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Short communication

Host-derived annexin II at the host–parasite interface of the *Echinococcus granulosus* hydatid cyst

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The hydatid cyst, caused by infection with the larval stages of *Echinococcus granulosus*, is bound by the two-layered hydatid cyst wall (HCW). The innermost (germinal) layer, which is the live parasite tissue, is responsible for the synthesis of the outer (laminated) layer, a massive extracellular structure that endows the cyst with mechanical resistance.

Surprisingly, little is known about the composition of the HCW and of the laminated layer in particular, considering that it constitutes the host–parasite interface in hydatid disease. Ultrastructurally, the laminated layer is composed of irregularly arranged microfibrillar material in which irregular electron-dense bodies occur (reviewed in [1]). The major carbohydrate monomers in this structure are galactose and aminosugars (mostly galactosamine) [2–4]. The existence of structural proteins with disulphide bonds has been suggested [5], but no molecular species have been defined. The presence of the major diagnostic antigens (antigen 5 and antigen B) in the laminated layer is controversial (reviewed in [6]). In fact, the most abundant protein species in HCW extracts are host albumin and immunoglobulins [2,7]. This reflects the fact that the HCW is very permeable to host-derived macromolecules [8,9] and can bind certain of these [10].

Taken together, the available evidence allows us to envisage the laminated layer as a meshwork of parasite-derived carbohydrate and protein inside which both occluded and bound parasite- and host-derived macromolecules can occur. Here we show that one of the most abundant bound proteins in the HCW of bovine hydatids is the phospholipid- and Ca^{2+}-binding protein, annexin II, and that this protein derives from the host granulomatous inflammatory cells.

Abbreviations: HCW, hydatid cyst wall.

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Extracts of HCWs from fertile hydatid cysts from naturally infected cow's lungs were obtained as described earlier [10]. The germinal and laminated layers were not separated prior to extraction. In consequence, it is to be expected that most of the material extracted will be derived from the bulkier laminated layer.

Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and Western blot analysis of HCW extracts showed that most of the proteins in these were derived from the host plasma (not shown). A major band running at 31 kDa under reduced conditions (and corresponding to a 34 kDa band apparent under non-reducing conditions), was not recognised by anti-(bovine serum) antibodies in Western blots (not shown). This band was reproducibly present in several HCW extracts, representing approximately 1% of the total extractable protein in the HCW. The band was most abundant in the extract obtained by treating the phosphate buffered saline (PBS) insoluble pellet left after pulverising the structure under liquid nitrogen with 2 M NaCl. This extract, in which the band under study represented 10–20% of the total protein (Fig. 1b), was used to obtain N-terminal sequence information for the protein component. As shown in Fig. 1a, the 13N-terminal amino acids of the protein species showed complete identity with residues 30–42 of bovine (and other mammalian) annexin II. In addition, a minor sequence corresponded to residues 33–40 of the same bovine protein. Comparison of the available sequences (Fig. 1a) strongly suggested that this was the host-derived annexin II rather than a parasite homologue because our stretch of sequence had complete identity with all known mammalian annexin IIs. In contrast, the known avian and amphibian homologues diverge considerably from the mammalian sequences over the same stretch (four or five differences in 13 residues). No helminth annexin II sequences are available for comparison.

Annexin II is known to be an abundant 36 kDa protein that is mostly intracellular. The protein is

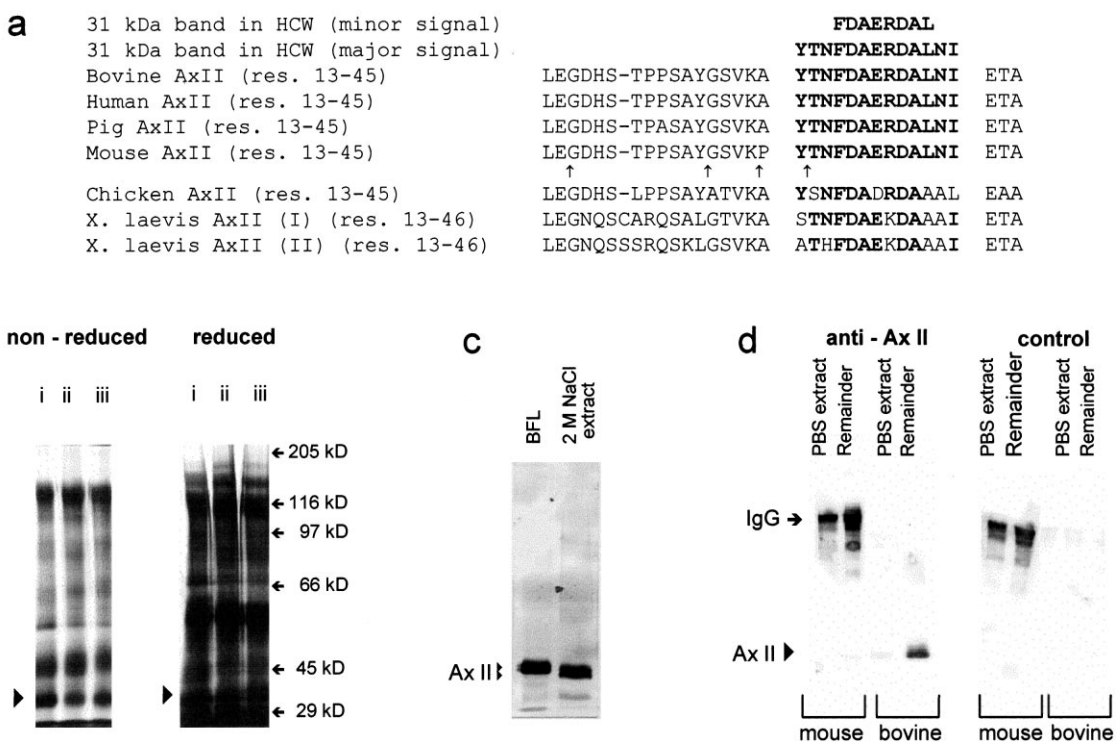

Fig. 1.

composed of a 3 kDa N-terminal domain and a 33 kDa C-terminal core composed of four annexin repeats, which are common to the annexin family of proteins (reviewed in [11,12]). The peptide sequences for the band in our extracts correspond to the beginning of the 33 kDa annexin II core protein; the estimated molecular mass of the band in our extracts is in good agreement with the molecular mass of this core. The stretch linking the annexin II core protein to the N-terminal domain is known to be particularly susceptible to proteolysis. As shown in Fig. 1a, the protein in our extracts has been cleaved at particular peptide bonds different from those reported earlier [13,14]. Annexin II exists in most, but not all, situations as a heterotetramer comprising two annexin II chains tightly linked in a non-covalent manner to two 11 kDa chains (known as p11) belonging to the S-100 family of proteins. The site of interaction with p11 on annexin II resides on the N-terminal extension (reviewed in [11]), which is absent from the truncated protein found in our extracts. No attempt was made in this work to ascertain whether the p11 component was present in the HCW.

The results above were confirmed by Western blotting, using a bovine annexin II-specific monoclonal antibody. Annexin II was detected in the same extract used to derive the N-terminal sequence as a 30–33 kDa doublet (Fig. 1c); annexin II from a bovine fibroblast lysate (used as a positive control) appeared as a 33–36 kDa doublet, also probably reflecting the susceptibility of the protein to limited proteolysis. In addition to its presence in several different HCW extracts, annexin II was detected by immunoblot at very low levels in hydatid cyst fluid; it was undetectable in a preparation from protoscoleces (not shown).

The question of the host/parasite origin of annexin II in the HCW was further addressed by comparing, with the help of the same monoclonal antibody, cysts from cattle infections with cysts developed after intraperitoneal infection of mice

Fig. 1. Identification, by N-terminal sequencing and immunoblot, of a major protein band in extracts of *E. granulosus* cyst walls as host annexin II. Extracts were prepared from HCWs, obtained either from natural bovine infections or from experimental (secondary, intraperitoneal) mouse infections. HCWs were washed with PBS, pulverised under liquid nitrogen with mortar and pestle, and extracted with PBS; in some cases, the insoluble remainders were further extracted with 2 M NaCl. A cocktail of proteinase inhibitors was used at all steps during extraction. A major 31 kDa band in the extracts from bovine HCWs was subjected to N-terminal sequencing. In (a) the two sequences obtained have been aligned with the homologous stretches of sequence (translated from cDNAs) from the bovine (P04272), human (P07355), pig (P19620), mouse (P07356), rat (Q07936), chicken (P17785), *Xenopus laevis* type I (P27006) and *X. laevis* type II (JQ1298) annexin II (AxII) proteins. The stretches of sequence shown correspond to the end of the N-terminal 'tail' of the annexin II molecule (up to residue 30 in the mammalian annexin II numeration), plus the beginning of the annexin core. The peptide sequences obtained from the cyst material correspond to the annexin II core protein. The positions of proteolytic removal of the N-terminal tail observed in previous works [13,14] for mammalian (porcine) annexin II are indicated with arrows. To obtain the peptide sequences, the 2 M NaCl extract from bovine HCWs was run on SDS-PAGE (12.5% w/v acrylamide and reducing conditions) and then electroblotted onto PVDF. The band under study was excised and sequenced using an Applied Biosystems 494A 'Procise' sequencer. The silver-stained SDS-PAGE (7.5% w/v acrylamide) profiles (non-reducing and reducing conditions) of the 2 M NaCl extract from bovine HCWs are shown in (b); three independent batches of extract have been run in parallel (I, II, III), and the band under study is indicated with arrowheads. In (c), the same extract as in (b) has been analysed by Western blot (after 7.5% acrylamide SDS-PAGE under reducing conditions), in parallel with a bovine fibroblast lysate (BFL), used as a positive control. The blot was probed with a mouse monoclonal antibody against bovine annexin II (Zymed Labs. Inc., San Francisco, CA, USA) followed by sheep anti-mouse IgG coupled with alkaline phosphatase. Recognition of the 31 kDa band by the antibody confirmed its identification as annexin II. A control blot in which an isotype-matched mouse monoclonal (LPM19C, directed against human CR3, a kind gift of Dr SK Alex Law, from the MRC Immunochemistry Unit) was substituted for the anti-annexin II showed no staining (not shown). In (d), HCW preparations from bovine and murine cysts were analysed comparatively by Western blot, to confirm that annexin II is of host, rather than parasite, origin. HCWs from both the hosts were washed, pulverised and extracted with PBS in identical fashion. Portions of both PBS-soluble extracts (3 μg total protein in each case) and the proportional portions of the insoluble remainders (90 μg total dry weight each) were loaded onto SDS-PAGE wells (8.5% w/v acrylamide, non-reducing conditions), electrotransfered and probed with anti-annexin II or control antibody, as above. Only cysts of bovine origin contain annexin II clearly recognised by the mouse anti-annexin II antibody. The strong staining in lanes loaded with murine material corresponds to mouse IgG (150 kDa), which is bound by the second antibody, as evidenced by the control blot.

with protoscoleces. The latter were obtained from natural bovine infections of the same origin as the cysts from which the bovine HCW extracts were made: hence, no parasite strain differences could exist between the two types of material. HCWs of the two host origins were homogenised and extracted in an identical fashion, and equal amounts of the preparations so obtained were loaded onto SDS-PAGE wells for Western blot analysis. As shown in Fig. 1d, annexin II was clearly detected only in the preparations from bovine host origin. The preparations from murine cysts showed only an extremely weak band at the molecular mass of annexin II, plus strong staining corresponding to host IgG, which is obviously bound by the second antibody, as is evident from the control blot. If annexin II was a parasite structural component, it is to be expected that it would be present in similar amounts in the two samples. The most likely explanation for the contrast in annexin II reactivity between the two types of sample is that the protein derives from a host reaction that takes place to very different degrees in the two different hosts species/cyst locations. Results presented below indicated that annexin II in bovine cysts arises from the cells of the host granulomatous response, and cysts growing free in the peritoneal cavity of mice, as opposed to cysts growing in bovine organ parenchymas, do not evoke granulomatous responses (Breijo M and Ferreira AM, Cátedra de Inmunología, University of Uruguay, pers. commun.).

It is known that in the bovine host, the local inflammatory response to the hydatid cyst does not resolve. This results in a well-organised granulomatous infiltration surrounding the cyst [15,16].

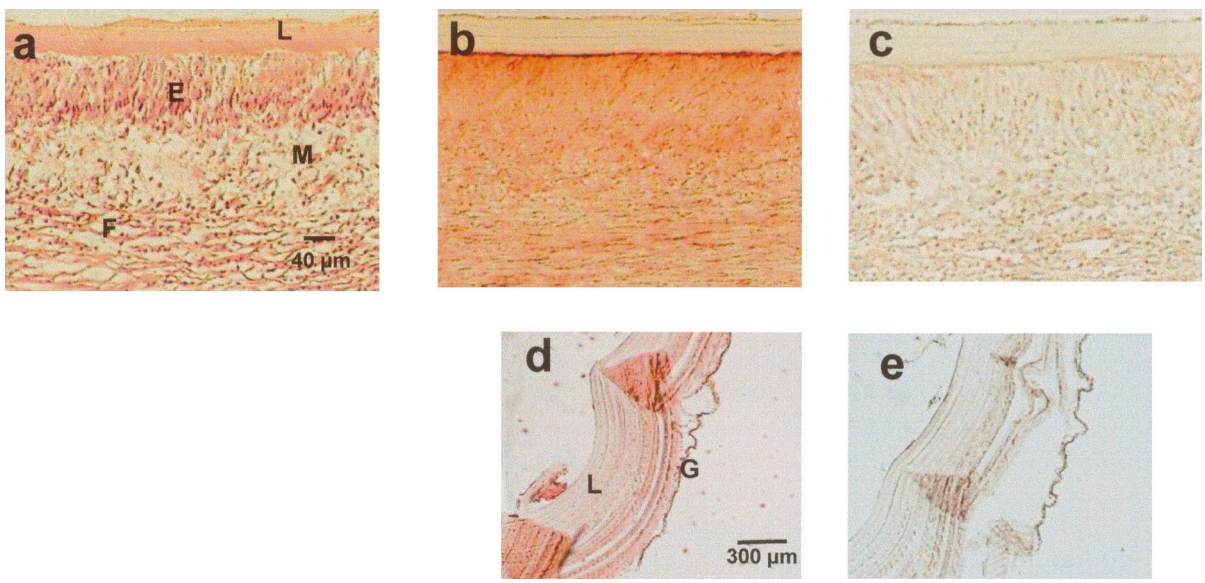

Fig. 2. Immunohistochemical localisation of host annexin II in the host–parasite interface of bovine hydatid cysts. Cysts from natural bovine infections were fixed and paraffin-embedded. Tissue sections were stained with haematoxilin-eosin (a) or probed with anti-annexin II monoclonal antibody (b, d) or control antibody (c, e). Sections were treated with 1% (w/v) H_2O_2 to inactivate endogenous peroxidase activity, and blocked with 10% (v/v) sheep serum. The first antibodies (as in the legend to Fig. 1) were diluted to 10 µg ml^{-1} in TBS with 1% (v/v) sheep serum. Development used biotin-coupled sheep anti-mouse IgG, followed by streptavidin–peroxidase, and 3-amino 9-ethylcarbazole substrate. Sections including the host inflammatory reaction are shown in (a–c). Annexin II localised to the whole of the host reaction, but was detected most strongly in the epitheiloid cell layer. Immunohistochemical detection of proteins in the laminated layer is generally difficult (our own observations). Antigen-retrieval procedures, which disrupt the tissue slightly, can surmount this difficulty. In (d) and (e), the isolated HCW has been subjected to such a procedure (consisting of microwaving the sections in citrate buffer, pH 6.0). Annexin II was detected on the striations of the laminated layer; it was also present in the germinal layer. The main features on the panels are indicated with capital letters as follows. L, laminated layer; G, germinal layer; E, epithelioid cells; M, mononuclear cell infiltrate; F, fibroblasts.

As shown in Fig. 2b and c, immunohistochemical analysis using the anti-annexin monoclonal antibody proved that the major sources of annexin II were the epithelioid cells of the inflammatory reaction. Epithelioid cells are specialised macrophages which constitute the hallmark of immunological granulomas [17,18]. In the inflammatory response to the hydatid cyst from natural infections, these cells, together with the related giant multinucleated cells, form a continuous palisading rim, which is apposed to the external side of the laminated layer [15,16] (see Fig. 2a). We had shown earlier that a product of these epithelioid cells, the proteinase cathepsin K, associates with the HCW [19]. Although annexin II has a broad cellular distribution, its expression varies over different cell types (reviewed in [11]); a specific association of this protein with epithelioid macrophages had not been reported before. We could also, as expected, detect the annexin II in the HCW; the specific staining formed a pattern of striations in laminated layer, and was also present in the germinal layer (Fig. 2d and e). No staining at all was observed in sections of protoscoleces from bovine cysts (not shown).

It is not clear how annexin II is released from the inflammatory cells or how it associates with the HCW. The protein does not have a leader peptide, but it is thought to be secreted under certain conditions (reviewed in [11]), and it is known to be released from some cell types as part of 'ectocytic' (out-side-out) vesicles [20]. In our context, both release from viable cells or after cell death may account for the observations made. Most of the binding activities reported for annexin II are Ca^{2+}-dependent (reviewed in [11,21]). However, we were unable to demonstrate a differential solubilisation of the protein (detected by Western blotting) from the pulverised HCW by buffers containing EDTA versus buffers without chelating agent (not shown).

The striking amounts of annexin II found in the HCW may play a role in the host–parasite relationship. Annexin II has long been shown to have anti-inflammatory and anticoagulant activities as a consequence of its capacity to sequester phospholipids (reviewed in [11]). Low-affinity Fcγ receptor activity in the human placenta has also been reported for extracellular annexin II [22]; it is conceivable that part of the IgG found associated with the HCW may be bound to annexin II. Particularly interesting in the context of hydatid disease is the precedent that extracellular annexin II plays an important role in the calcification of cartilage [23], by virtue of its Ca^{2+}-binding properties. The retention of annexin II by the HCW may well contribute to calcification, an outcome often associated with an intense local response to the hydatid cyst [15,16].

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