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Review

Control of host complement activation by the *Echinococcus granulosus* hydatid cyst

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Abstract

Cystic hydatid disease is caused by the multicellular parasite *Echinococcus granulosus*. The hydatid cyst, being a long-lived, large, antigenic structure lodged in the host's internal organs, could potentially elicit major inflammatory responses. However, in practice, the cyst causes only minimal local inflammation. The complement system is a major pathway to immune-mediated inflammation. Recent results have shown that the host-exposed structure of the cyst, the hydatid cyst wall (HCW), fails to trigger the complement system strongly. We have carried out a wide survey for the mechanisms making the cyst wall relatively complement-inert. The results of those studies are summarised in this work, with emphasis on the most recently identified of the complement inhibitory mechanisms. This is based on a non-protein heat-stable, parasite inhibitor of the activation of host complement factor B.

Keywords: *Echinococcus*; Hydatid; Parasite; Complement; Factor B

The larval stages of the cestode parasite *Echinococcus granulosus* cause cystic hydatid disease, which affects humans and a range of livestock animals (for review on the biology of the parasite, see Thompson, 1995). The disease is characterised by the growth in the host's internal organs (mainly, but not only, liver and lungs) of large, fluid-filled, slowly growing bladder-like structures. These cysts cause pathology mainly by the pressure exerted on the host organs. Infection of humans and livestock comes about through the ingestion of eggs (oncospheres) passed out with the faeces of infected dogs; dogs are hosts to the adult (intestinal worm) stage of the parasite. Hydatid cysts reproduce asexually by budding towards the inside, giving rise to protoscoleces (PSC), the infective stage for the dog host. PSC are also capable of reverse development, and they can give rise to secondary cysts if released by cyst rupture or unsuccessful surgical intervention. Human hydatid disease is a serious health problem in areas of South America, East Africa and China (reviewed by Schantz et al., 1995). Livestock infection has an important economic impact, through losses in meat, milk and wool production (reviewed by Schwabe, 1986).

Abbreviations: AP, Alternative pathway; CP, Classical pathway; HCW, Hydatid cyst wall; PSC, Protoscoleces

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The basic structure of a hydatid cyst is shown in Fig. 1a. The cyst is filled with hydatid cyst fluid, which contains both parasite and host-derived proteins, and is surrounded by a two-layered hydatid cyst wall (HCW). The innermost layer of this structure is the live parasite tissue (germinal layer), which is only a few cell bodies wide. The germinal layer lays down around it a massive (up to 2 mm thick), acellular, carbohydrate-rich, mechanically resistant structure termed the laminated layer. This layer, often referred to as an exaggerated, modified glycocalix, protects the cyst from direct attack by host immune cells. The laminated layer is to a large extent permeable to host macromolecules (Coltorti and Varela-Díaz, 1974).

Hydatid cysts can attain tens of centimeters in diameter and can survive for as long as their hosts. They elicit readily detectable antibody and cellular immune responses in their hosts (reviewed by Craig, 1988; Lightowlers, 1990; Nieto et al., 1994; Heath, 1995; Dixon and Jenkins, 1995a,b; Dixon, 1997). This parasitic disease therefore presents some pecu-

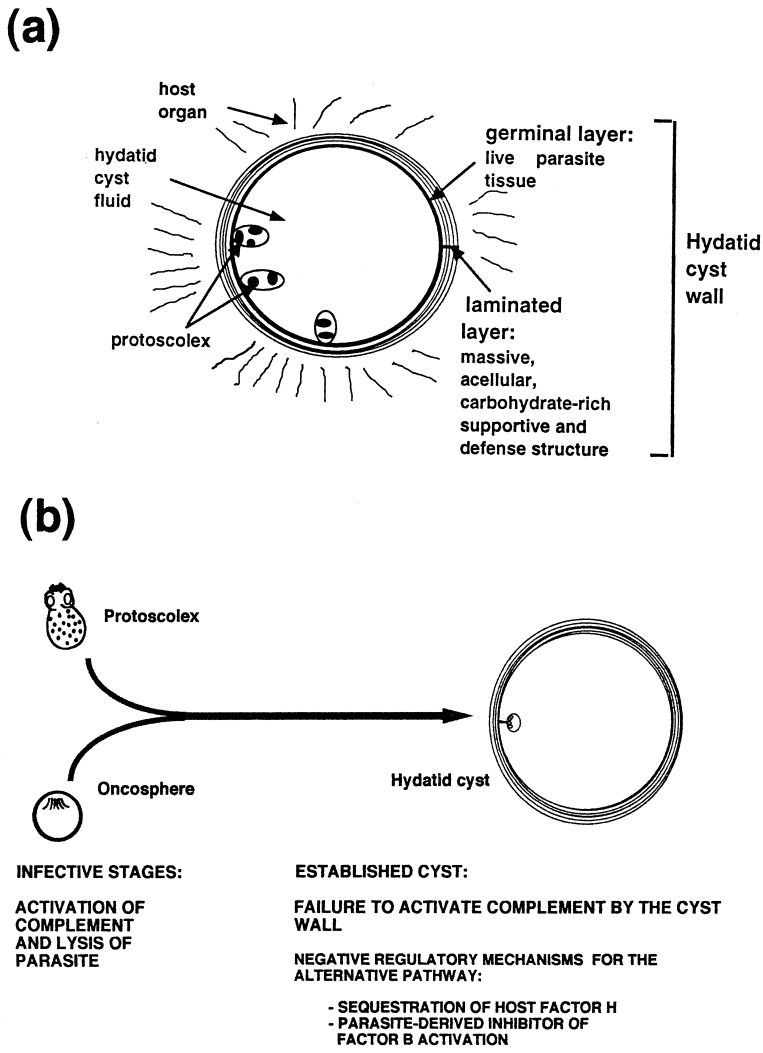

Fig. 1. (a) Diagram showing the basic organisation of a hydatid cyst. (b) Proposed model of the interaction of the parasite with the host complement system.

liar characteristics, including the fact that the hydatid cyst is probably the largest antigenic structure which lodges inside human tissues. In principle then, hydatid cysts should have formidable potential for eliciting host inflammatory responses.

From the histological evidence, however, hydatid cysts elicit minimal local inflammation. In humans in particular, hydatid cysts are generally surrounded by a poorly infiltrated or non-infiltrated collagenous capsule, around which a mild inflammatory infiltrate may sometimes occur (see, for example, Mufarrij et al., 1990). On the outside, the host organ parenchyma is generally minimally disrupted. This apparently balanced situation is thought to arise after the resolution of an initial, more intense, local response. In certain other host species, particularly in cattle, the initial response tends not to resolve, a chronic granulomatous response becoming established around the cyst. Across different host species, persistence of the local granulomatous inflammation correlates with cyst infertility and cyst death, whereas the subsidence of that response is associated with thriving parasites (Yamashita et al., 1957; Nieberle and Cohrs, 1967; reviewed by Smyth and Heath, 1970; Rao and Mohiyuddin, 1974; Thomas and Kothare, 1975; Slais and Vanek, 1980; reviewed by Thompson, 1995). As suggested by Thompson (1995), the parasite may have evolved into a balanced relationship only with those hosts in which the inflammation resolves. In addition, histological evidence suggests that parasite death, possibly a result of the inflammatory response, is in its turn followed by exacerbated local inflammation. Thus, a general picture arises that can be summarised as: (i) because of its size, location and immunogenicity, the parasite has extraordinary potential to elicit inflammation; (ii) if unchecked, the host local response can damage the parasite, and (iii) the live parasite can control the host inflammatory response, and seems to do this more effectively in some host species than in others.

The complement system is one of the most important pathways to both innate and adaptive immune inflammation. The hydatid cyst can be envisaged as having powerful potential triggers to initiate complement activation. The cyst wall, which constitutes the host–parasite interface, is very rich in neutral carbohydrate (Kilejian et al., 1962; Korc et al., 1967; Kilejian and Schwabe, 1971; Khoo et al., 1997), usually a trigger for alternative pathway (AP) activation (reviewed by Sim and Malhotra, 1994). Further, host immunoglobulin is found strongly bound to the parasite cyst wall (Varela-Díaz and Coltorti, 1973; Coltorti and Varela-Díaz, 1974), where it could potentially activate the classical pathway (CP).

The recently recognised lectin pathway of complement is activated by the binding of mannan binding lectin (MBL) to target carbohydrates on pathogens (reviewed by Malhotra et al., 1994). MBL belongs to the collectin family of soluble C-type lectins with collagenous domains, which act in innate immunity (reviewed by Holmskov et al., 1994). Since no information was available on whether the potential triggers for the lectin pathway were present on the hydatid cyst, we studied the presence of host MBL in HCW extracts of bovine origin. No trace of MBL or the related circulating collectins, conglutinin and CL-43, were found in a comprehensive range of well characterised HCW extracts (see Díaz et al., 1997 for a description of the preparation of extracts), by a sensitive Western blotting assay. Antisera against the bovine collectins were kindly provided by Dr. U. Holmskov, University of Odense, Denmark. The major monosaccharide constituents of the HCW, namely galactose and *N*-acetylgalactosamine (Kilejian et al., 1962; Korc et al., 1967; Kilejian and Schwabe, 1971) are known not to be major targets of the collectins under study. On the other hand, *N*-acetylglucosamine, mannose and fucose, also present in the HCW (references above, and Khoo et al., 1997), are known to be bound by the collectins. It seems likely that one of the survival strategies of *E. granulosus* is to expose on the HCW sugars which, by virtue of their monomer composition, or because of the spatial distribution of these monomers, cannot be recognised by the collectins. Poor extravasation and/or permeability across the laminated layer of the larger-sized collectins (MBL and conglutinin, but not CL-43) might also contribute to this observation.

Before summarising our results on some of the ways in which the established cyst avoids activating complement, in spite of the potential triggers for activation of the AP and CP, we will digress briefly to discuss the infective stages of *E. granulosus*. These offer an interesting comparison with the established cyst, in as much they do activate complement. Both oncospheres and PSC are killed by complement

in vitro, even in the absence of specific antibody, by the action of the membrane attack complex on their tegumental membrane (Herd, 1976; Kassis and Tanner, 1976; Rickard et al., 1977; Ferreira et al., 1992; Heath et al., 1994). In the case of PSC, it is well established that complement activation takes place by the AP (Ferreira et al., 1992). Possibly reflecting the behaviour of the intact organisms, lysed PSC or soluble extracts from these are strong activators of complement (Kassis and Tanner, 1976; Irigoín et al., 1996).

Strong AP activators are also present in hydatid cyst fluid (Hammerberg et al., 1977; Perricone et al., 1980, 1984; Ferreira and Nieto, 1991; Ferreira et al., 1995). Cyst fluid is sequestered inside the cyst, and release of its macromolecular components from the cyst probably does not take place in vivo at a rate large enough to cause major complement activation. Traumatic cyst rupture, which does result in cyst fluid release, is often accompanied by anaphylactic shock in the host. Host plasma macromolecules do find their way in vivo into cyst fluid, but in concentrations which are sufficiently low (typically 3–4 orders of magnitude lower than in plasma; see for example Coltori and Varela-Díaz, 1974) to make complement activation in this medium unlikely.

Both in cyst fluid and in PSC, complement activators are high molecular weight carbohydrate-rich

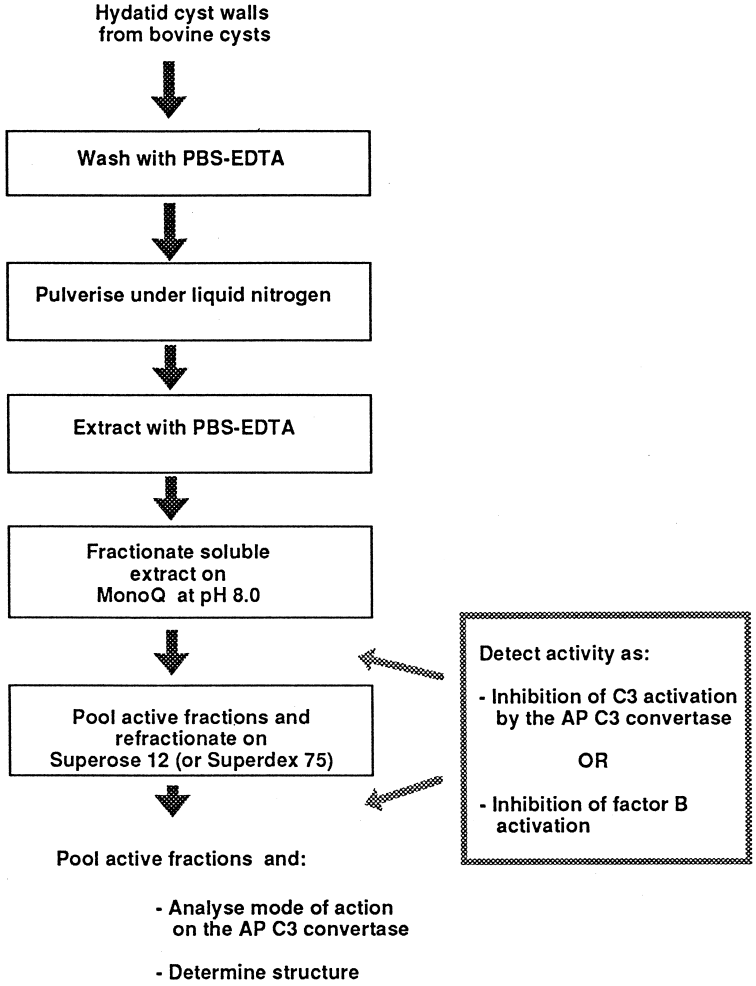

Fig. 2. Summary of the extraction and purification of the novel inhibitor of factor B activation from *E. granulosus*.

components (Ferreira and Nieto, 1991; Ferreira et al., 1995; Irigoín et al., 1997). In addition, part of the complement activating capacity of cyst fluid is due to immune complexes (Ferreira and Nieto, 1991).

In vivo, a large proportion of individual parasites in experimental inocula of *E. granulosus* PSC is killed soon after infection (Breijo et al., 1998). On development to the cystic form, surviving parasites must change to expose a surface that does not activate complement (Fig. 1b). Unchecked complement activation by the cyst would cause intense inflammation and, in view of the permeability of the laminated layer to macromolecules, would put the outer syncytial tegument of the germinal layer at risk of damage from the action of the membrane attack complex, just as it happens in the case of oncospheres and PSC.

Experimental evidence supports the idea of inertness to complement acquired by the parasite during development. While studying PSC apparently in the process of developing towards miniature cysts in vitro, Kassis and Tanner (1976), reported that parasites that lost their external 'hook' structures and started to vesiculate became refractory to complement-mediated killing. Further, whole extracts of the HCW are very poor complement activators in comparison with extracts from PSC and hydatid cyst fluid (Irigoín et al., 1996).

We have searched for the mechanisms that make the HCW relatively complement-inert. Activation of the AP is in essence a default mechanism, fuelled by an inbuilt amplification loop: the activated form of C3, C3b, is a precursor of the C3 convertase, the enzymic complex that generates more C3b. The crucial physiological negative regulator for the AP is factor H. Factor H has affinity for moieties present in mammalian cells but absent from many pathogens, in particular, for certain negatively charged sugars. We showed that the HCW sequesters host factor H in vivo, that this factor H is active as a cofactor for the inactivation of C3b by complement factor I, and that the whole of the factor I-cofactor activity found in the HCW is due to the sequestered host regulator (Díaz et al., 1997). The parasite molecules responsi-

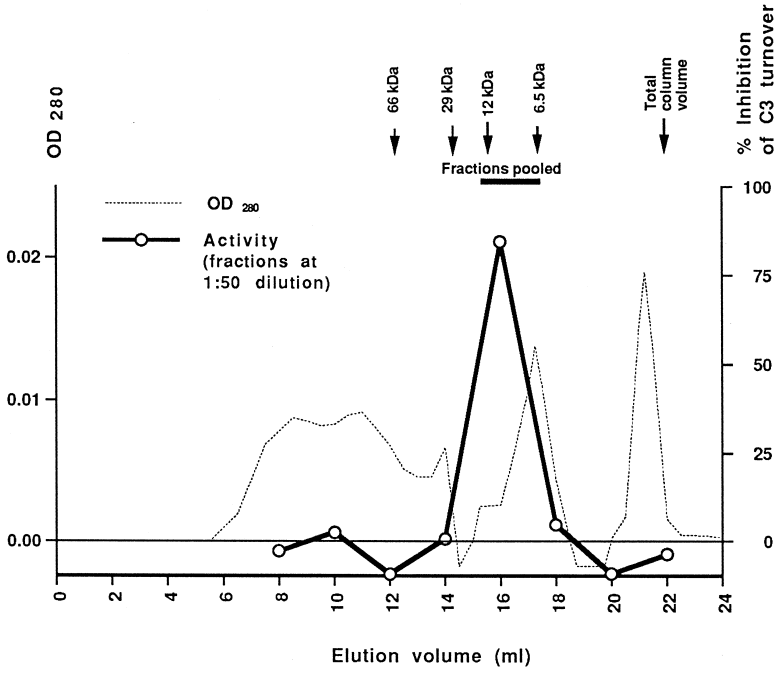

Fig. 3. Purification of the *E. granulosus* inhibitor of factor B activation. Gel filtration step. Pooled active fractions from the anion exchange step were refractionated on Superose 12 HR 10/30 and fractions assayed for the inhibition of C3 activation by the AP C3 convertase.

ble for binding factor H have not been identified; although it seems likely that negatively charged sugars are responsible for this, direct evidence is lacking.

The search for molecules other than host factor H inhibiting activation of the AP yielded a novel, parasite-derived, non-protein, heat-stable inhibitor. This was first detected as a potent, factor H-independent activity inhibiting the function of the AP C3 convertase. In these assays, the AP C3 convertase was formed from purified components ($C3(NH_3)$, used as a C3b analogue, factor B, and factor D), and its activity measured in terms of the activation (cleavage) of ^{125}I-labelled C3 (as summarised in Fig. 4). The extraction and purification of the inhibitor from the HCW is summarised in Fig. 2. In the initial Mono Q anion exchange step, the inhibitory activity eluted as a broad peak between approximately 0.3 and 0.5 M NaCl at pH 8. Upon refractionation by gel filtration on Superose 12 HR 10/30, the activity eluted as a sharp peak corresponding to a molecular size of 11 kDa, as compared with protein standards (Fig. 3). When this gel filtration step was carried out instead on a Superdex 75 column, the inhibitor still eluted as a sharp peak, but at a volume corresponding to a size of 4 kDa (i.e., after the 6 kDa aprotinin standard), possibly as a result of interactions with the matrix. In spite of its apparently small molecular size, the inhibitor was retained by ordinary 10 kDa-cutoff dialysis membranes.

The material obtained after the two chromatographic steps, which represented approximately 0.7% of the total dry weight of the HCW, yielded very simple ^{1}H and ^{13}C NMR spectra, suggestive of a fairly homogeneous composition. This material was used to further define the mode of action of the inhibitor. The inhibitor was found, contrary to factor H, not to act on the AP C3 convertase once this is formed. Rather, its inhibition of convertase function closely paralleled inhibition of the activation of factor B into Bb, the active serine proteinase of the convertase. Therefore, as summarised in Fig. 4, the novel inhibitor did not destabilise the C3bBb complex, but prevented its formation by stopping the activation of factor B. Physiologically, factor B activation (reviewed by Volanakis, 1989) by the proteinase factor D can only take place when factor B is bound to C3b. Thus, it is either the C3b–factor B

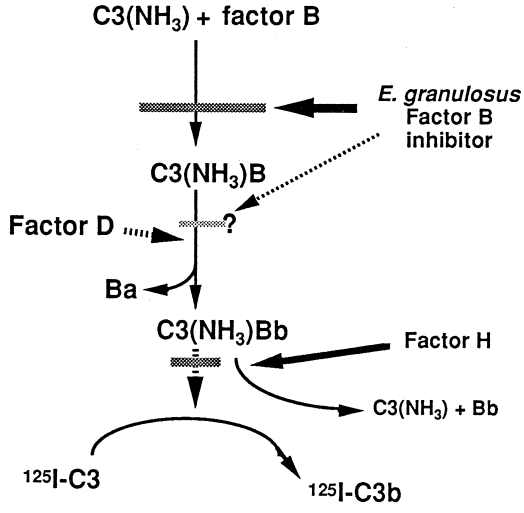

Fig. 4. Summary of the proposed mode of action of the *E. granulosus* inhibitor of factor B activation. In our assay, the fluid-phase AP C3 convertase is formed from the purified human components $C3(NH_3)$ (used as a C3b analogue), factor B and factor D. Then, convertase activity is detected as the activation of ^{125}I-labelled human C3, detected by SDS-PAGE and autoradiography. Inhibitors interfering in any way with the formation of the AP C3 convertase and/or with its stability/activity can be potentially detected by this assay. The basic assay employs a one-step incubation, where convertase components, potential inhibitors and the ^{125}I-labelled C3 substrate are added in a single step in a buffer containing Mg^{2+} or Ni^{2+} ions. Inhibitors acting on convertase formation can be discriminated from those acting on convertase stability/activity by a variation of the assay in which the C3 convertase is first formed and then EDTA, substrate and inhibitors added. Human factor H acts in this assay system entirely by destabilising the convertase, rather than by preventing its formation. The novel parasite inhibitor prevents convertase formation, and this is most likely the result of interference with factor B binding to $C3(NH_3)$.

interaction or the activity of factor D which is inhibited by the parasite inhibitor. Our results suggest that the inhibitor interferes with the former, since an excess of factor D was not able to reverse the inhibition phenomenon. It is possible that the inhibitor may bind to the Ba portion of factor B, which is known to be necessary for the initial interaction with C3b. A pattern of activity formally analogous to that of this parasite inhibitor (i.e., prevention of the formation of the AP C3 convertase without destabilisation of the preformed complex) has been described for a monoclonal antibody directed against Ba (Ueda et al., 1987).

Our recent preliminary results from NMR analysis and determination of total phosphorus content suggest that the inhibitory molecule has a repetitive structure, built up from phosphorylated, 5-carbon polyhydroxylated monomers. Definitive structural elucidation is under way.

Thus, two mechanisms inhibiting AP activation on the HCW have been outlined. These mechanisms have the potential to act synergistically, since they both act on the AP C3 convertase, but do so by different mechanisms.

We also sought mechanisms inhibiting the activation of the CP. A very important checkpoint in the physiological regulation of this pathway is located at the initial step of its activation cascade. This is the inhibition of the activated complement serine proteinases C1r and C1s by the serpin family inhibitor C1 inhibitor (reviewed by Sim and Reboul, 1981; Davis et al., 1993). Serpin family inhibitors form covalent complexes with their target proteinases (reviewed by Wright, 1996). Taking advantage of this property, we used ^{125}I-labelled activated human C1s (as described by Sim et al., 1979) to attempt the detection of serpin-type inhibitors of this complement enzyme in *E. granulosus* extracts. In spite of the high sensitivity of the assay for mammalian C1 inhibitors, no evidence of C1s-inhibiting serpins in parasite extracts was found. In a wider-scope search, we assayed the parasite extracts for direct inhibition of the enzymatic activity of activated human C1s on a synthetic substrate (N^{α}-carbobenzoxy-L-lysine-*p*-nitrophenol ester; Sim and Reboul, 1981) or on the ^{125}I-labelled form of one of its natural substrates, human complement C4. Again, no evidence of inhibition was found.

It seems likely that the parasite has the means to avoid activation of the classical complement pathway. In this context, the search for inhibitors of the CP C3 convertase should be carried out; the possibilities of sequestration of the host regulator C4bp, and of inhibition by the same or a similar molecule as that found to inhibit AP activation should be explored. Independently of any specific CP inhibitors, interference with the AP can be expected to impact upon the CP, since the AP acts as an amplification mechanism for the CP.

We feel that the avoidance of triggering of a full-blown host inflammatory response by a structure such as the hydatid cyst is a formidable feat. The study of the mechanisms underlying this avoidance should yield interesting and useful findings.

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