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Eosinophil cationic protein damages protoscoleces *in vitro* and is present in the hydatid cyst

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SUMMARY

Eosinophils are locally recruited during the establishment and chronic phases of cystic hydatidosis. This study provides evidence that eosinophil cationic protein (ECP), one of the major components of eosinophil granules, can damage Echinococcus granulosus *protoscoleces (PSC). The toxicity of ECP was investigated* in vitro *by following parasite viability in the presence of this protein. ECP was found to damage PSC at micromolar concentrations; the effect was blocked by specific antibodies and heparin, and was more severe than the one caused by similar concentrations of RNase A, suggesting that the cationic nature of ECP, and not its ribonuclease activity, is involved in toxicity. This observation may highlight the capacity of eosinophils to control secondary hydatidosis, derived from PSC leakage from a primary cyst. To further assess the relevance of the previous result during infection, the presence of eosinophil proteins was investigated in human hydatid cysts. ECP was found to be strongly associated with the laminated layer of the cyst wall, and present at micromolar concentrations in the hydatid fluid. Overall, these results demonstrate that eosinophils degranulate* in vivo *at the host–parasite interface, and that the released ECP reaches concentrations that could be harmful for the parasite.*

Keywords *cestodes,* Echinococcus granulosus*, eosinophil cationic protein, eosinophil, hydatid cyst, protoscolex*

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INTRODUCTION

The larval stage or metacestode of *Echinococcus granulosus* (hydatid cyst) causes cystic hydatidosis in mammalian hosts (livestock animals and humans). One of the main features of this infection is chronicity, which reflects the great ability of the parasite to establish and persist within host viscera (mainly liver and lungs), dealing with host defences. It is currently accepted that, among other adaptation mechanisms, strategies to control host inflammation are essential to achieve a stable host–parasite relationship. Thus, when this control is not reached, inflammation develops towards a granulomatous-type reaction that has been associated with parasite degeneration and death (reviewed by (1)). Eosinophils are one of the cell types present in hydatid granulomas, and some pieces of evidence suggest that they contribute to host defences against the hydatid cyst: (i) eosinophil numbers around cysts from infected bovines were correlated with the degree of cyst degeneration (2), and (ii) mice unable to attract eosinophils via C5a (C5-deficient mice) showed lower eosinophil infiltrates at the early and chronic phases of infection and were more susceptible to infection than C5-sufficient mice (3). Animal models, including IL-5 or IL-5 receptor knock-out mice, have been widely used to assess the contribution of eosinophils to host defences against various helminth parasites (4); however, these models have not been used to examine the role of eosinophils during hydatid infection, nor has the capacity of eosinophils or their toxic products to damage *E. granulosus* been assayed *in vitro*.

Eosinophil cationic protein (ECP) is one of the major components of eosinophilic granules (5–7). It is a small (16–21 kDa, depending on its glycosylation level), extremely cationic protein (pI ≅11) that belongs to the pancreatic RNase A (EC 3·1·27·5) superfamily. ECP has poor ribonuclease activity in comparison with other members of the family; it is 50–100-fold less active than eosinophil-derived neurotoxin (EDN) (8), a related ribonuclease with similar molecular weight but less cationic than ECP (pI ≅9; (5)). The determination of the crystal structure of ECP has revealed

substitutions of amino acid residues in the catalytic subsites consistent with its weak RNase activity (9). ECP is toxic for a variety of pathogens including bacteria (10), single-stranded RNA respiratory syncytial virus (11), and trematode and nematode parasites (reviewed by (12)). Interestingly, although ECP is more toxic for parasites than EDN, the latter is a more potent antiviral agent (13). Comparison of the structure–function relationship between ECP and EDN has led to suggestions that ECP cytotoxicity is associated with its cationic nature rather than its ribonuclease activity (9).

In this study, we investigated the capacity of ECP to damage and kill *E. granulosus* protoscoleces (PSC). We examined the effect of recombinant human ECP (rhECP) on the viability of PSC *in vitro*. This may be relevant in terms of the capacity of eosinophils to limit the establishment of a secondary hydatid infection. In addition, because the ECP released by eosinophil degranulation could penetrate into the cyst and reach the germinal layer and PSC inside the cyst, we looked for evidence of *in vivo* eosinophil degranulation at the host–parasite interface, by determining the presence of eosinophil proteins in walls and hydatid cyst fluid (HCF) recovered from natural infections. Taken together these studies contribute to understand the molecular events that may derive from the inflammatory response against the hydatid cyst.

MATERIALS AND METHODS

Preparation of recombinant human ECP (rhECP) and anti-rhECP antibodies

Human eosinophils were isolated from the blood of healthy donors using differential centrifugation and a magnetic particle-based method of negative selection. Leukocytes were initially separated from erythrocytes and plasma by gravity sedimentation through hetastarch and the eosinophil-enriched fraction was obtained using Percoll (Amersham-Pharmacia, USA) step gradient centrifugation (14). Eosinophils were subsequently purified by negative selection of CD16-positive cells. Briefly, anti-human CD16 mAb (clone B-E16, Biosource International, USA) was incubated with the cells for 20 min at 23°C; then, goat anti-mouse IgG coated magnetic beads (Polysciences Inc., USA) were added to the suspension and mixed for 30 min at 23°C. Finally, eosinophils (> 99% pure as assessed by light microscopy after eosin Y staining) were recovered by centrifugation after separation of CD16-positive cells with a magnetic device (15).

The cDNA encoding ECP was amplified by RT-PCR from total eosinophil RNA (isolated with Trizol, Gibco-BRL, USA) using forward 5′-GGG ATC CAC AGA CCC CCA CAG TTT ACG AGG GCT CAG-3′ and reverse 5′-G GTC GAC TTA GAT GGT GGT ATC CAG GT-3′ primers. The PCR product was purified and initially cloned into pCR II (Invitrogen, USA); it was then subcloned into the expression plasmid pQE32 (Qiagen, USA), which encodes a hexahistidine at the N-terminus of the cloned product. The recombinant plasmid was used to transform *Escherichia coli* (strain JM 101). Bacteria were grown, induced for expression with 0·1 M isopropyl thiogalactoside, harvested by centrifugation, and lysed by sonication and freezing with thawing in 10 mM imidazole pH 7·4, 0·3 M NaCl, 10 mM EDTA, 2 mM benzamidine, 0·1% Triton X-100. Inclusion bodies containing the rhECP were recovered by centrifugation at 7000 ***g*** for 10 min. The pellet was suspended in 10 mM HEPES, 1 mM MES pH 6·5, 0·15 M NaCl, 8 M urea and centrifuged to remove insoluble material. rhECP was purified using metal ion affinity chromatography on nickel-charged tris(carboxymethyl)ethylenediamine-sepharose (16). rhECP was eluted with 40 mM sodium acetate pH 4·5, 6 M guanidine-HCl and dialysed against 0·5 M KCl, followed by HEPES-buffered saline (HBS; 10 mM HEPES, 0·15 M NaCl; pH 7·3).

A rabbit anti-ECP antiserum was prepared with the purified protein by Strategic Biosolutions (USA). New Zealand white rabbits were inoculated three times with rhECP (200 µg protein) in adjuvant over a 6-week period. At the end of the protocol, the animals were euthanased, the serum was collected and the IgG fraction purified by 33% ammonium sulphate precipitation, followed by chromatography on DEAE-sephacel in 0·15 M sodium bicarbonate. Anti-rhECP IgG (1 mg/mL in 0·15 M $NaHCO_3$, pH 9·0) was biotinylated by incubation with biotinamidocaproate N-hydroxysuccinimide ester (120 µg/mg of protein) for 4 h at room temperature. The biotinylated IgG was dialysed against phosphate-buffered saline (PBS; 0·01 M phosphate, 0·15 M NaCl; pH 7·2) overnight at 4°C and stored at –20°C.

Parasite material

Echinococcus granulosus PSC were obtained from fertile cysts in lungs and livers of naturally infected cattle (Uruguay) and processed under aseptic conditions. Briefly, PSC were recovered by aspiration of the cyst content, concentrated by sedimentation and washed extensively with PBS followed by culture medium (RPMI 1640, 10 mM HEPES, 2 mM glutamine, 10 mM pyruvate, 1·5 g/L sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% foetal calf serum). They were cultured in the same medium at 37°C and 5% CO_2. Only PSC batches with an initial viability of at least 95% as measured by eosin exclusion were used. For eosin exclusion, equal volumes of PSC suspension and 1% (w/v) eosin solution (Fluka Chemie AG, Sweden) were mixed, and the parasites observed immediately under the optical microscope (Olympus BH-2, Olympus America Inc., USA).

Echinococcus granulosus hydatid cyst walls of human origin (hHCW) were obtained from hepatic cysts surgically

removed from four patients (Hospital Militar, Montevideo, Uruguay). Cysts were classified according to morphological features, determined by imaging techniques, following the criteria standardized by the World Health Organization (WHO, http://www.medicalweb.it/aumi/echinonet/echinonews97). Due to surgical procedures, the HCF could be recovered only from two non-calcified cysts. hHCW were washed with sterile PBS, and either processed immediately or stored at –20°C until use.

Evaluation of the toxicity of rhECP for PSC

PSC (15–25) were incubated at 37°C with 100 μL of rhECP in HBS (3, 10 and 37·5 μM) or buffer as a control and their integrity assessed after 3, 6 and 72 h by following tegument morphology and viability (determined through eosin exclusion, motility and activity of flame cells, (17)). Microscopic images were captured using the Image-Pro plus program, 4·0 version (Media Cybernetics, USA).

The involvement of rhECP in PSC damage was assessed by comparing parasite integrity after incubation with the protein in the absence or presence of anti-rhECP IgG. Briefly, PSC (15–25) were incubated for 3 h with 100 μL of a solution containing rhECP (40 μM) and different concentrations of anti-rhECP IgG (10, 50 and 200 μM). After incubation, parasite damage was evaluated as described above. Similarly, the relevance of ECP basic residues for toxicity was studied by comparing parasite integrity after incubation for 3 h with rhECP (40 μM) in the absence or presence of heparin (40 and 150 μM; Laboratorios Galien S.A., Uruguay). The participation of ribonuclease activity in ECP toxicity was evaluated under identical conditions using bovine pancreatic RNase A (Ambion Inc., USA).

Preparation of extracts from hHCW

Fractionation of hHCW was performed using a previously described multistep procedure (18), with slight modifications. This allows discrimination of weakly adsorbed host macromolecules (including plasma components such as albumin) from those that are strongly bound to HCW. In brief, each hHCW was washed (30 min) with PBS containing protease inhibitors (PI: 1 mM phenyl methyl sulphonyl fluoride, 0·5 mM iodoacetamide, 5 mM EDTA, 1 mM benzamidine). The wash was centrifuged (1 h, 20 000 ***g***, 4°C) to separate the supernatant (PBS_w) and the pellet was sequentially extracted (30 min, 4°C), firstly with 0·05% Tween-20 in PBS-PI, and then with 2% Triton X-100 in PBS-PI. The Tween (Tw_E) and Triton (T_{E1}) extracted materials were separated by centrifuging as before. The pellet was frozen and pulverized with a mortar and pestle under liquid nitrogen. The homogenate was thawed and extracted with PBS-PI for 10 min. After centrifugation (30 min, 20 000 ***g***), a supernatant called PBS_{E1} was obtained. This step was repeated twice to recover supernatants PBS_{E2} and PBS_{E3}. The remaining insoluble material was divided into three aliquots, which were extracted (45 min) with either: (i) 2% Triton X-100 in PBS-PI, (ii) 1 M guanidinium hydrochloride in 0·2 M Tris, pH 8·0, containing PI, or (iii) 2 M NaCl. The solubilized material (designated T_{E2}, Gua_E and $NaCl_E$, respectively) was recovered after centrifugation, leaving a small residue behind. Extracts were extensively dialysed against PBS containing 5 mM EDTA and stored at –20°C. The protein concentration of the extracts was determined by the bicinchoninic acid assay (Pierce, USA), using bovine serum albumin as a standard.

Western blot analysis of ECP/EDN

The presence of ECP/EDN in HCW extracts was analysed by Western blot using anti-rhECP IgG. Samples were run by SDS-PAGE (10% polyacrylamide gels, reducing conditions) according to Laemmli (19). When necessary, they were concentrated by precipitation with trichloroacetic acid (18) before electrophoresis. Gels were stained with silver nitrate (20) or transferred to nitrocellulose membranes at 0·8 mA/cm^2 for 1 h, using a semidry electrotransfer system. Blots were blocked overnight at 4°C in PBS containing 0·1% Tween-20 and 5 mM EDTA, and then incubated with anti-rhECP IgG (10 μg/mL in PBS, 0·05% Tween-20, 0·05% gelatin, PBS-TG) for 90 min at 37°C. They were washed with PBS, 0·05% Tween-20 and incubated for 1 h at 37°C with anti-rabbit IgG-alkaline phosphatase conjugate (diluted 1 : 5000 in PBS-TG; Calbiochem, Novabiochem, USA). After washing, blots were developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma, USA).

Quantification of ECP/EDN by capture ELISA

Microplates (Nunclon, Denmark) were coated overnight at 4°C with 100 μL/well of anti-rhECP IgG (10 μg/mL in 0·1 M sodium carbonate pH 9·6) and blocked with 1% gelatin in PBS for 1 h at 37°C. Duplicates of the samples (undiluted or diluted at 1 : 2 to 1 : 100 in PBS-TG) and rhECP (8–300 ng/mL in the same buffer) were incubated for 1 h at 37°C. After washing, plates were incubated overnight at 4°C with an adequate dilution in PBS-TG of biotinylated anti-rhECP IgG, washed and incubated with 1 : 2000 dilution in PBS-TG of streptavidin-peroxidase (Sigma) for 1 h at 37°C. They were developed using 2,2′-azino-bis[ethylenbenzothiazoline-6-sulphonic acid] (Roche Diagnostics, Germany), and the optical density (OD_{415}) was measured after 15 min in a Labsystems Multiskan MS ELISA reader. The detection limit of the assay was 10 ng/mL of ECP. The content of ECP/EDN was calculated for each hHCW by adding the ECP/EDN content of extracts obtained after pulverization of

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HCWs to exclude any plasma-derived ECP/EDN. The ECP/EDN content for each hHCW was normalized by the total extracted protein.

Isolation of ECP and EDN using heparin-coated acrylic beads

This was carried out according to the method of Gleich *et al.* (21). Two mL of heparin acrylic beads (Sigma) were equilibrated with 20 mL of 10 mM phosphate pH 7·2, containing 75 mM NaCl. The suspension was centrifuged (100 ***g***, 5 min), and the beads were incubated with a solution of the sample for 1 h at 37°C. After centrifugation, the supernatant was separated, and the beads were washed with equilibration buffer. EDN and ECP were sequentially eluted using 0·5 M and 2 M NaCl, respectively. Each eluate was extensively dialysed against PBS and analysed by Western blotting.

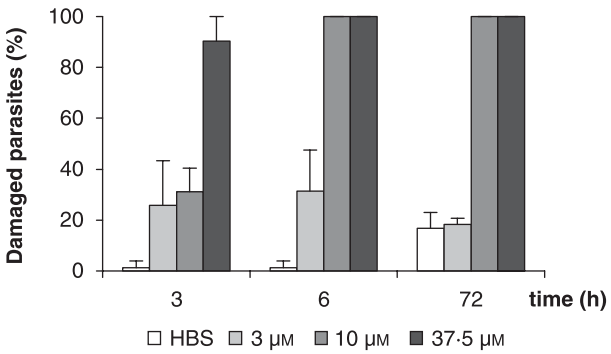

Figure 1 Dose-dependent damage of recombinant human eosinophil cationic protein (rhECP) against protoscoleces (PSC). PSC were incubated with rhECP at 0 μM, 3 μM, 10 μM and 37·5 μM in HEPES-buffered saline (HBS). PSC damage was determined after 3, 6 and 72 h on the basis of tegument integrity and parasite viability. Results are shown as means (± SE) of three independent experiments.

RESULTS

rhECP damages *E. granulosus* PSC *in vitro*

rhECP was capable of damaging PSC in a dose-dependent manner (Figure 1). At 3 μM, it caused punctuate alterations in the tegument, but did not kill the parasites (Figure 2); interestingly, these alterations were reversible, disappearing after 72 h (not shown). Higher concentrations of rhECP (10 and 37·5 μM) were lethal, and the effect was more severe and rapid at 37·5 μM. At the highest concentration assayed, rhECP led to a complete disintegration of the parasite, initiated by the formation of bubbles in the tegument and followed by the release of material, including PSC hooks (Figure 2c,f). Evaginated and invaginated PSC were equally susceptible to damage, with tegumental alterations developing mainly at the posterior region of both forms. Equimolar or higher concentrations of anti-rhECP significantly (~60%) reduced PSC damage (Figure 3a). The relevance of basic

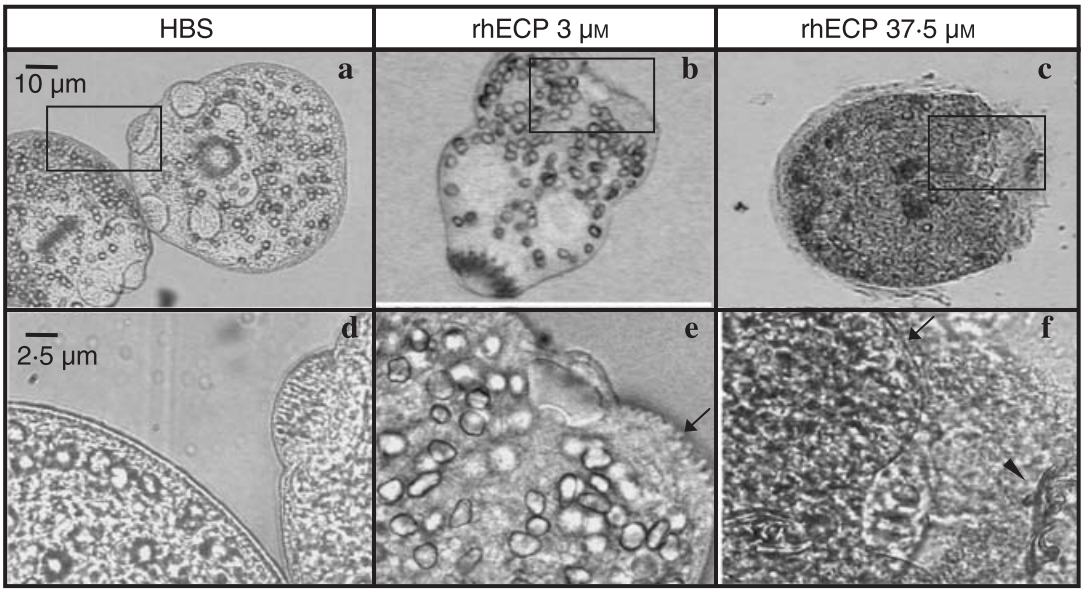

Figure 2 Changes in PSC morphology induced by rhECP. PSC were incubated for 6 h with buffer (HBS, a, d), rhECP at 3 μM (b, e), or 37·5 μM (c, f) in HBS. The arrows show alterations in PSC tegument and the arrowhead shows a hook released from a disintegrated PSC. The bottom panels include a higher magnification of a section of the upper ones, as indicated.

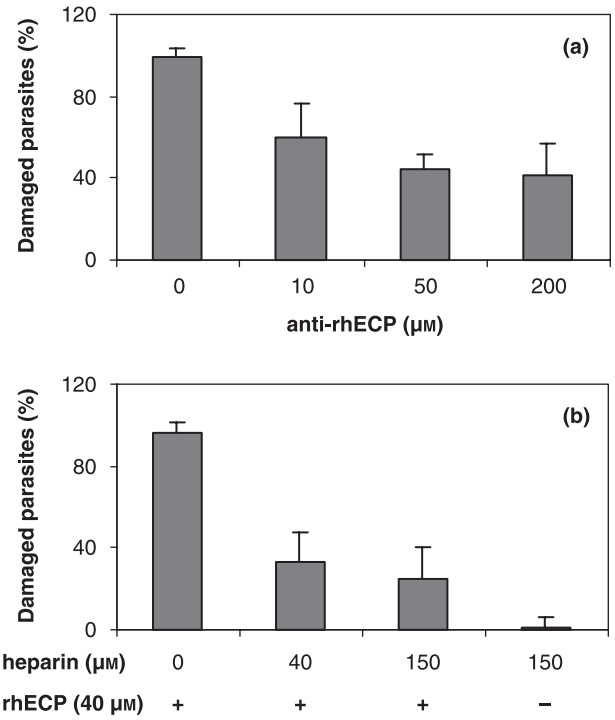

Figure 3 Effect of specific antibodies and heparin on rhECP damage to PSC. PSC were incubated with rhECP (40 μM) in the presence of various concentrations of anti-rhECP IgG (a) or heparin (b). Damage was determined after 3 h on the basis of tegument integrity and parasite viability as described in Materials and Methods. Results correspond to means (± SE) of two independent experiments.

residues for ECP toxicity was studied by co-incubation with heparin, whose binding to ECP is known to block its toxicity (reviewed by (12)). The presence of equimolar or higher concentrations of heparin did lead to ~70% reduction of parasite damage (Figure 3b), suggesting that the cationic nature of ECP is involved in toxicity against PSC. In addition, the effects induced by RNase A on PSC were less severe than those provoked by rhECP. RNase A increased PSC permeability to eosin only when it was used at 37·5 μM, and this effect was not associated with the formation of tegument bubbles nor with parasite disintegration, even after 72 h incubation (data not shown), indicating that the ribonuclease activity of ECP would not play a major role in PSC damage.

Eosinophil proteins bind to the HCW during natural infections

The presence of ECP in hHCW extracts was examined by Western blotting and ELISA using an anti-rhECP antibody. This antibody was found to cross-react with human EDN (Figure 4). Similar blots were obtained for the extracts from all four HCW; a representative one is shown in Figure 5a.

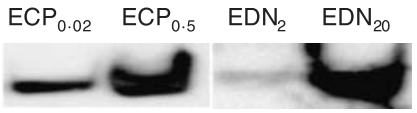

Figure 4 Eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) reactivity with anti-rhECP. Unreduced samples of recombinant human ECP (0·02 and 0·5 μg) and EDN (2 and 20 μg) were electrophoresed on 9% polyacrylamide gels, transferred to a nitrocellulose membrane, and probed with rabbit anti-rhECP antibody.

ECP and/or EDN were indeed present in hHCW: a band reacting specifically with anti-rhECP and migrating as rhECP (Figure 5a) was observed in $NaCl_E$ and Gua_E, which were almost free of plasma proteins, as judged by the absence of albumin (Figure 5c). Quantification of ECP/EDN by ELISA revealed that between 65 and 70% of ECP/EDN in hHCW was recovered in these extracts, indicating the protein(s) was (were) predominantly bound to the HCW through strong ionic interactions. This is consistent with the fact that both proteins, and particularly ECP, are highly positively charged at physiological pH. A minor component, with a slightly lower electrophoretic mobility, was also recognized by anti-rhECP, but it was not further analysed.

The ECP/EDN present in PBS_w is most likely plasma-derived, since significant amounts of albumin were observed in this initial wash and also in Tw_E, T_{E1} and PBS_{E1} (Figure 5c). Considering that we did not detect ECP/EDN in Tw_E and T_{E1} (Figure 5a), the content of these proteins in hHCW was estimated from the amount determined by ELISA in the extracts obtained after T_{E1}. ECP/EDN contents were normalized by the total extracted protein to allow comparison between samples. As shown in Table 1, the hHCW1, which derived from an inactive cyst (with signs of calcification, type CE5), contained a higher level of ECP/EDN than the samples recovered from active cysts (unilocular, simple cysts, with no signs of calcification, type CE1).

Fractionation of the $NaCl_E$ on heparin acrylic beads was carried out to separate ECP and EDN according to the method described by Gleich *et al*. (21). Fractions eluted at 0·5 M and 2 M NaCl showed components with similar electrophoretic mobility to rhECP and were recognized by the anti-rhECP antibodies, indicating the presence of both ECP and EDN in hHCW (Figure 6).

In addition, we detected ECP/EDN in the HCF from two cysts (type CE1) by Western blot (not shown) and estimated ECP/EDN concentration to be around 5 μM by ELISA.

DISCUSSION

Blood eosinophilia is not a typical feature of hydatid infections (22), but studies on experimental and natural infections have

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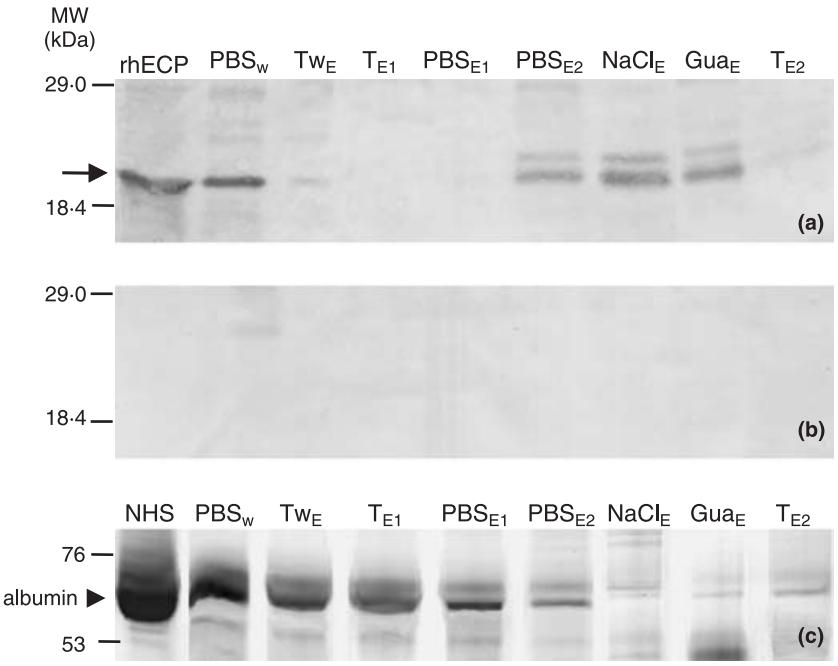

Figure 5 ECP/EDN are present in hydatid cyst walls of human origin (hHCW). rhECP (0·5 μg) and aliquots of hHCW extracts (reflecting their proportion in the hHCW) were loaded in a 10% polyacrylamide gel (reducing conditions), transferred to nitrocellulose membranes and probed with rabbit anti-rhECP antibodies (a) or non-immune rabbit immunoglobulins as control of nonspecific binding (b). A similar SDS-PAGE gel was stained with silver nitrate; rhECP was substituted by normal human serum (NHS) to control the presence of albumin as a marker of host proteins in hHCW extracts (c). The arrow indicates the components recognized by the antiserum.

Table 1 Eosinophil cationic protein (ECP)/eosinophil-derived neurotoxin (EDN) content in samples of hydatid cyst walls of human origin (hHCW)

	hHCW1	hHCW2	hHCW3	hHCW4
Type of cyst[a]	CE5 inactive partially calcified	CE1 active	CE1 active	CE1 active
mg (ECP/EDN)/mg total protein[b]	0·31 ± 0·06	0·12 ± 0·01	0·05 ± 0·01	0·15 ± 0·04

[a]Based on WHO ultrasound classification of hydatid cysts (CE, cystic echinococcosis). [b]Results are expressed as mean value ± standard deviation of two independent determinations.

shown that eosinophils are locally recruited throughout the establishment (3,23) and during the chronic phase of hydatidosis (2,24), mainly in situations where the host inflammatory response is not efficiently controlled by the parasite. Little is known about the contribution of eosinophils to host response against the hydatid cyst. One of the aims of this work was to examine the capacity of ECP to damage *E. granulosus* PSC, which could indicate to what extent eosinophils contribute to prevent secondary hydatidosis derived from the leakage of a primary cyst. Our results (Figures 1 and 2) demonstrated that rhECP damages PSC *in vitro*; the effect is dose-dependent and inhibited by specific antibodies (Figure 3a). Recombinant ECP is toxic for PSC in the micromolar range, similar to the level of native ECP that was found to be toxic for the trematode *Schistosoma mansoni* (25) and the nematodes *Brugia malayi* and *Brugia pahangi* (26). Interestingly, similar concentrations of rhECP were neither capable of killing human and mouse carcinoma cell lines nor normal mouse epithelial and rat smooth muscle cell lines (27). Thus, despite its relative lack of specificity against parasites, the toxicity of ECP has some level of selectivity, supporting the concept that it has evolved in response to pathogens (5).

Regarding the structure–toxicity relationship of ECP, its oligosaccharides do not seem to play a role in toxicity, since the non-glycosylated recombinant protein was found to kill bacteria and viruses (10,11); this was also the case for PSC. Comparison of the effects of rhECP and RNase A on PSC suggested that ribonuclease activity was not associated with ECP toxicity; RNase A, which is far more active as ribonuclease (70–200-fold depending on the substrate used; (28)), was less toxic than rhECP at the same molar concentration. In contrast, the effect of rhECP on PSC was blocked by heparin (Figure 3b), showing that basic side chains are involved in the interaction with PSC. These data support the idea that toxicity is associated with the high positive charge

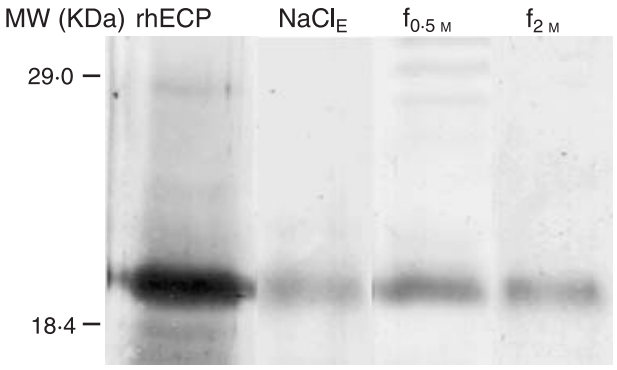

Figure 6 ECP and EDN are present in hHCW. An ECP/EDN-rich extract ($NaCl_E$) was incubated with heparin acrylic beads. Bound proteins were eluted using steps of increasing ionic strength. rhECP (0·5 μg), $NaCl_E$, and the fractions recovered at 0·5 M NaCl ($f_{0·5\ M}$) and 2 M NaCl ($f_{2\ M}$) were loaded in a 10% polyacrylamide gel (reducing conditions), transferred to a nitrocellulose membrane, and probed with rabbit anti-rhECP antibodies.

distributed throughout the surface of ECP (9). Moreover, it is in accordance with the hypothesis that ECP is a peculiar member of the eosinophil-associated ribonuclease (EAR) family. It is currently considered that EARs have diverged at a high rate in response to pathogenic single-stranded RNA viruses (13); ECP could have emerged in a similar manner, and its selection been driven by the need to respond against multicellular pathogens, which are susceptible to its cationic nature rather than to its ribonuclease activity.

Our results do not allow us to draw conclusions about the capacity of ECP to damage the hydatid cyst; this analysis would require the standardization of suitable methods to follow cyst viability. However, because the cellular structure of the PSC tegument is similar to the germinal layer (the latter is indeed continuous with the PSC tegument through the brood capsules wall, (29)), it is likely that ECP could also damage the germinal layer of the cyst. The external laminated layer would normally prevent a direct interaction between the germinal layer and eosinophils or their granular proteins. Nevertheless, considering that host plasma proteins are known to be present inside the cyst (30–32), it is conceivable that, if released around the cyst, ECP could diffuse through the laminated layer and reach the germinal layer. We thus investigated the presence of ECP in HCWs recovered from natural infections. Using antibodies reacting with ECP and EDN, and a procedure to separate the two proteins, we found they are both present in hHCW (Figures 5 and 6), suggesting they are released *in vivo* as a result of eosinophil degranulation at the host–parasite interface. Moreover, eosinophil proteins (ECP and/or EDN) were found at micromolar concentration inside hydatid cysts, in the HCF; this level is similar to the value reported for ECP in inflammatory sites associated with allergic disorders (μM range) and higher than values in the plasma of normal donors and asthmatic patients (nM range; (33,34)). Taken together, our results suggest that ECP could be released at the host–parasite interface, diffuse throughout the laminated layer and damage the germinal layer and PSC. EDN would be less harmful, because its toxicity is associated with ribonuclease activity and RNase A was only poorly cytotoxic against PSC. This is consistent with the available information regarding EDN toxicity for helminths; in fact, higher concentrations of EDN than ECP (about 10^{-4} M) were found to be required to kill *B. malayi* and *B. pahangi* microfilariae (26). Other eosinophil granular proteins as well as oxygen and nitrogen-derived toxic metabolites could synergistically act with ECP/EDN for parasite damage, as has been observed for the *in vitro* killing of schistosomula (25).

Interestingly, when comparing the ECP content of active (type CE1) and inactive (type CE5) cysts, we found that the hHCW from an inactive cyst had a higher content of eosinophil proteins than those derived from active cysts (Table 1), in agreement with the stronger inflammatory response usually associated with cyst degeneration. It is worth noticing that such response could also be a consequence of parasite death. In fact, in an experimental model of hydatid infection, dead parasites, but not live ones, were found to be potent triggers of inflammation (Martín Breijo and Ana M. Ferreira, Cátedra de Inmunología, Uruguay, unpublished results).

Although eosinophils are considered to be a hallmark of helminth infections (reviewed by (4,35)), their role in immunity and/or pathology remains controversial. Helminth parasites include a wide range of multicellular pathogens with complex life-cycles and they are capable of occupying diverse niches within their hosts; the contribution of eosinophils to host defences may thus differ when infections caused by organisms belonging to different taxa and/or living in distinct niches are considered. *Echinococcus granulosus* is particular among helminths for its excellent ability to adapt to inflammation in suitable intermediate hosts. The resolution of inflammation is reflected by the formation of a typical fibrous capsule around fully developed cysts, the adventitial layer, which reduces the interaction of cells, including eosinophils, with the cyst. The control of host inflammation is extremely demanding for the parasite, because inflammation may be triggered by several factors and because the cyst dwells and grows inside host viscera. Thus, under circumstances impairing this tight control, inflammatory cells would not be prevented from damaging the cyst. In this context, our results allow us to propose that eosinophils may be involved in cyst damage as they degranulate in the interface and their products (notably ECP) are harmful for PSC and most likely for the germinal layer. This proposal is in agreement with observations in natural infections. Firstly, as already

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mentioned, eosinophil numbers around cysts from infected bovines were found to correlate with the degree of cyst degeneration. In addition, the Th2-type response reported in hydatid patients with active/transitional cysts (36) would favour eosinophil recruitment. It also fits with the current idea about the putative role of these cells in defence against the larval stages of most helminth parasites; a hypothesis derived mainly from studies with nematode larvae (reviewed by (4,37)). Eosinophils are also currently associated with the development of pathology in helminth infections (reviewed by (4)). In the case of hydatidosis, inefficiently removed dead parasites would enhance inflammation; and, consequently, the contribution of eosinophils to inflammatory lesions cannot be discarded.

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