activity on the suckers and tegument of E. granulosus *protoscoleces. A fraction (PSA-Fc$^+$) from protoscolex somatic antigens was isolated by affinity chromatography on human Fc-γ1-Sepharose. Analysis by SDS-PAGE of PSA-Fc$^+$ showed that it contained two major components. Using mouse F(ab')2-human Fc chimaeric monoclonal antibodies we verified that PSA-Fc$^+$ bound mainly to human Fc-γ1 and Fc-γ3 isotypes. In addition, one of the components of PSA-Fc$^+$ showed proteolytic activity. Both, Fc-binding and proteolytic activities localized on the protoscolex surface, may play a relevant role in the host-parasite interaction.*

Keywords E. granulosus, *hydatid disease, parasite Fc-receptors, host-parasite interaction*

INTRODUCTION

The larval stage of *E. granulosus* causes hydatid disease which is a worldwide distributed zoonosis (McManus & Smyth 1986). The immune response of infected hosts is unable to eliminate the parasite which produces a long lasting infection. It has been proposed that one evasive mechanism could be the adsorption of host proteins on the parasite surface, which would thus help to disguise parasitic antigens and prevent binding of specific antibodies (Kalinna & McManus 1993). Indeed, immunoglobulins non-specific for parasite antigens were found on the surface of the bloodfluke *Schistosoma mansoni* (Kemp, Merrit, Bogucki *et al*. 1977, Kemp, Merrit & Rosier 1978, Tarleton & Kemp 1981, Auriault, Ouassi, Torpier *et al*. 1981), the cestodes *Taenia solium* (Flisser, Espinoza, Tovar *et al*. 1986), *Taenia crassiceps* (Kalinna & McManus 1993) and *Taenia taeniaeformis* (Kwa & Liew 1978), as well as within the genus Echinococcus on *E. multilocularis* (Alkarmi, Alshakarchi & Behbehani 1988). The results obtained in all those cases suggest that the immunoglobulins are adsorbed on the parasite surface through interactions with Fc-receptors (Kemp *et al*. 1977). Parasite Fc-binding molecules might be involved in some of the immune evasion strategies either by masking parasite antigens or by impairing Fc-mediated effector mechanisms of defence. In this work we report the presence of Fc-binding molecules on the surface of *E. granulosus* protoscoleces which react mainly with human Fcγ1 and Fcγ3 and also present proteloytic activity.

MATERIALS AND METHODS

Protoscolex somatic antigen

Protoscoleces were obtained by aseptic puncture of fertile bovine hydatid cysts as previously described (Baz, Hernández, Dematteis *et al*. 1995). Protoscoleces viability was

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determined by eosin exclusion (Robinson & Arme 1985) and flame cell activity (Gurri 1963). Protoscolex somatic antigen (PSA) was prepared by sonication of viable protoscoleces (Míguez, Baz & Nieto 1996) in the presence of 2 mM phenylmethylsulphonyl fluoride (PMSF, Sigma St. Louis, MO, USA) and 10 mM EDTA (Marco & Nieto 1991). Protein concentration of PSA preparations was determined with the bicinchoninic acid reagent (BCA, Pierce, Rockford, USA).

Rabbit anti-peroxidase antibodies

An adult New Zealand rabbit was primed and boosted with 400 μg and 200 μg of peroxidase (HRP, Sigma) respectively, following standard protocols. Blood samples were taken according to the stipulated guidelines on removal of blood from laboratory mammals (Morton, Abbot, Barcalay *et al*. 1993). Specific antibodies were purified by affinity chromatography on Sepharose-immobilized HRP.

Detection of Fc-binding activity on *Echinococcus granulosus* viable protoscoleces

Protoscoleces were incubated overnight, at 4° or 37°C, with rabbit anti-HRP immunoglobulins. After washing, protoscoleces were incubated with 0·1% H_2O_2 for 15 min to block endogenous peroxidase activity. Following, a solution of 10 μg/ml of HRP was added and incubated for one h at room temperature. After several washings, parasites were incubated with di-amino benzidine (Sigma) in 0·05 M Tris buffer, pH 7·6 containing 0·01% H_2O_2. Additional aliquots of protoscoleces were incubated with HRP to detect non-specific binding of the enzyme to the parasite surface.

Detection of Fc-binding activity on histological sections of *Echinococcus granulosus* protoscoleces

Viable protoscoleces fixed in 10% buffered formalin were embedded in paraffin following standard protocols. Sections (5 μm) were deparaffined, rehydrated, treated with 0·1% H_2O_2 for 15 min and then incubated overnight at 4°C with rabbit anti-HRP antibodies. After incubation with HRP and development as described above sections were counter stained with haematoxylin. Controls were made by incubation of correlative sections either with HRP followed by DAB and H_2O_2 or with substrate alone.

Purification of Fc-binding molecules from protoscolex somatic antigens

Protoscolex Fc-binding molecules were purified by affinity chromatography using a protocol previously described (Kalinna & McManus 1993) with some modifications. Briefly, PSA was adsorbed overnight at 4°C onto bovine serum albumin immobilized to Sepharose-4B. The non-bound fraction was then incubated overnight at 4°C with human-γ1-Fc-fragment coupled to CNBr-Sepharose-4B (Fcγ1 fragments obtained from a patient with γ heavy chain disease were kindly provided by Dr F. Goñi, Immunology Department, Faculty of Chemistry, Montevideo, Uruguay). The retained fraction (PSA-Fc$^+$) was eluted, dialized against Milli-Q water and freeze-dried.

Sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE)

The fraction PSA-Fc$^+$ was reconstituted in phosphate buffered saline (PBS) and analysed by sodium dodecyl sulphate-7% polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) in a Phast-system electrophoresis equipment (Pharmacia, Sweden) under non-reducing conditions. After electrophoresis, silver staining was performed following the manufacturer's recommendations (Phast System, Pharmacia).

ELISA for detection of Fc-binding activity in PSA-Fc$^+$

We designed an ELISA based on an immunodot protocol previously described to detect soluble Fc-receptors (Kristoffersen, Matre, Ulvestad *et al*. 1994). Briefly, microtitre plates (NUNC, Roskild, Denmark) were coated with PSA-Fc$^+$ or with PSA, and blocked with 1% bovine serum albumin-PBS. Serial dilutions of rabbit anti-HRP antibodies or normal rabbit immunoglobulins were incubated overnight at 4°C. After washings, a solution of 10 μg/ml of HRP was dispensed and incubated for 90 min at room temperature. The plates were then washed and the substrate solution containing 3-methyl 2-benzothiazolinone hydrazone hydrochloride; 3-dimethyl-aminobenzoic acid and H_2O_2 (Sigma) (Ngo & Lenhoff 1980) was added. Optical densities at 600 nm (OD 600) were measured in a Titertek Multiskan Plus Reader (Labsystem Flow Laboratories, Helsinki, Finland). Non-specific adsorption of HRP was simultaneously determined following the above protocol but rabbit immunoglobulins were substituted by 1% BSA in PBS-0·05% Tween.

Binding of PSA-Fc$^+$ to different human Fc isotypes

Microtitre plates were sensitized with PSA-Fc$^+$ and blocked with BSA. After washings, plates were incubated overnight at 4°C with serial dilutions of chimaeric antibodies (mouse F(ab′)2-human Fc) of isotypes γ1, γ2, γ3, γ4, ϵ or α2 specific for the 5-iodo-4-hydroxyl-3-nitrophenacetyl hapten

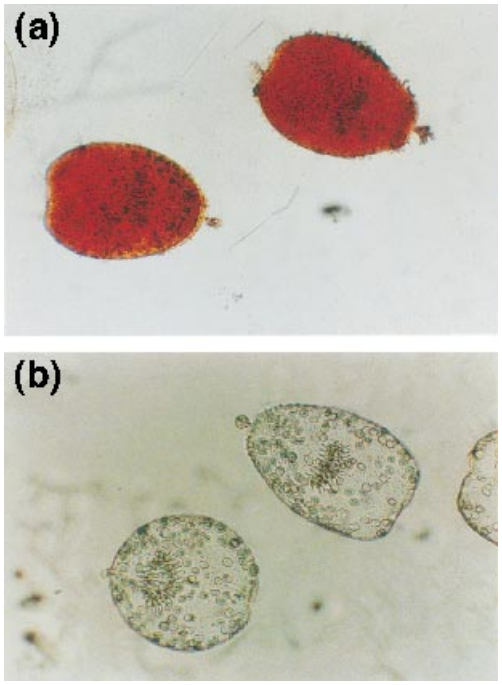

Figure 1 Viable protoscoleces were incubated with rabbit anti-HRP antibodies (A) or with PBS-T (B) overnight at 4°C. After washings, HRP was added and incubated for 1 h at room temperature followed by addition of DAB and H_2O_2. Magnification ×40.

(NIP) (Dunne, Richardson, Jones *et al*. 1993). An appropriate dilution of $F(ab')_2$-rabbit anti-mouse antibodies biotin labelled (Sigma) was added and incubated for 90 min at room temperature, followed by incubation with extravidin-peroxidase (Sigma) for 90 min at room temperature. Reaction was developed as was described above and OD 600 was determined.

Detection of proteolytic activity of Fc-binding molecules from protoscolex somatic antigens

Proteolytic activity of PSA-Fc^+ and PSA was determined in 7·5% (w/v) acrylamide gels containing 0·2% (w/v) gelatin as substrate as previously described (Marco *et al*. 1991).

RESULTS

Localization of Fc-binding activity on protoscoleces

Three independent experiments showed that rabbit anti-HRP immunoglobulins bound to protoscoleces while maintaining the ability to recognize HRP (Figure 1). Interestingly, we observed a positive reaction when the antibodies and the protoscoleces were incubated at 4°C but not at 37°C. The putative Fc-binding molecules were localized in the parasite tegument and suckers (Figure 2).

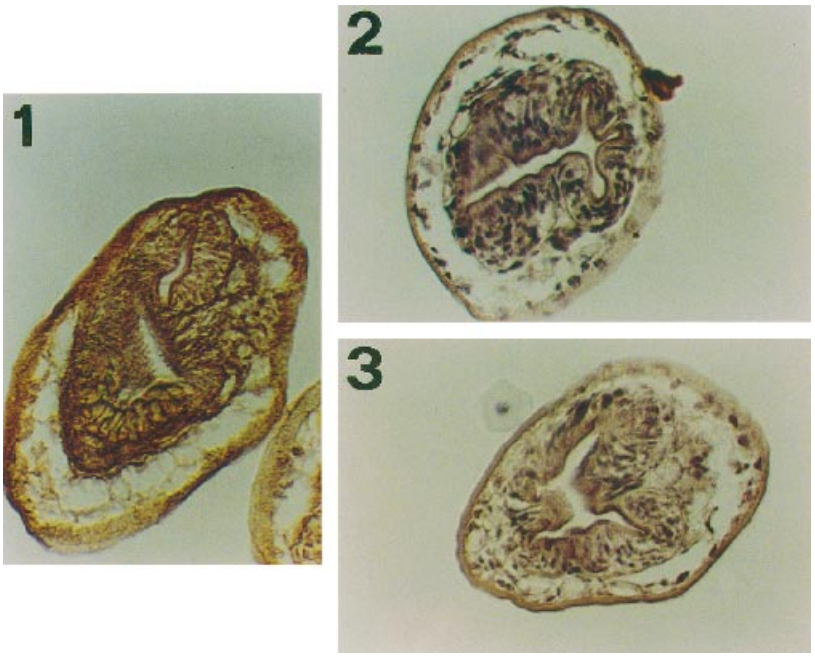

Figure 2 Paraffin embedded 5 μm section of *E. granulosus* protoscoleces incubated with rabbit anti-HRP antibodies (1) or with PBS-T (2) and (3), overnight at 4°C. After washings, a solution of HRP was added to slides (1) and (2) and incubated for 1 h at room temperature. The three slides (1), (2) and (3) were then exposed to DAB and H_2O_2 and counter stained with haematoxilin. Magnification ×40.

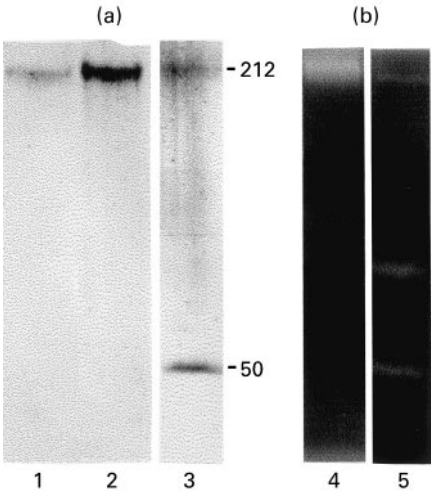

Figure 3 Molecules with Fc-binding activity (PSA-Fc$^+$) resolved by sodium dodecyl sulphate-7·5% polyacrylamide gel electrophoresis and silver stained (a). Three different PSA-Fc$^+$ fractions obtained from different batches of protoscolex somatic antigens (PSA) as starting material (lanes 1, 2 and 3). Proteolytic activity of PSA and PSA-Fc$^+$ detected by SDS-PAGE on gels containing gelatine (b). The component of PSA-Fc$^+$ that has less mobility shows an intense proteolytic activity (lane 4). On lane 5 the proteolytic activity pattern of whole PSA is shown.

Purification of Fc-binding molecules from protoscolex somatic antigens

Human-Fcγ1-binding molecules from protoscolex somatic antigens showed two major peptidic components (Figure 3). Only those two components were observed in several PSA-Fc$^+$ fractions independently prepared, however, the relative proportion of these components varied with the batch of protoscolex somatic antigens used (Figure 3).

Detection of Fc-binding activity of PSA-Fc$^+$ by ELISA

Results from six different preparations of PSA-Fc$^+$ showed that they were all able to bind rabbit anti-HRP antibodies and that these antibodies retained their ability to specifically react with HRP (OD600 was between 1·3 and 0·5 for PSA-Fc$^+$ at an antibody concentration of 50 μg/ml). On the contrary, binding of rabbit immunoglobulins to crude protoscolex somatic antigens was not detected (OD 600 was lower than 0·05 at an antibody concentration of 50 μg/ml).

Binding of different human Fc isotypes to PSA-Fc$^+$

To investigate whether different classes and subclasses of antibodies could equally bind to PSA-Fc$^+$ we used chimaeric antibodies with identical mouse (F(ab′)$_2$ portions (specific to NIP) and human Fc regions of different isotypes. Results

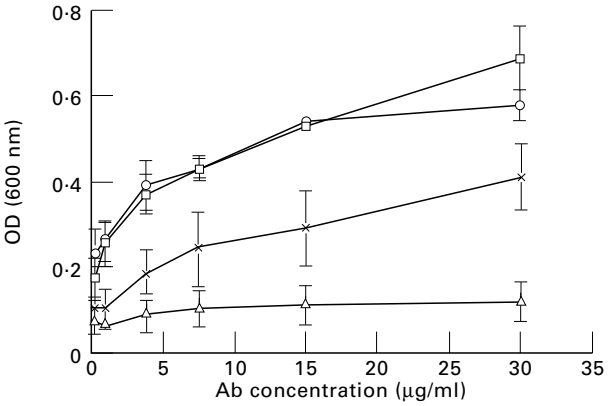

Figure 4 Three different preparations of PSA-Fc$^+$ were tested for their ability to recognize human Fc-regions of different isotype. Plates were coated with PSA-Fc$^+$, blocked and incubated with different concentrations of anti-NIP chimaeric antibodies mouse F(ab′)2-human Fc of isotypes γ1, γ2, γ3, γ4, ϵ or α2. As second antibody rabbit anti-mouse-F(ab′)2 biotinylated antibodies were used and plates were later incubated with extravidin-peroxidase. Reaction was developed by adding DMAB, MBTH and H_2O_2 and optical density at 600 nm (OD 600) was measured. Values of OD 600 corresponding to each PSA-Fc$^+$ batch *vs* the concentration of each chimaeric antibody are represented, except for antibodies with ϵ and α2 isotypes since OD 600 were very low to be plotted. □ IgG1; △ IgG2; ○ IgG3; × IgG4.

depicted in Figure 4 show that human Fcγ1 and Fcγ3 bound mainly to PSA-Fc$^+$. The other isotypes were less (Fcγ4, Fcγ2) or not recognized at all (Fcϵ and Fcα2) by PSA-Fc$^+$.

DISCUSSION

Numerous reports providing evidence for the presence of Fc-receptors on the surface of multicellular parasites have been reported (Kemp *et al*. 1977, 1978, Kwa & Liew 1978, Auriault *et al*. 1981, Tarleton & Kemp 1981, Flisser *et al*. 1986, Alkarmi *et al*. 1988, Kalinna & McManus 1993). In this work we present evidence of Fc-binding activity on the surface of *E. granulosus* protoscoleces, using an experimental approach similar to those successfully used with other pathogens. Our results show that *E. granulosus* protoscoleces possess surface molecules that selectively bind antibodies through the Fc-region (Figure 1), although we cannot discern whether these Fc-binding molecules are synthesized by the parasite or host-derived. The interaction of rabbit immunoglobulins Fc-region with *E. granulosus* protoscoleces was detected at 4°C but not at 37°C. It was reported that adsorbed immunoglobulins on *S. mansoni* are shed from the surface at 37°C in a very short time (Kemp *et al*. 1977). This same phenomenon could also take place on *E. granulosus* protoscoleces.

Once Fc-binding activity was detected on the protoscoleces, the corresponding molecules were purified by affinity chromatography on immobilized human-Fc fragments from soluble somatic antigens. A similar experimental approach was used to isolate Fc-binding molecules from *T. crassiceps* antigens (Kalinna & McManus 1993). We obtained a fraction (PSA-Fc$^+$) that contained two major components (Figure 3). The relative proportions of these components varied with the batch of protoscolex somatic antigens used to purify PSA-Fc$^+$. These results do not allow us to rule out either the possibility of aggregation of the low molecular weight component or autoproteolysis of the high molecular weight component.

The Fc-binding activity of six different preparations of PSA-Fc$^+$ was confirmed by ELISA. To rule out the possibility that PSA-Fc$^+$ could bind to the antibody molecule through any other region different from the Fc, we used human-mouse chimaeric monoclonal antibodies (Dunne, Richardson, Jones *et al*. 1993). According to our results (Figure 4) PSA-Fc$^+$ bound mainly to Fc-γ1 and Fc-γ3. Lack of binding to other isotypes (Fcϵ and Fcα2) demonstrates that PSA-Fc$^+$ did not interact with the chimaeric antibody through the Fab, since all the chimaeric antibodies were identical in that region. The binding of PSA-Fc$^+$ to Fc-γ1 and Fc-γ3, suggests that PSA-Fc$^+$ could interact with the residues 233 to 237 where IgG1 and IgG3 have identical sequences (Jefferis 1990a). These residues have been proposed to be crucial to the recognition of IgG1 and IgG3 by human Fcγ RI and Fcγ RII. Both subclasses of antibodies have been implicated in many effector functions such as phagocytosis, ADCC, oxidative burst and complement activation (Jefferis 1990b, Wiener 1990, Raghavan & Bjorkman 1996). It is tempting to speculate that PSA-Fc$^+$ could impair the effector functions mediated by IgG1 and IgG3, however further experiments must be performed to demonstrate whether this actually happens *in vivo* and results in any benefit for the parasite survival.

It has been demonstrated that binding of IgG through the Fc-region to *S. mansoni* is followed by the cleavage of the antibody into small peptides (Auriault *et al*. 1981). A similar proteolytic mechanism has been described in *Tetrahymena pyriformis* (Eisen & Tallan 1977). As binding and cleavage of immunoglobulins might also contribute to the immune escape mechanisms of parasites (Auriault *et al*. 1981), we investigated whether the PSA-Fc$^+$ fractions possessed proteolytic activity. Results showed (Figure 3) an intense proteolytic activity associated with the high molecular weight component of PSA-Fc$^+$ but not with the other component. The possibility that the protoscoleces bind and cleave IgG as a mean to acquire nutrients or for other purposes not related to immune evasion should also be taken into consideration.

In conclusion, our results provide evidence for the presence of Fc-binding molecules on the surface of *E. granulosus* protoscoleces. The purification of these molecules yielded a fraction (PSA-Fc$^+$) which contained two components and bound mainly human γ1 and γ3 isotypes. In addition, the high molecular weight component present in PSA-Fc$^+$ had an intense proteolytic activity. Whether these molecules could interfere with the effector functions mediated by IgG1 and IgG3 antibodies and whether they are synthesized by the parasite or host-derived, will be the subject of future investigations.

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Abbreviations: HRP—horse radish peroxidase; Fc—cristallizable fragment of immunoglobulins; Fab—antigen binding fragment of immunoglobulins; PSA—protoscolex somatic antigens; BSA—bovine serum albumin; NIP—5-iodo-4-hydroxyl-3-nitrophenacetyl hapten.

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