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Host serum proteins in *Echinococcus multilocularis*: complement activation via the classical pathway

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Summary. The presence of host serum proteins, including IgG and IgM, within the cyst membranes and on the surface of protoscoleces of *Echinococcus multilocularis* was demonstrated by the use of HRPO-conjugated purified antisera. Viable protoscoleces incubated either in EDTA- or EGTA-fresh human sera were not lysed; the addition of calcium restored the protoscolecidal activity of the EGTA serum. It is concluded that the complement-mediated lysis of this metazoan organism proceeds via the classical pathway of complement activation. The results are discussed in relation to the ability of this parasite to survive in the immunologically hostile environment of the host.

INTRODUCTION

Larvae of cestodes belonging to the genus *Echinococcus* characteristically produce fluid-filled cysts in their intermediate hosts and in man. We (Kassis, Goh & Tanner, 1976; Kassis & Tanner, 1967a, and b) have studied the immune response evoked by these metacestodes, expecially *E. multilocularis*, since this parasite produces a rapidly growing, tumour-like, metastatic cluster of cysts. The proliferation of these parasites is controlled by mechanisms which have an immunological component since the protoscoleces of both *E. granulosus* and *E. multilocularis* are lysed by complement proteins without supplementary treatment with antibodies (Kassis & Tanner, 1976a). Our results have lead us to propose a simple, effective immuno-therapy procedure for hydatid disease (Kassis & Tanner 1976b) and they have also stimulated our curiosity concerning the nature of the complement activators on the surface of these protoscoleces.

The presence of host-like proteins in the cyst tissues and in the fluid of the larval stage of *Echinococcus* sp. and the permeability of the cyst membranes to these proteins have been the subject of a number of reports (Cameron & Stavely, 1957; Kagan & Agosin, 1968; Coltorti & Varela-Diaz, 1972, 1974, 1975; Varela-Diaz & Coltorti, 1972, 1973). The aim of the present study was to determine whether the complement-mediated lysis of protoscoleces by fresh normal serum (Kassis & Tanner, 1976a) can be ascribed to IgG and IgM immunoglobulins. To this purpose, horseradish peroxidase (HRPO) was coupled to specific anti-immunoglobulin antisera and used to identify the class-specific antibodies associated with the parasite tissues; EDTA (ethylenediaminetetraacetic acid) and EGTA (ethyleneglycoltetraacetic acid) were used to chelate the magnesium

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and/or the calcium in fresh human serum to inhibit the alternate and/or the classical complement activation pathways (Fine, Marney, Colley, Sergent & Des Prez, 1972). Our present and previous findings are discussed in relation to the ability of protoscoleces and hydatid cysts to survive within the tissues of immunologically-competent hosts.

MATERIALS AND METHODS

Parasite

Thin-walled, fertile cysts of *E. multilocularis* (0·5–2 cm in diameter) were obtained from the peritoneal cavity of cotton rats (*Sigmodon hispidus*) which had been inoculated 60 days previously with about 2000 protoscoleces; these cysts were used as a source of protoscoleces and cyst membranes. The cysts were washed five times with 50 ml of phosphate-buffered saline, pH 7·2 (PBS) to remove any loosely adsorbed host serum proteins, embedded in Ames O.C.T. compound (Miles Laboratories, Indiana) and frozen on metal blocks at −20°. Sections 6–8 μm thick were cut in a cryostat, placed on microscope slides, air dried at room temperature and used in the conjugation studies (see below).

Protoscoleces to be used to demonstrate the presence of host serum proteins were fixed in absolute methanol and stored at −70°. When needed, approximately 100 of these larvae were pipetted onto a glass microscope slide and air dried. These larvae adhered strongly to glass and, consequently, no additional treatment was needed to fix the protoscoleces on the slides to prevent them from falling off during the staining procedure.

Antisera

Pooled sera from normal cotton rats were fractionated on a 2·5 × 100 cm Sephadex G-200 column; the fractions under either the 19S or the 7S globulin peaks were pooled, concentrated to approximately 10 mg protein/ml by ultrafiltration through a Diaflo PM 30 membrane (Amicon Corporation, Lexington, Massachusetts), sterilized by Millipore filtration (0·45 μm) and stored at −20°. Antisera to each of these globulins, as well as to whole cotton rat serum, were prepared in female New Zealand white rabbits by subcutaneous injections containing 1–2 mg protein emulsified in an equal volume of Freund's complete adjuvant and given once a week for 3 weeks; 5 weeks after the final injection, each animal was boosted by a single i.m. inoculation of the appropriate antigen (1–2 mg) and bled 10 days later by cardiac puncture. The antisera were heat inactivated (56° for 30 min), sterilized by Seitz filtration and stored at 4°.

Normal cotton rat 7S globulin (prepared as indicated above) or whole normal cotton rat serum from which the IgG had been removed by filtration through a QAE-Sephadex A-50 column (Joustra & Lundgren, 1969) were each covalently bound to cyanogen-bromide-activated Sepharose 4 B resin (Pharmacia), as recommended by the manufacturers; each of these two preparations was packed into 1 × 20 cm columns (Columns I and II respectively). Rabbit antiserum to the normal cotton rat 19S globulins was filtered through Column I to remove any antibodies to IgG whereas antiserum to cotton rat IgG was filtered through Column II to remove any contaminating antibodies to IgM and albumin. In both cases, the non-adsorbed antiserum was pooled and the protein concentration adjusted to 10 mg/ml. The elution profiles of the immunoadsorbent columns and the specificity of the purified antisera as assessed by immunoelectrophoresis are shown in Figs 1 and 2.

The conjugation of horseradish peroxidase (HRPO)

Two millilitres of each of the three rabbit antisera, as well as 2 ml of whole normal rabbit serum, were precipitated three times with an equal volume of a saturated ammonium sulphate solution whose pH had been freshly adjusted to 7·8 (Campbell, Garvey, Cramer & Sussdorf, 1970); following the third precipitation, each sediment was redissolved in 2 ml of 0·01 M sodium carbonate buffer, pH 9·5. The solutions were dialysed against 200 vol. of the same buffer at 4° (Herbert & Pittman, 1965); protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) and each solution was adjusted to contain 5 mg/ml.

The recently described method of Nakane & Kawaoi (1974) was used to conjugate HRPO to the immunoglobulins. Briefly, 0·4 ml of a 1% solution of 1-fluoro-2,4-dinitrobenzene (Eastman Organic Chemicals, Kodak) in absolute ethanol was added to 20 mg of HRPO (Sigma; RZ = 2·65) in freshly prepared 0·3 M sodium bicarbonate and mixed gently for 1 h at room temperature. Four millilitres of 0·08 M sodium metaperiodate (Fisher) was then added, mixed for 30 min and the reaction was stopped by the addition of 4 ml of 0·16 M ethylene glycol. The solution was stirred for an additional 60

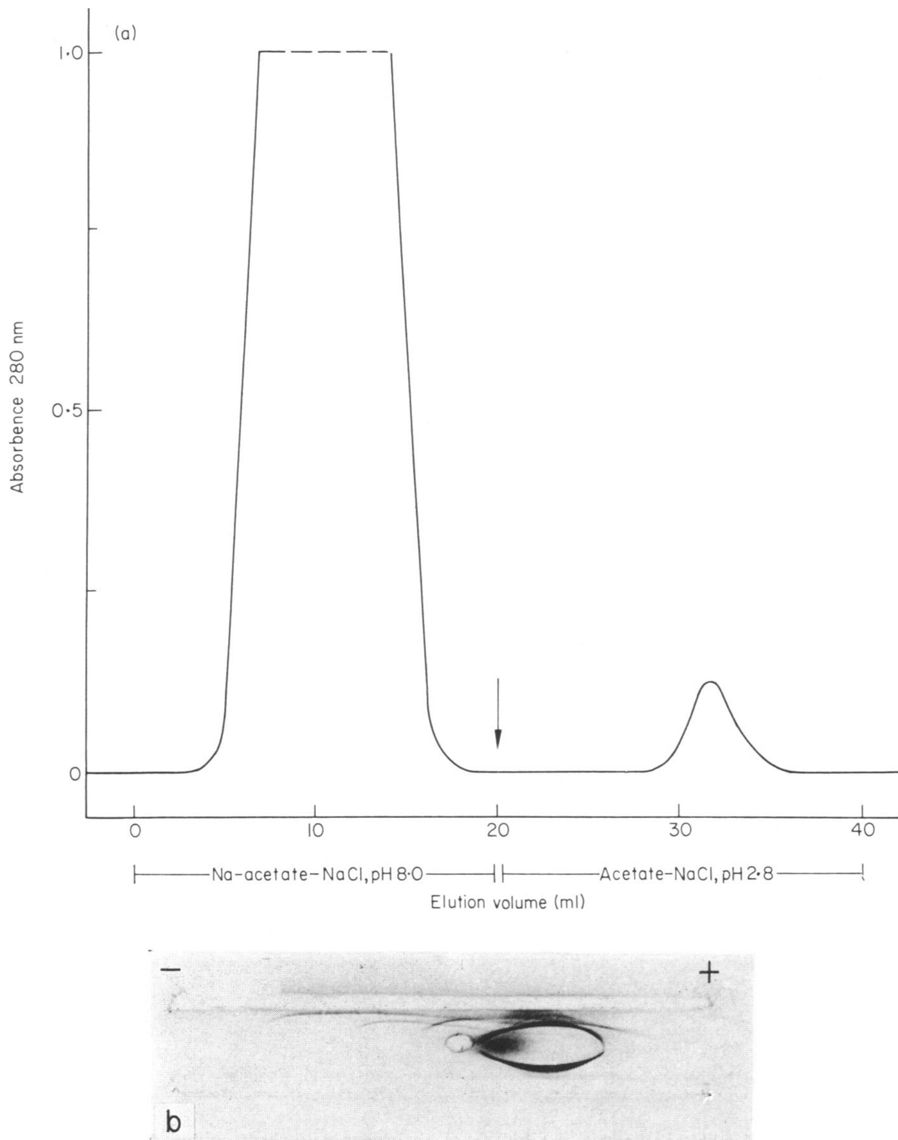

Figure 1. (a) Elution profile of rabbit anti-cotton rat IgM from an immunoadsorbent column to which cotton rat 7S immunoglobulins were covalently bound. (b) Immunoelectrophoresis of the antibody before (top trough) and after (bottom trough) affinity chromatography. Well: normal cotton rat serum (NRCS).

min and dialysed extensively overnight at 4° against 0·01 M sodium carbonate buffer, pH 9·5. Five milligrams of the appropriate purified immunoglobulin was reacted with 2 ml of the dialysed HRPO-solution for 3 h at room temperature. Five milligrams of sodium borohydride (Fisher) was then added to each mixture, mixed thoroughly, left at 4° overnight and then dialysed against an excess of 0·01 M PBS, pH 6·8. These mixtures were fractionated through a Sephadex G-200 column (2·5 × 70 cm); the fractions eluting under the first peak were pooled, concentrated to 10 ml by ultrafiltration through a PM 30

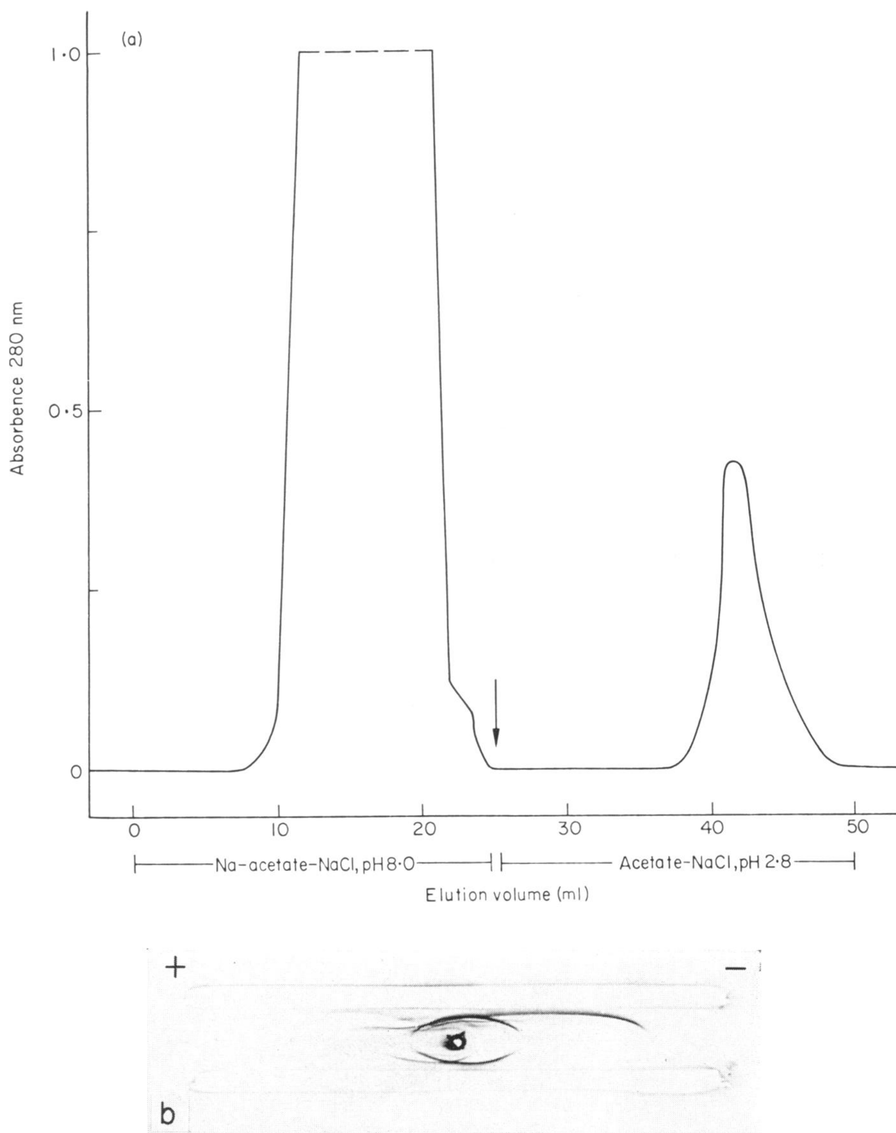

Figure 2. (a) Elution profile of rabbit anti-cotton rat IgG from an immunoadsorbent column to which cotton rat IgG-free serum was covalently bound. (b) Immunoelectrophoresis of the antibody before (top trough) and after (bottom trough) affinity chromatography. Well: NCRS.

Diaflo membrane and stored at −70° in 1-ml aliquots containing 10 mg crystalline bovine serum albumin (NBC).

Staining and microscopy

Parasite tissues were fixed in 0·074% HCl in absolute ethanol for 15 min at room temperature (Weir, Pretlow, Pitts & Williams, 1974a, b) and then incubated, also at room temperature, for 1 h in a humidified atmosphere with the peroxidase-labelled conjugates. The slides were washed three times, 30 min each, with PBS (pH 7·4), incubated in absolute ethanol for 15 min and gently washed in tap water. After the final wash, the slides were placed in 90 ml of freshly filtered solution of 0·03% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 0·05 M

citrate-dibasic ammonium phosphate, pH 5·0, and 10 ml 0·05% hydrogen peroxide were added (Graham & Karnovsky, 1966). After 6 min, the slides were washed with tap water and mounted in buffered glycerol (pH 7·4) for examination by light microscopy. The specificity of the staining by the conjugated antisera was indicated by the fact that the pre-incubation of the parasite tissues with unlabelled antisera abolished the staining reaction.

Chelation studies

Protoscoleces were aspirated from thin-walled fertile cysts and washed extensively with Medium 199 on a 40 μm mesh Teflon grid. Viable protoscoleces were transferred into Lab-Tek 8-unit tissue culture chamber slides (20–50 protoscoleces per chamber) and incubated for 60 min at 37° in 0·1 ml fresh unpooled human serum (from volunteers) which had been chelated with either EDTA (disodium ethylenediaminetetraacetic acid, Fisher) or EGTA (ethyleneglycol-bis (beta-amino-ethyl ether) N,N′-tetraacetic acid, Sigma) in a final concentration of approximately 10 mM (Fine *et al.*, 1972). Controls consisted of human serum with either saline substituted for the chelator or with the chelator saturated by the addition of 0·1 ml of 100 mM calcium chloride per ml of serum.

RESULTS

The presence of host IgM and IgG was demonstrated within the cyst membranes and on the surfaces of protoscoleces of *E. multilocularis*, with HRPO-conjugated antibodies rendered class specific by the use of affinity chromatography (Figs 1 and 2). The specificity of the reaction was indicated by the absence of staining in parasite tissues pre-incubated with unlabeled antisera. The photographs presented in Fig. 3 show that the laminated cyst membrane stains extensively with the labelled antisera raised

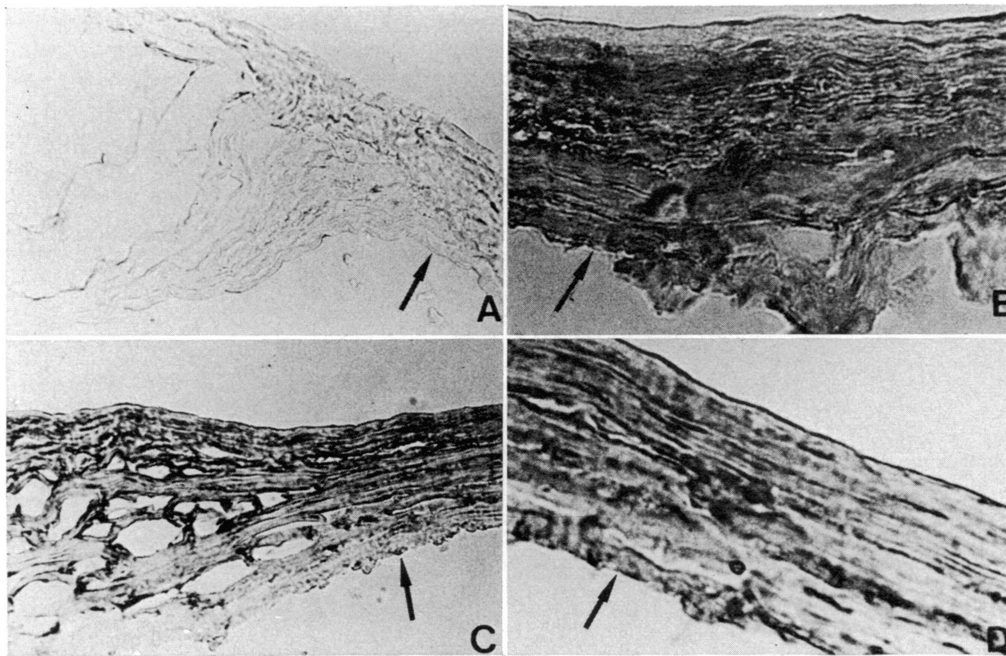

Figure 3. Photomicrographs of sections of the cyst wall of *E. multilocularis* exposed to horseradish peroxidase (HRPO)-conjugated antibodies. The arrows indicate the thin germinal membranes. Above this membrane, the striations of the laminated membrane can be easily seen.

(A) HRPO-normal rabbit serum (Magnification ×264.)
(B) HRPO-rabbit anti-cotton rat serum (Magnification ×525.)
(C) HRPO-rabbit anti-cotton rat IgG (Magnification ×247.)
(D) HRPO-rabbit anti-cotton rat IgM (Magnification ×525.)

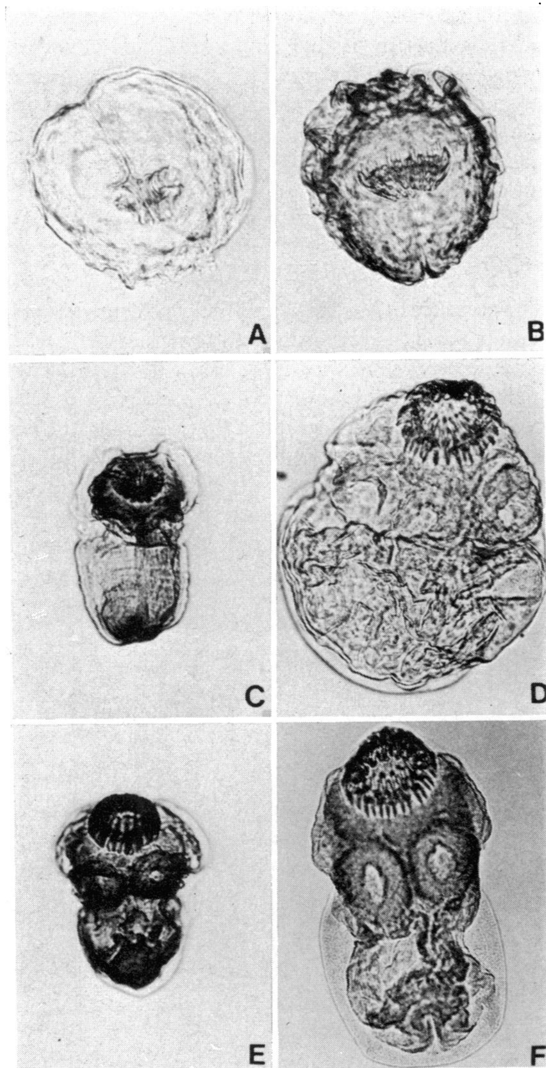

Figure 4. Photomicrographs of *E. multilocularis* protoscoleces exposed to HRPO-conjugated antibodies. (Magnification ×247.)
(A) HRPO-normal rabbit serum.
(B) HRPO-rabbit anti-cotton rat serum.
(C) HRPO-rabbit anti-cotton rat IgG.
(D) Same as C (vesiculating protoscolex).
(E) HRPO-rabbit anti-cotton rat IgM.
(F) Same as E (vesiculating protoscolex).

against whole host serum or against cotton rat IgG and IgM globulins indicating that these proteins form an important component of the structure of the parasite cyst.

The distribution of the host proteins in *E. multilocularis* cyst membranes is mainly restricted to concentric lines within the laminated membrane (Fig. 3B, C and D), similar to the results obtained by Coltorti & Varela-Diaz (1974) with *E. granulosus.* These authors did not detect the presence of any immunoglobulins on the germinal membrane of *E. granulosus*; our results, however, indicate that *E. multilocularis* contains host proteins on this membrane (Fig. 3B, C and D). This contradiction in results might be due either to an inherent difference between these two parasite species or, more likely, to the autofluorescence of the germinal membrane (Panaitesco, 1965) which could have camouflaged the fluorescence emitted by the FITC-conjugates used by Coltorti & Varela-Diaz (1974). The use of HRPO-ligands was not without its own problems since natural peroxidase activity is present in the parasite tissues; this activity was effectively inhibited (Figs 3A and 4A) by fixing the tissues in 0·074% HCl in ethanol (Weir *et al.*, 1974a). Natural peroxidase activity in the cyst membranes of *E. granulosus* was detected by Coltorti & Varela-Diaz (1974) while examining hydatid cysts for permeability to peroxidase. Since these authors did not find it necessary to inhibit this activity in their studies, the tissues of *E. granulosus* presumably contain less natural peroxidase activity than those of *E. multilocularis.*

Protoscoleces stained extensively following the incubation with HRPO-anti-whole cotton rat serum (Fig. 4B) or with HRPO-anti-cotton rat IgG and IgM (Fig. 4C, D, E, and F), indicating the presence of these host immunoglobulins on the surface of the larvae. However, it was observed that the intensity of the staining of the protoscoleces decreased with

Table 1. Effect of chelating agents on the lytic action of fresh human serum on protoscoleces of *E. multilocularis in vitro*

Chelator	Protoscolecidal activity
–	+*
EDTA	–
EGTA	–
EGTA + $CaCl_2$†	+

* + Indicates lysis of protoscoleces.
† 10 μl (100 mM).
Twenty to fifty protoscoleces were incubated for 1 h at 37° in 0·1 ml undiluted fresh human serum containing 10 μl of chelator (100 mM).

increase in size and with vesiculation of these metacestode larvae (Fig. 4D and 4F).

The *in vitro* lysis of viable protoscoleces by fresh human serum reported earlier (Kassis & Tanner, 1976a) is inhibited when the serum is chelated with either EDTA or EGTA (Table 1). The addition of calcium to EGTA-chelated serum restores the protoscolecidal activity of the serum.

DISCUSSION

Hydatid disease is a cyclozoonotic helminth infection which is caused by the larval fluid-filled cysts of *Echinococcus* sp. The cyst wall consists of an inner, highly nucleated, thin germinal membrane of parasite origin and an outer, nonnucleated, thick laminated membrane of host-parasite origin. The cysts are often fertile, containing large numbers of small larvae (the protoscoleces) which can differentiate either into adult worms in the intestine of canine or feline hosts or into cysts when inoculated parenterally into a susceptible animal (experimental secondary hydatidosis) (Lubinsky, 1960). It is also believed that protoscoleces can vesiculate and develop into cysts which are identical to the parent cyst following the rupture of a fertile hydatid cyst in the infected host (natural secondary hydatidosis. Since hydatid cysts develop in the presence of specific anti-*Echinococcus* antibodies and an unimpaired cellular response (Baron & Tanner, 1976; Rau & Tanner, 1976), it was of interest to study the host parasite interaction to clarify the ability of this parasite to survive in an immunocompetent environment.

Host serum immunoglobulins have been repeatedly demonstrated in the cyst tissues of *E. granulosus* (Coltorti & Varela-Diaz, 1972, 1974, 1975; Varela-Diaz & Coltorti, 1972, 1973). Our present results indicate that hydatid cysts survive in spite of the constant exposure of the germinal membrane to antibodies and suggest that such antibodies have no functional antigenic targets on the external surface of the membrane. The internal surface of the germinal membrane is, however, susceptible to relatively large quantities of anti-*Echinococcus* antibodies since the injection of fresh serum from infected animals into *E. granulosus* cysts *in vivo* causes the regression and death of the parasite (Kassis & Tanner, 1976b). Thus it can be stated that the germinal membrane alone is involved in protecting hydatid cysts against the humoral responses of the host. The laminated membrane, on the other hand, may well be responsible for a non-specific walling off of the germinal membrane from the cell mediated immune responses of the host.

We had demonstrated earlier that fresh human serum, as well as fresh sera from a number of different animal species, is lytic to the protoscoleces of *Echinococcus* sp. *in vitro* and that heat inactivation of the serum at 56° rendered it non-cytolytic (Kassis & Tanner, 1976a). In our present work, EDTA- or EGTA-treated human sera lose their protoscolecidal activity and the addition of calcium to EDTA-treated serum restores its ability to lyse these larvae, i.e. conditions under which calcium ions needed for complement activation via the classical pathway, are provided (Sandberg & Osler, 1971; Fine *et al.*, 1972). Therefore, the activation of the complement proteins by this metazoan parasite is mediated through the classical pathway and can not proceed via the alternate pathway; hence, the subsequent demonstration of host IgM and IgG on the surface of protoscoleces (Fig. 4) was not surprising.

Recently, Coltorti & Varela-Diaz (1975) concluded that the penetration of IgG into hydatid cysts might be a discontinuous or a random process since gerbil IgG was detected in the fluid of only 29·5% of cysts that had been transplanted from mice into the peritoneum of gerbils. In this paper, however, we demonstrate conclusively that host IgG and IgM are present on protoscoleces collected from more than fifty different cysts thus indicating that host macromolecules penetrate *all* hydatid cysts *in vivo*. Moreover, the presence of IgM with a mol. wt of about 900,000 daltons shows that the permeability of the cyst wall is not restricted to macromolecules below 200,000 daltons as suggested by Petithory (1969).

Since vesiculating protoscoleces have less antibodies bound to their surface than non-vesiculated larvae (Fig. 4), the previous demonstration that vesiculated protoscoleces are not susceptible to the lytic action of complement (Kassis & Tanner, 1976a) could be due to the fact that these latter larvae have little, if any, complement-fixing antibodies bound to their surface. Vesiculation of protoscoleces within a hydatid cyst, therefore, offers a mechanism by which *the parasite* can circumvent the effects of complement should a cyst rupture within the host. On the other hand, the attachment of complement-fixing antibodies to protoscoleces *in vivo* could protect *the host* against the effects of cyst rupture since these larvae would be killed by the complement proteins of the infected animal.

Since the protoscoleces are lysed by complement alone (Kassis & Tanner, 1976a), previous sensitization of a host is not necessary to limit a secondary infection; complement, therefore, plays a major role in determining the number of protoscoleces which will develop and controls the eventual parasite load. The few larvae that vesiculate rapidly and are thus resistant to lysis by complement (Kassis & Tanner, 1976a) escape this primary humoral defence line and are able to establish an infection in a naïve animal. On the other hand, natural secondary hydatidosis is unlikely to be induced by protoscoleces released from a ruptured hydatid cyst since complement, serum and cells from infected animals are cytotoxic to these larvae *in vitro* (Baron & Tanner, 1976; Kassis & Tanner, 1976a; Rau & Tanner 1976). It is possible, however, that natural secondary hydatidosis could either result from the growth of small foci previously suppressed by a pre-existing large hydatid cyst (Rau & Tanner, 1973) or by the growth of complement-resistant vesiculated larvae.

The lesions produced by the complement proteins on red blood cells, bacteria and viruses seem to be formed of a central hole surrounded by raised edges and have an average size of about 900 nm (Humphrey & Dourmashkin, 1969). Whether or not these pits are 'functional holes' is still a matter of conjecture. On the other hand, the lesions produced by complement on the surface of protoscoleces, when studied with the aid of electron microscopy (Kassis *et al.*, 1976), are much larger (70,000–310,000 nm) but are of similar doughnut shape. Moreover, sections through these complement-lysed protoscoleces show extensive disruption of the plasma membrane surrounding the larvae, indicating that, at least in this metacestode, the complement proteins can produce 'functional holes' in the plasma membrane. This system, therefore, offers a new model to study the lesions induced by complement and could help to define the mode of action of these cytotoxic proteins on metazoan organisms. This is the first report, to our knowledge, that the complement mediated lysis of a metazoan organism is activated by antibodies via the classical pathway. However, further studies are required to fully define the mechanisms by which these cestodes can exist in immunologically competent hosts.

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