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Research note

Assessment of *in vivo* complement activation on the *Echinococcus granulosus* hydatid cyst wall

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SUMMARY

The larval stage of the parasite Echinococcus granulosus *causes hydatid disease. The hydatid cyst is potentially capable of activating host complement, since it is a large, persistent, carbohydrate-rich structure, coated with host immunoglobulins, and localized in the host's internal organs. Nonetheless,* in vitro *studies have suggested that the cyst surface, the hydatid cyst wall (HCW), is a poor complement activator. In this study, we assessed the occurrence of* in vivo *complement activation on the hydatid cyst by measuring the levels of two complement activation products, C3d and complexes bearing a C9 activation neoepitope (TCC/MAC), in extracts from HCW of human origin. Low amounts of C3d and TCC/MAC were found in HCW in comparison with their levels in normal human plasma and activated human sera, suggesting that* in vivo *complement activation on HCW is efficiently down-regulated. This regulation may contribute to limit host inflammation which has been observed to correlate with parasite degeneration and death.*

Keywords Echinococcus*, hydatid cyst, complement activation, C3d, TCC/MAC*

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INTRODUCTION

The larval stage of the cestode parasite *Echinococcus granulosus* causes hydatid disease, a chronic infection which affects humans and a wide range of livestock animals. The larva develops in the host's internal organs (mainly liver and lungs) as an unilocular, fluid-filled cyst. The cyst wall (HCW) is the parasite surface exposed to host defences, and consists of an inner nucleated or germinal layer and an external acellular laminated layer, which acts as a barrier preventing the direct contact of host cells, but not of host macromolecules, with the parasite tissue. The biology of *Echinococcus* has been reviewed elsewhere (1).

Comparison of the inflammatory reaction which surrounds fully developed cysts between suitable and non-suitable hosts has suggested that host inflammation is potentially harmful for the parasite, and that the parasite in suitable hosts manages to control it (1). The control of host inflammation by the hydatid cyst may include mechanisms of inhibition of complement activation on the HCW, since complement has a pivotal role in driving the early inflammatory reaction around the parasites [our unpublished observations using the murine chamber model of secondary infection in mice (2)], and since C5-dependent mechanisms, likely involving eosinophil recruitment, are deleterious for parasite establishment and growth (3).

In vitro studies have suggested that during the differentiation process from infective stage to cyst, the parasite adapts to host complement (4). In spite of these observations, the extent to which complement is activated *in vivo* on HCW has not been evaluated. In this work, we assessed the occurrence of *in vivo* complement activation on HCW by measuring the presence of two complement activation products: C3d, the factor I-resistant C3 activation fragment (32 kDa) that remains covalently bound to activators, and

complexes bearing a C9 neoepitope that results from the activation of the lytic pathway, the terminal complement complex (TCC) and/or the membrane attack complex (MAC).

Human-derived hydatid cyst walls (HHCW) were obtained from hepatic, hialine, noncalcified, relapsed cysts surgically removed from two patients. Extracts from each HHCW were prepared according to Díaz *et al*. (5) with slight modifications. Each HHCW was first washed with 10 mM phosphate, 150 mM NaCl, pH 7·2 (PBS) containing protease inhibitors (PI; 1 mM PMSF, 0·5 mM iodoacetamide, 5 mM EDTA) and the wash (PBS-wash) was separated by centrifugation (1 h, 20000 ***g***, 4°C). The pellet was then sequentially extracted (45 min, 4°C) with Tween-20 (0·5% w/v in PBS-PI) and Triton X-100 (2% w/v in PBS-PI). The soluble Tween- (Tween-ext) and Triton- (Triton-ext) extracted materials were separated by centrifugation (1 h, 20000 ***g***, 4°C). The pellet was frozen and pulverized with a mortar and pestle under liquid nitrogen. The homogenate was thawed into PBS-PI. Extraction with PBS-PI followed by centrifugation (30 min, 20000 ***g***) was carried out three times, and supernatants were called PBS-ext_1 to PBS-ext_3. The insoluble material was extracted (45 min, at room temperature) with 8 M guanidinium hydrochloride in 0·2 M Tris, pH 8·0, containing PI; the extracted materials (Guanidine-ext) were centrifuged (10 min, 20000 ***g***) leaving a small residue. All extracts were extensively dialysed into PBS containing 5 mM EDTA, and stored thereafter at −20°C.

Complement activation was examined in HHCW extracts, normal human plasma (NHP) and complement-activated human serum (AHS) by measuring the levels of C3d and TCC/MAC. NHP was obtained from healthy donors ($n = 5$, Hospital Militar, Montevideo, Uruguay); they were made 1 mM in benzamidine and maintained at −70°C until use. AHS was prepared by incubation (2 h, 37°C) of a pool of normal human sera with zymosan (3 mg/ml serum) or inulin (12·5 mg/ml serum). For C3d analysis in HHCW extracts, samples were previously incubated (2 h, 37°C) in 0·1 M methylamine (or hydroxylamine) in Tris-HCl pH 8·8, to release C3d molecules covalently bound, via ester bonds, to the solubilized HHCW components, or in buffer without methylamine as control. Quantification of C3d was performed according to Peakman *et al*. (6); briefly, C3d was measured by a capture ELISA in the supernatant obtained after precipitation of samples with 11% w/v polyethylene glycol 6000 (PEG); this procedure separates C3d from other C3-derived fragments of higher molecular mass. The total content of covalently bound C3d in each HHCW sample was calculated by adding the amounts of C3d released from all sequential extracts obtained for that sample. This figure was then normalized for total HHCW dry mass. TCC/MAC levels in HHCW extracts, NHP and AHS were measured by a capture ELISA as described by Würzner *et al*. (7) with slight modifications. Briefly, the anti-C9 neospecific monoclonal antibody (IgG1, WU 7-2 mAb, kindly donated by Dr Reinhard Würzner, Institut für Hygiene, Innsbruck, Austria) was used for coating, and detection was carried out using biotinylated antihuman C6 (Calbiochem, La Jolla, CA, USA) followed by peroxidase-labelled streptavidin (Sigma, St Louis, MO, USA). For each HHCW sample, the total content of extracted TCC/MAC was calculated and normalized for total HHCW dry mass.

Covalently bound C3d was mainly recovered from components extracted during the first PBS wash; lower amounts of C3d were recovered after detergent and guanidine extractions (Figure 1a). Host serum albumin, an indicator of weakly adsorbed host plasma proteins, showed a similar pattern to C3d being mostly removed in PBS-wash to PBS-ext (analysed by Western blot, data not shown). Thus, the majority of C3d recovered from HHCW came from easily removed macromolecules which probably include host plasma contaminants. This suggests that C3d extracted cannot be attributed entirely to complement activation on the cyst surface; binding of C3d to host plasma components is most likely a consequence of the physiological level of C3 activation in extracellular fluids. In contrast, TCC/MAC complexes were essentially removed in the later steps of the extraction procedure (PBS-ext_1 and Guanidine-ext) (Figure 1b) suggesting that these complexes are strongly bound to HHCW components as a result of complement activation on the cyst wall. Immunolocalization of TCC/MAC complexes in HHCW, using the WU 7-2 mAb or an irrelevant IgG1 mAb against *Tripanosoma cruzi* cysteine-proteinase (generously donated by Dr Gualberto González, Cátedra de Inmunología, Montevideo) as a control, revealed that these complexes were deposited on both laminated and germinative layers (data not shown).

Evaluation of the degree of complement activation on HCW would ideally require the comparison of the level of complement activation products deposited on HCW and on a normal host tissue. Since appropriate control tissues were not available, we compared the total content of C3d and TCC/MAC (normalized per mass of dry tissue) in HHCW with their corresponding values in NHP (normalized per dry mass). This comparison was based on the fact that normal arteries and normal plasma are know to contain similar amounts of TCC (8). C3d and TCC/MAC were present in HHCW at lower (12-fold and two-fold, respectively) levels than in NHP (Figure 1c,d). The levels of deposition of TCC/MAC complexes on HHCW were similar to those reported on normal myocardium (estimated on the basis that the content of MAC complexes is very low) (9):

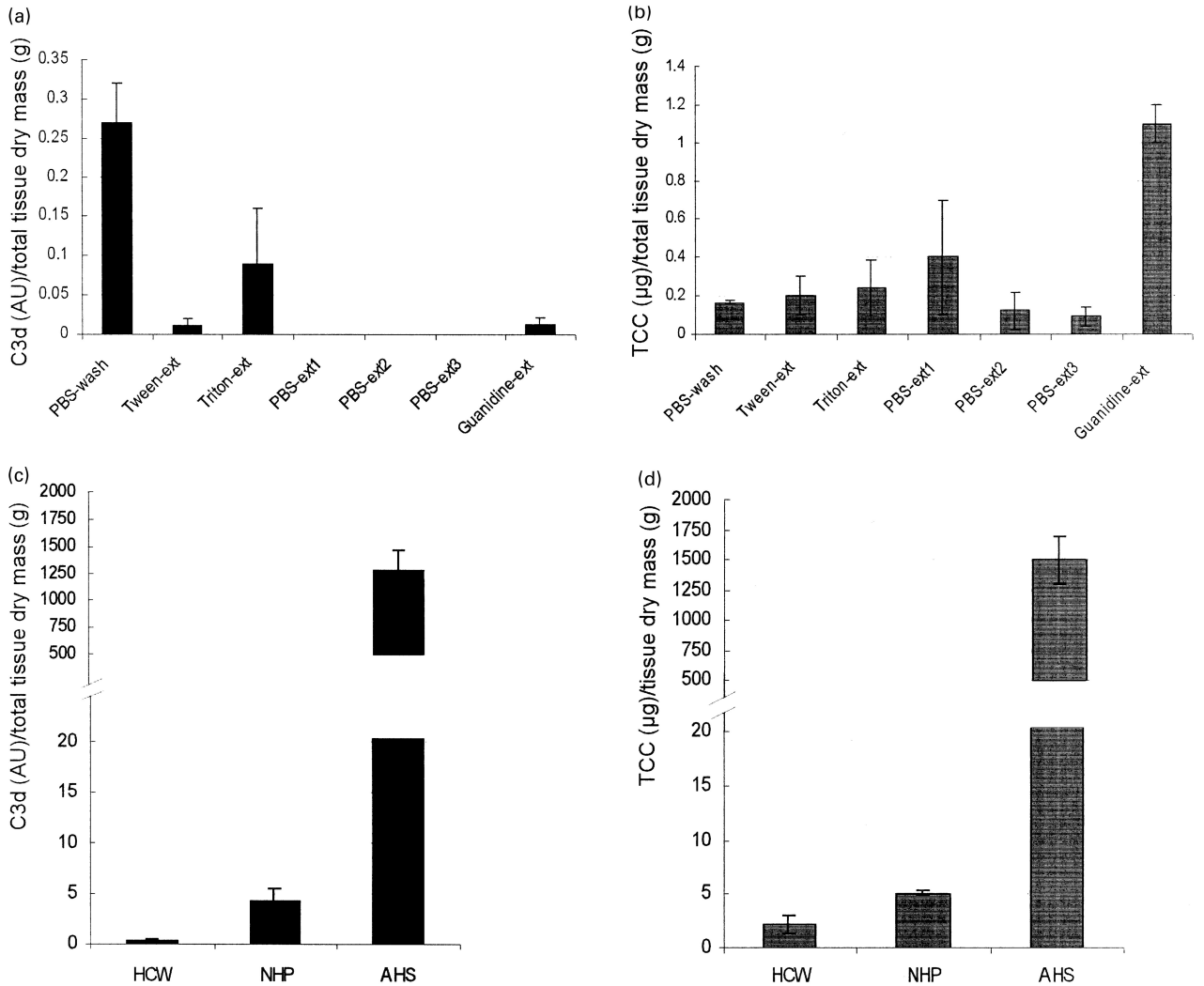

Figure 1 Content of covalently bound-C3d and TCC/MAC in HHCW. C3d levels were measured by ELISA and referred to a standard (the PEG precipitation supernatant of inulin-AHS) which was assigned a value of 100 arbitrary units (AU). ELISA plates were coated with antihuman C3d (DAKO, used at 0·15 µg/well), samples were incubated for 2 h at 37°C and detection was carried out using the same antibody labelled with biotin (at 0·12 µg/well) followed by peroxidase-labelled streptavidin (Sigma, diluted 1 : 1000). For each HHCW extract, the level of C3d covalently bound to HHCW components was calculated by subtracting the C3d level of the untreated sample from that obtained after methylamine treatment. TCC/MAC levels were determined by ELISA; ELISA plates were coated with the anti-C9 neospecific monoclonal antibody WU 7-2 mAb (at 1 µg/well), samples were incubated overnight at 4°C and detection was performed using biotinylated antihuman C6 (at 0·5 µg/well, 2 h at 37°C) followed by peroxidase-labelled streptavidin (diluted 1 : 1000, 1 h at 37°C). Levels of covalently bound C3d (a) and TCC/MAC (b) in HHCW extracts, referred to the total tissue dry mass, are expressed as the mean ± SD ($n = 2$). The total content of C3d (c) and TCC/MAC (d) in HHCW (normalized per mass of dried tissue) are expressed as the mean ± SD ($n = 2$), and plotted in parallel with those in NHP ($n = 5$) and AHS ($n = 2$) (normalized per dry mass) for comparison.

0·3 ± 0·1 and 0·46 µg/g tissue wet mass, respectively. It is worth mentioning that activation of complement on infarcted myocardium leads to a three-fold increase in TCC/MAC deposition (9). Taken together, our results suggest that the hydatid cyst is capable of down-regulating the activation of host complement on its surface, thus contributing to the evasion of inflammation.

Multiple mechanisms are probably required to control complement activation on the cyst surface since the nature of the HCW components, rich in neutral sugars (10), and the important amounts of host immunoglobulins bound to the HCW (11) would provide the triggers for the alternative, lectin and the classical pathways. While two strategies may account for inhibiting the alternative complement pathway (4) − the sequestration of host factor H and the existence of high amounts of IP_6, a potential C3

convertase inhibitor (Irigoín *et al*. unpublished observations) − the mechanisms involved in the inhibition of the lectin and classical pathways have not been elucidated and constitute interesting areas of study.

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