The schistosome egg: development and secretions

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(*Received 7 May 2000; revised 7 September 2000; accepted 7 September 2000*)

SUMMARY

We have investigated the development of the schistosome egg and its secretions in order to understand how it migrates through gut tissues and also initiates pathology in the liver. We show by electron microscopy that the subshell envelope is absent in the newly deposited egg, but appears very early and differentiates as development progresses. In the mature egg, this nucleated envelope contains extensive endoplasmic reticulum, suggestive of a protein synthetic capacity. Furthermore, Reynolds' layer only appears between the envelope and the egg-shell in the mature egg and may represent its accumulated secretions. We have biosynthetically labelled and collected the secretions (ESP) released by mature but not immature eggs during culture. Their fractionation by SDS–PAGE reveals a simple pattern of 6 bands, differing markedly in composition from soluble egg antigen preparations. Electrophoresis in casein substrate gels demonstrates the presence of 2 distinct proteases in the egg secretions. By immunocytochemistry, ESP localized predominantly to the envelope of the mature egg, suggesting that this layer rather than the miracidium is the source of egg secretions.

Key words: schistosome egg, ultrastructure, development, secretion, protease.

INTRODUCTION

Adult worm pairs of the blood fluke *Schistosoma mansoni* reside in the hepatic portal system where the females deposit undeveloped eggs in the vessels of the gut wall. These eggs must undergo development, traverse the gut tissue to reach the lumen and be voided with the faeces to continue the life-cycle. Little is known about the secretions from eggs which facilitate their passage through the tissues but the process is known to be dependent on the immune response, in both rodents (Doenhoff *et al*. 1986) and humans (Karanja *et al*. 1997). From these observations it can be inferred that eggs release immunogens to mediate the inflammatory response which assists their tissue migration. Furthermore, it is plausible that such secretions from viable eggs embolized in the liver might initiate granuloma formation (Hang, Warren & Boros, 1974) which drives the pathogenic processes of schistosomiasis.

To date, there has been only 1 major ultrastructural study of the *S. mansoni* egg (Neill *et al*. 1988). The relative impermeability of the egg-shell is an obstacle to investigations, preventing the infiltration of embedding resins, and was circumvented by slam freezing and freeze substitution. This study revealed in the mature egg a subshell layer (Reynolds' layer) comprising microfibrils in a granular matrix. Beneath this and completely surrounding the miracidium and other structures was a thin envelope (von Lichtenberg's envelope) of squamous cellular origin. This is equivalent to the 'vitelline membrane' described in other trematode eggs (Wilson, 1967), and was believed by Neill *et al*. (1988) to represent a barrier to simple passive diffusion between the host extracellular fluids and the developing miracidium. They concluded that the envelope might regulate the transport of egg antigens and other embryonic products out of, and of nutrients into, the milieu surrounding the miracidium.

As part of a wider investigation of schistosome egg biology, we have examined the structures of both immature and mature schistosome eggs after conventional fixation and embedding procedures. We describe the development and differentiation of the subshell envelope and associated Reynold's layer. We have collected and characterized the egg secretions and shown that they are different in composition to the standard soluble egg antigen (SEA; Boros & Warren, 1970) preparation used by schistosome researchers and contain at least 2 proteases. Furthermore, the envelope possesses protein synthetic machinery and we demonstrate that egg secretions localise abundantly to this layer.

MATERIALS AND METHODS

Generation of parasite material

Groups of MF1 or CBA × C57Bl/6 (F1 cross) mice each were infected with 200 cercariae of *S. mansoni*. Seven weeks later they were sacrificed, their livers were removed, homogenized and digested with

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trypsin for 3 h at 37 °C. After incubation, the livers were sieved, the eggs collected by sedimentation and cleaned by washing 6 times in RPMI-1640 (Gibco). Mature and immature cleaned eggs were separated by layering on top of a mixture of 6 ml of Percoll (Pharmacia), 0·6 ml of 9% saline and 3·4 ml of RPMI-1640 and centrifuging for 15 min at 250 ***g***. The mature eggs collected at the bottom of the tube with immature eggs and some tissue debris remaining at the interface. The separate egg fractions were then washed a further 6 times in RPMI-1640 to remove the Percoll.

Preparation of soluble egg antigens (*SEA*)

Cleaned eggs were resuspended in 1 ml of PBS and sonicated for 4 × 1 min on full power with 1 min on ice between each sonication, centrifuged at 100 000 ***g*** for 60 min and the supernatant taken as SEA. The protein concentration was measured using a BioRad protein assay kit based on the technique of Bradford (1974).

Preparation for electron microscopy

Mature and immature eggs were prepared separately. Primary fixation was performed in 2·5% glutaraldehyde/4% paraformaldehyde in 100 mM phosphate buffer, pH 7·2, at 4 °C overnight. Groups of eggs were washed thoroughly in PBS and placed into a Petri dish on a dissecting microscope. Individual eggs were hand-pricked with a fine entomology pin, to allow the penetration of resin into the shell, and collected into an Eppendorf tube. Secondary fixation was by incubation in a 1% solution of osmium tetroxide in 100 mM phosphate buffer, pH 7·2, for 1 h on ice. The eggs were then embedded in Spurr's resin, 0·075 μm sections were cut, stained with lead citrate and uranyl acetate and examined on a Jeol JEM-1200EX transmission electron microscope.

Culture of mature eggs

After separation and cleaning, approximately 500 000 mature eggs were placed into a tissue culture flask with 30 ml of RPMI-1640 culture medium containing penicillin (300 U/ml), streptomycin (300 μg/ml) and gentamycin (500 μg/ml), but in the absence of serum supplements. Eggs were maintained for 72 h at 37 °C in 5% CO_2 and the culture medium was collected and concentrated in an Amicon Ultrafree-15 centrifugal ultrafiltration cell (5 kDa molecular weight cut-off) to a final volume of approximately 1 ml to give the preparation referred to as egg secreted protein (ESP). Viability of the eggs after the culture period was better than 85% as assessed by flame cell activity or muscular contraction of the miracidia. The protein content of the ESP was measured as before. Some aliquots of eggs were biosynthetically labelled by adding 0·3 μl/ml (0·158 MBq/ml) of ^{35}S-labelled methionine and cysteine (ProMix, Amersham) to the medium during the culture period. The counts incorporated into egg proteins were determined by sonicating known numbers of eggs for 1 min in the presence of 100 μl of 1% bovine serum albumin as a carrier protein and 200 μl of 10% trichloroacetic acid (TCA). The amounts of labelled protein secreted by immature and mature eggs during the culture period were determined on TCA-precipitated aliquots of culture medium, and related to the number of eggs present.

Gel electrophoresis

ESP proteins were separated on a 4–12% Bis–Tris SDS–PAGE gel (Novex) according to the manufacturer's instructions, with and without the presence of reducing agents in the sample buffer and the resulting gel stained with Coomassie Blue. Molecular weights were calculated on a GelDoc system (FlowGen), using Precision Protein Standards (BioRad) as calibrants. Dried gels of radio-isotope labelled protein preparations were exposed to autoradiography film for 16 h at −80 °C.

Protease substrate gels

ESP proteins were separated on a 15% SDS-PAGE gel using a BioRad Mini-Protean II system, without reducing agent. The gel included 0·1% casein to act as a protease substrate (Lockwood *et al*. 1987). After electrophoresis, the gels were washed twice for 30 min in 2·5% Triton X-100 (Sigma), to remove the SDS and renature the proteins. The gel was then incubated in a 50 mM Tris, pH 7·5 buffer, containing 200 mM NaCl, 5 mM $CaCl_2$ and 5 μM $ZnSO_4$, at 37 °C overnight and stained with Coomassie Blue as normal.

Preparation of antisera

One rabbit was immunized with 200 μg ESP in TiterMax Gold adjuvant (Sigma), bled after 4 weeks and serum prepared by centrifugation after clotting. The titre (50% maximum OD) of the antiserum was 1 in 3000 as assessed by ELISA.

Immunocytochemistry

Pieces of mouse liver were removed 7 weeks post-infection and frozen in OCT compound (Histological Equipment Ltd, Nottingham) by immersion in thawing isopentane. Sections (10 μm thick) were cut on a cryostat, air dried and fixed in acetone for 30 min at −20 °C. After drying, the sections were blocked by incubation in 10% normal goat serum in

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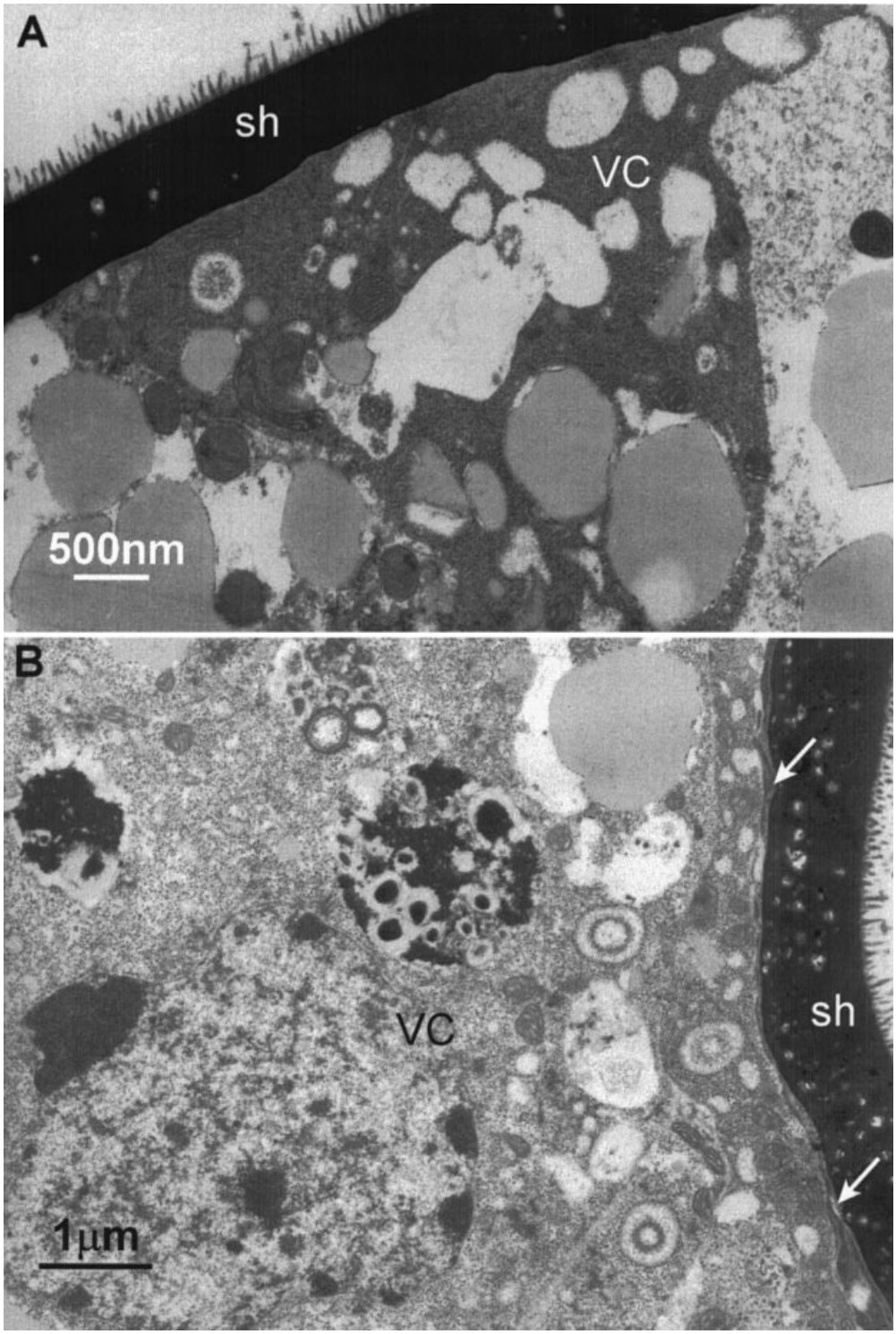


Fig. 1. (A) Transmission electron micrograph through the outer layers of an immature egg of *Schistosoma mansoni*. The only structures visible are the shell (sh) and the enclosed vitelline cells (VC). (B) Transmission electron micrograph through the outer layers of an intermediate egg, showing the presence of cytoplasmic plates (arrowed) interposed between the egg contents and the shell.

PBS–Tween for 30 min. The sections were then incubated in the anti-ESP antiserum diluted 1:300 with 10% normal goat serum in PBS–Tween for 90 min, washed 3 times in PBS–Tween and incubated for 30 min in an FITC-conjugated goat anti-rabbit IgG antibody (Vector Laboratories, Peterborough), diluted 1:100 in PBS–Tween with 10% normal goat serum. After a final wash, the sections were mounted in CitiFluor antifade mountant (CitiFluor, London). As a control, sections were incubated with a 1:300 dilution of the pre-immunization serum from the test rabbit, prior to incubation with secondary antibody, or with secondary antibody alone. To reduce the excessive

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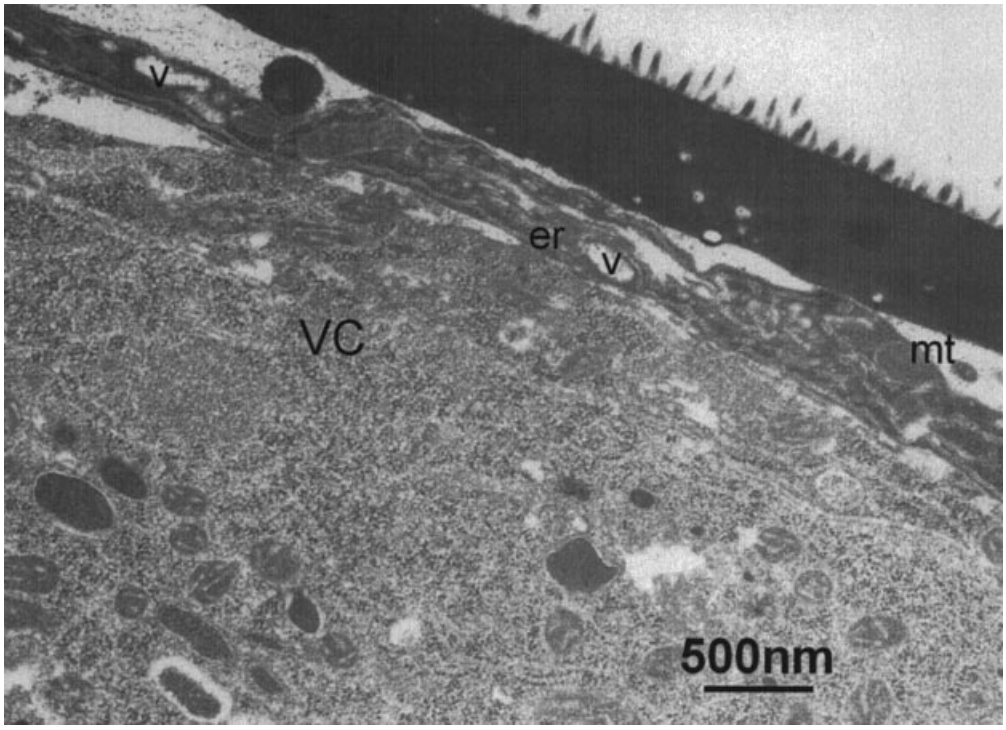


Fig. 2. Transmission electron micrograph through the outer layers of an intermediate egg. The vitelline cells (VC) are still present within the egg, but the envelope is now thicker and contains small mitochondria (mt), endoplasmic reticulum (er), ribosomes and vesicles (v).

yellow autofluorescence contributed by the egg-shell, sections were viewed on a Nikon Labophot fluorescence microscope fitted with a narrow band-pass filter for the green emissions.

Circumoval precipitation

Aliquots of mature and immature eggs were washed in PBS and 50 μl was combined with 50 μl of rabbit anti-ESP serum. This mixture was then pipetted into a chamber on a microscope slide, sealed with vaseline, and incubated for 24 h at 37 °C before examination and photography.

RESULTS

Newly deposited eggs lack complex subshell structures

Viewed by light microscope the newly deposited egg is small ($\bar{x} = 111{\cdot}9 \pm$ S.E.$2{\cdot}2 \times 44{\cdot}0 \pm 0{\cdot}5$ μm, $n = 10$) with highly granular contents and no visible sign of the complex arrangement of membranes and vesicles which surrounds the miracidium in the mature egg ($\bar{x} = 148{\cdot}8 \pm$ S.E.$1{\cdot}4 \times 68{\cdot}7 \pm 1{\cdot}4$ μm, $n = 10$). This lack of structures is confirmed by electron microscopy on undeveloped eggs (Fig. 1A). They are each bounded by the cross-linked protein shell bearing a dense array of microspines on its outer surface. The bulk of the egg contents comprises nucleated vitelline cells with cytoplasm rich in mitochondria, lipid droplets, vesicles of various kinds and granular inclusions, possibly glycogen. The plasma membranes of the vitelline cells lie in direct contact with the inner surface of the egg-shell with no sign of intervening material.

Subshell structures appear as eggs develop

It is difficult to classify precisely how far an individual egg has progressed in development, but by examining a number of eggs it is possible to construct a plausible sequence of events. At an early stage, very thin plates of cytoplasm become interposed between the egg-shell and the intact, metabolically active vitelline cells (Fig. 1B). These plates, bounded on both surfaces by plasma membranes, are presumably extensions from 1 or more parent cells (see Fig. 2) and in thicker parts, internal structure is apparent. As development proceeds, the plates become a continuous layer of cytoplasm 200–300 nm in thickness, separating the vitelline cells from the shell and completely enclosing the egg contents. As such, the layer must represent the proto-envelope. At this stage, the layer has a smooth inner and outer surface; its granular cytoplasm contains small mitochondria and lamellate membranous structures extending laterally throughout. A small amount of flocculent material is apparent in the space between the layer and the shell.

The subshell envelope is a synthetically active tissue

In the fully developed egg, the envelope attains a thickness of 500–600 nm, and has a highly differen-

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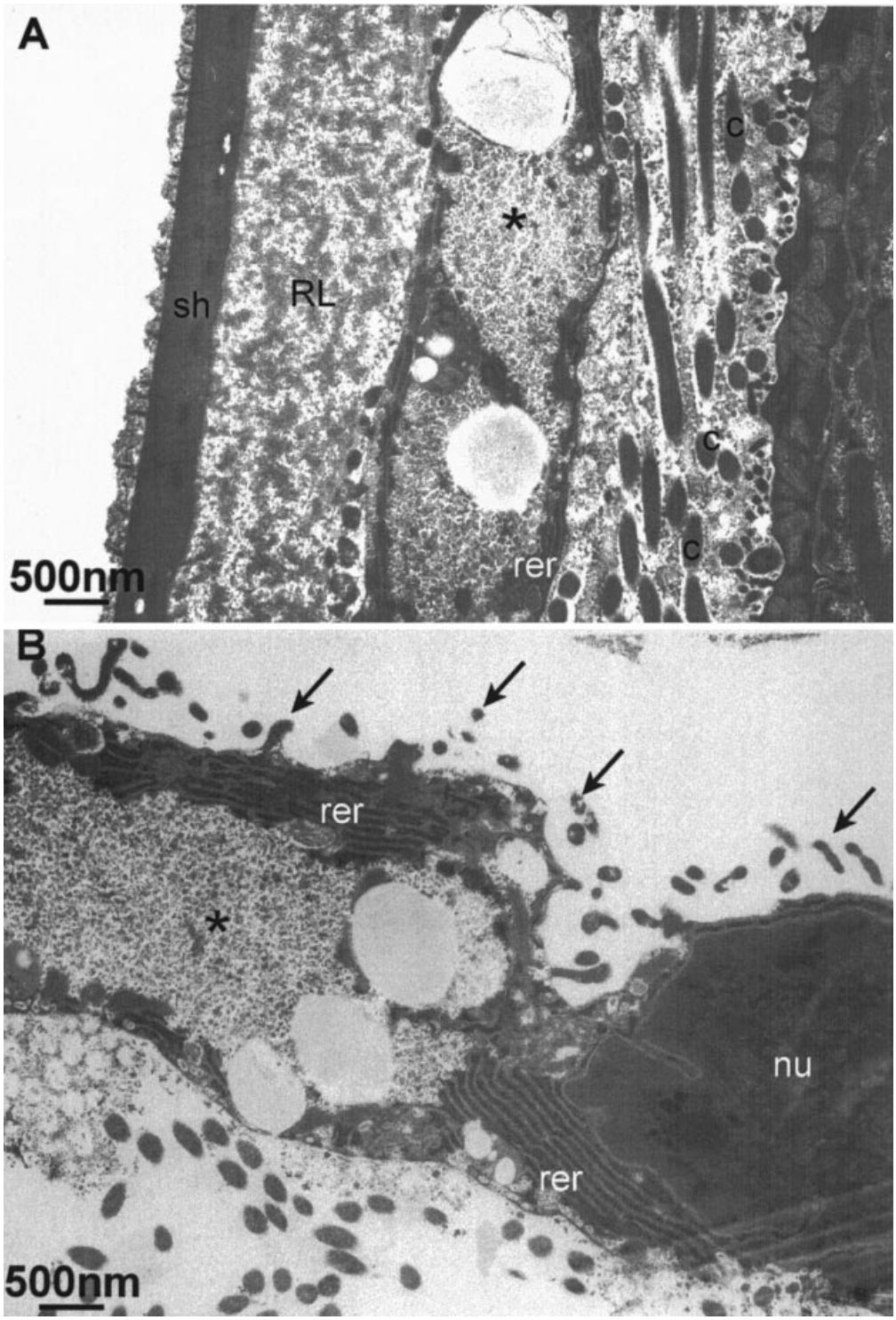


Fig. 3. Transmission electron micrographs through the outer layers of mature eggs. (A) The fully developed envelope lies between the shell (sh) and Reynolds' layer (RL) on the left and the cilia (c) of the miracidium on the right. The presence of large expanses of rough endoplasmic reticulum (rer) is indicative of a protein synthetic role for the envelope. (B) The contents have pulled away from the shell during preparation, highlighting the presence of the projections from the outer surface of the envelope (arrowed). This image also contains one of the nuclei (nu) of the envelope. Also notable in both images is the presence of large expanses of granular material within the envelope (*)

tiated cytoplasm (Fig. 3A, B). Nuclei can clearly be identified confirming the cellular (syncytial?) nature of the layer. The external plasma membrane is drawn out into numerous squat projections reminiscent of microvilli, whilst the inner membrane is smooth. The cytoplasm is divided into distinct regions. Large vesicles are present, probably for lipid storage, whilst the greatest area is occupied by micro-aggregates of granular material. The other most noticeable feature is the expanse of regular,

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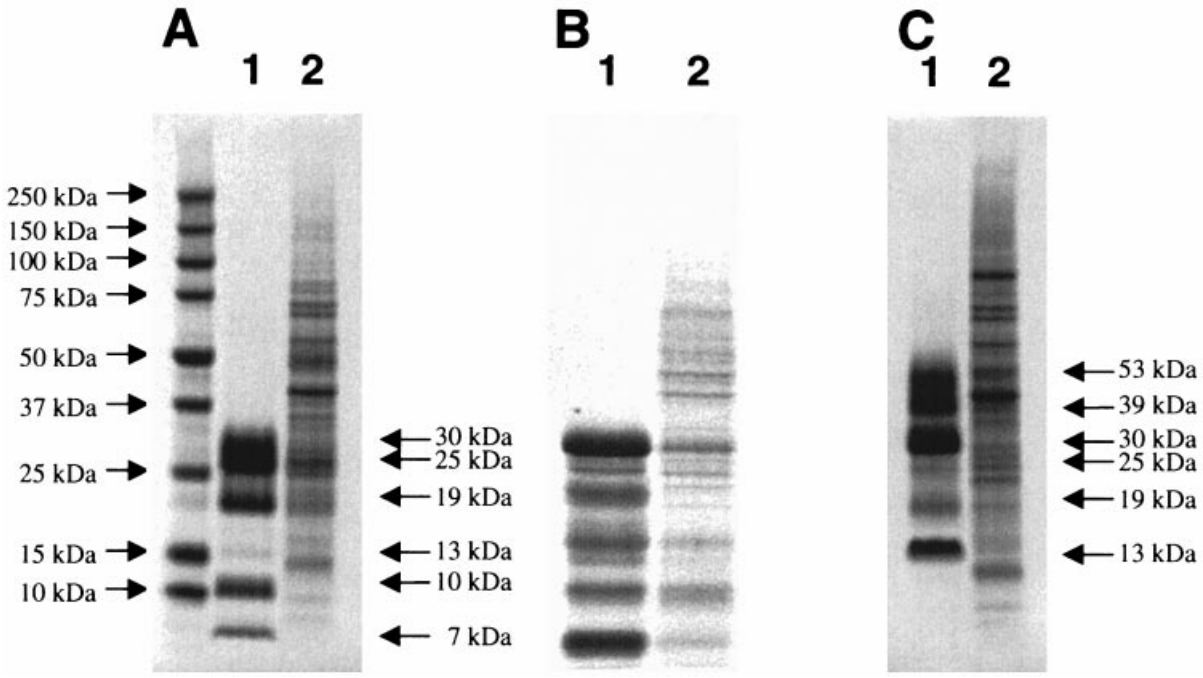


Fig. 4. (A) Coomassie-stained SDS–PAGE gel of ESP (lane 1) and SEA (lane 2). (B) Autoradiograph of an SDS–PAGE gel of ESP and SEA, showing the biosynthetic labelling of ESP during the culture period. (C) SDS–PAGE gel of ESP and SEA run without reducing agents in the sample buffer. Two bands are present in ESP which have higher molecular weights than any band in the reduced sample.

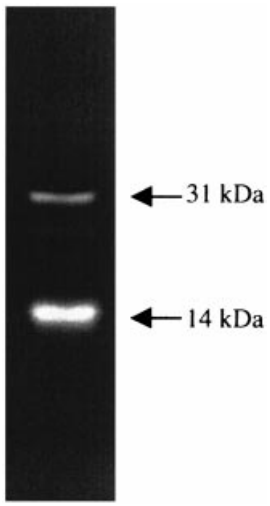


Fig. 5. Coomassie-stained protease substrate gel of ESP. The 2 clear bands indicate the presence of active proteases in the gel.

tightly packed, rough endoplasmic reticulum (ER), indicative of protein synthetic activity. The cytoplasm between the ER cisternae is very dense, and in places contains aggregates of small vesicles. It is possible that the extensive granular micro-aggregates lie in ballooned cisternae of the ER (reminiscent of mammalian plasma cells), rather than free in the cytosol. In the mature egg, the space between the envelope and the shell (Reynolds' layer) is extensive (1 μm wide) and filled with heterogeneous granular material. The microspines on the outer surface of the shell entrap a layer of material, very similar in appearance to the contents of Reynold's layer. The miracidium lies adjacent to the envelope with some of its cilia in direct contact, and is bathed in a milieu of free, dense material, different in texture from either the envelope contents or Reynolds' layer.

Cultured eggs synthesize and release a simple mixture of proteins, containing 2 proteases

Mature eggs isolated from livers and intestines, and cultured *in vitro* for a 3-day period in RPMI-1640, released significant amounts of protein (2·63 μg/1000 eggs) whereas immature eggs were much less active (0·32 μg/1000 eggs). When the filter-concentrated culture supernatants from mature eggs were fractionated by SDS–PAGE a simple pattern of 6 bands (M_r 7, 10, 13, 19, 25 and 30 kDa) was revealed, quite distinct from the very complex pattern characteristic of SEA (Fig. 4A). Indeed, the highly enriched released proteins are either not detectable or present at low abundance in SEA. Mature and immature eggs were cultured in RPMI containing ^{35}S-labelled amino acids, and the TCA-precipitable counts determined. In a typical experiment ($n = 3$) 1000 mature eggs incorporated 86752 cpm during culture whilst immature eggs incorporated a roughly similar 69252 cpm. During the culture period, 1000 mature eggs released 13299 cpm of TCA precipitable material whilst the same number of immature eggs released only 1122 cpm. When the material released by mature eggs was characterized by electrophoresis and detected by autoradiography, a similar pattern of

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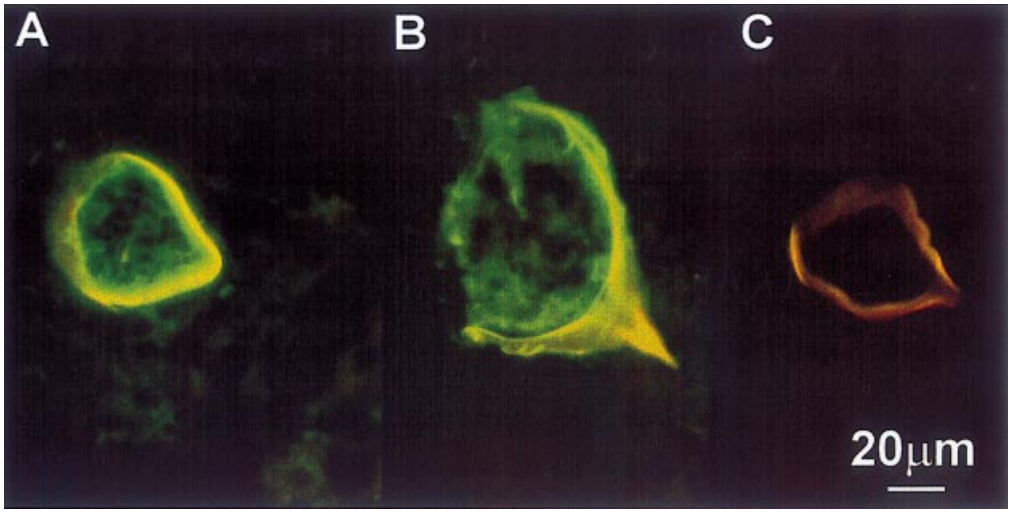


Fig. 6. Immunocytochemical localization using antisera raised against ESP. In addition to the yellow autofluorescence of the shell, the most intense positive (green) staining can be seen immediately beneath the shell, corresponding to the position of the envelope (A) or beneath the shell, and around the outer surface suggestive of release from the egg (B). Sections probed with pre-immunization rabbit serum show no positive reaction (C).

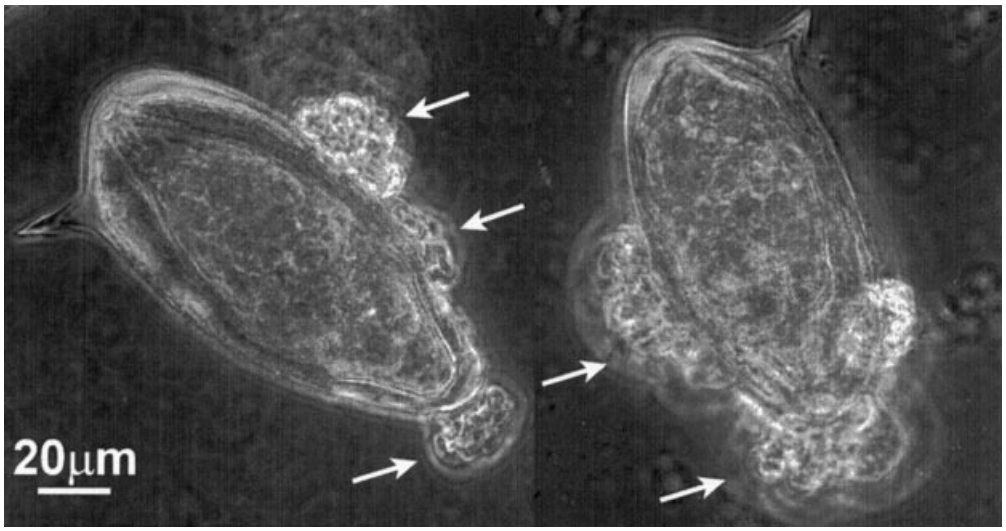


Fig. 7. Circumoval precipitation (arrowed) of ESP proteins by a rabbit anti-ESP antiserum demonstrates the active secretion of ESP from live mature eggs.

6 bands to that seen by Coomassie staining was observed (Fig. 4B), but with differing intensities. This demonstrates the *de novo* synthesis and release of proteins during the 3-day culture period. Furthermore, the pattern of labelled released proteins is closer to that obtained by fractionation of labelled SEA made from cultured eggs, suggesting that in mature eggs the major synthetic activity is the production of protein for secretion.

If the ESP proteins are separated without the presence of a reducing agent in the sample buffer (Fig. 4C), the pattern of bands is slightly different, with the 2 lowest molecular weight bands (7, 10 kDa) absent, and an additional 2 bands present at higher molecular weight (39, 53 kDa). This indicates that at least some of the proteins in the mixture exist as disulphide-bonded oligomers. Inclusion of casein as a protease substrate in the SDS–PAGE gel prior to electrophoresis allowed the detection of proteolytic activity in the ESP mixture (Fig. 5). The 2 distinct clear bands (molecular weights, 14 and 31 kDa, corresponding approximately with the bands at 13 and 30 kDa), where the substrate has been digested, demonstrate that there are at least 2 separate proteases secreted by the egg.

Immunocytochemistry reveals that egg secretions originate in the subshell envelope

Immunocytochemistry on cryostat sections of infected mouse liver revealed that, among all the subshell structures, the most intense staining was in the envelope (Fig. 6). Positive reactions were seen immediately outside the egg-shell and could also be discerned in the surrounding granulomatous tissue, suggestive of release and diffusion away of the

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secreted proteins. No reactivity was observed with either pre-immunization serum or with the secondary antibody alone.

Circumoval precipitation demonstrates secretion by live eggs

Incubation of live mature eggs for 24 h in the presence of rabbit anti-ESP serum resulted in the formation of characteristic circumoval precipitations (Fig. 7). These were localized to particular regions, rather than being uniformly distributed around the circumference of the egg. This implies variations in shell permeability, but with no obvious pattern between eggs. No such precipitations were observed around immature eggs or when normal rabbit serum was used as a control.

DISCUSSION

In view of the importance of the schistosome egg in pathogenesis, it is remarkable that its basic 'physiology' has received so little attention. The newly laid egg of *S. mansoni* shows a relatively simple organization, with a tanned protein shell surrounding a single ovum and 20–30 vitelline cells. The lack of a subshell envelope at this stage (so evident in mature eggs) is not perhaps surprising since its presence would impede the release of granules from the vitelline cells and their coalescence to form the shell. Thus, the plasma membranes of the numerous vitelline cells lie in direct contact with the egg-shell. When such eggs are cultured *in vitro* in defined medium plus a serum supplement they will develop to maturity in 7 days (Michaels & Prata, 1968). The absence of development in simple medium strongly suggests a need for an external source of nutrients and growth factors, in complete contrast to the egg of *Fasciola hepatica* which is self-contained and will develop to maturity in distilled water (Wilson, 1967). This need is confirmed by the uptake and incorporation by schistosome eggs of labelled amino acids into newly synthesized proteins, and by the increase in egg size during development. Comparison of the volumes of newly laid and mature eggs, using the formula for a prolate spheroid ($\frac{4}{3}\pi ab^2$), reveals a 3·24-fold increase during development. Our method for separating undeveloped and mature eggs, using Percoll density gradients relies on densities of $<1{\cdot}06$ and $>1{\cdot}09$ g/ml respectively. This implies that a schistosome egg increases in mass by a factor of 3·33 during development, i.e. more than 2/3 of the constituents of the fully mature egg are derived from external nutrients. The increased size also reveals that the egg-shell must have considerable elasticity.

We observed that early in development, cytoplasmic plates interpose between the vitelline cells and the shell. The subsequent growth and differentiation of this layer and the identification of nuclei within it, reveals its cellular origin. Indeed, in this respect, *S. mansoni* does not appear to differ from other digenetic trematodes, where this envelope is referred to as a vitelline membrane (strictly inaccurate, since it is not the direct secretion of a fertilized egg). Morphological studies on digeneans as diverse as *Fasciola* (Schubmann, 1905; Ortmann, 1908), *Paragonimus* (Chen, 1937) and *Parorchis* (Rees, 1940), showed that the envelope is derived early in development (after the first 7 or 8 divisions of the ovum) from cells which separate from the embryonic miracidium. These cells migrate to the inner surface of the shell where they flatten and extend to form the continuous lining. In our present study, this phase is represented by the partially differentiated egg, where mitochondria are evident within the envelope, implying metabolic activity; the first signs of an endomembrane system are also apparent. From the continuous and cellular nature of this layer, it is clear that it must exert significant control over the transport of materials into and out of the egg.

Our observations on the mature envelope reveal a more organized structure than reported by Neill *et al*. (1988). We can ascribe this in part to our use of glutaraldehyde as a primary fixative on isolated eggs, together with the puncture of the egg-shell to allow ingress of resin, compared with osmium fixation after slam freezing and freeze substitution of intestinal tissue samples in the earlier study. The presence of extensive rough ER indicates high levels of protein synthetic activity. The occurrence of extensive aggregates of granular material in the mature envelope may imply storage of the newly synthesized protein prior to exocytosis from the layer. An analogous situation is found in the egg of *Fasciola* where the microfibrils of the viscous cushion involved in egg hatching are laid down entirely within the confines of the equivalent vitelline membrane layer (Wilson, 1967). In the *S. mansoni* egg, since the vesicles in the envelope associated with the ER lie close to both the inner and outer bounding membranes, we cannot make any deductions from the morphology of this layer about the direction of export. The presence of microvillar-like extensions on the outer surface of the mature (but not immature) envelope supports an inward transport function for this layer, presumably of the large quantities of nutrients required to explain the increase in mass. The maximum thickness of the envelope and its full capacity for protein synthesis are only apparent in the mature egg.

The occurrence of macromolecular material between the egg-shell and the envelope is slight in the partially developed egg, but becomes very evident as the egg matures and is sufficiently prominent to be termed Reynolds' layer by Neill *et al*. (1988). The origin of this layer from outside the egg seems unlikely, since the shell is inert and so would not operate differentially in partially and fully developed

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eggs (unless the stretching of the shell as egg volume increased opened pores to allow the ingress of host proteins). A more likely explanation is that the material in Reynolds' layer originates either within the envelope itself, or from the internal milieu surrounding the miracidium. An origin within the envelope accords with the large expanses of granular material in the cytoplasm. Since the envelope and Reynolds' layer reach their maximum thickness and complexity as the egg matures, it is tempting to conclude that their formation is related to the process of egg transit through the intestinal tissues. Cultured mature eggs secreted much more protein than immature eggs, an observation confirmed by the relative amounts of biosynthetically labelled protein released. We suggest that this is a device to prevent premature expulsion of the eggs from the tissue environment which is clearly conducive to their development. In this context, the accumulation of Reynolds' layer and the slow diffusion of its constituents through the egg-shell pores (Race *et al.* 1969), suggest that the egg acts as a slow release capsule. Furthermore, the presence of similar granular material entrapped by the surface microspines may represent the sequestration of secretions in the immediate vicinity of the egg, thus focussing the host inflammatory response. We raised antibodies against ESP to investigate the relationship between egg development and secretion using the circumoval precipitation reaction as an indicator. Our observations confirm the abundant egress of proteins from the mature, but not the immature, egg.

The pattern of proteins secreted by mature eggs (ESP) was markedly different from the total egg homogenate represented by the standard SEA preparation. No constituent of ESP had an M_r of >40 kDa whereas constituents of SEA ranged up to >200 kDa. This could imply size selection by pores in the egg-shell, or partial degradation of proteins after release. The distinct patterns indicate a compartmentalization of egg proteins which could presage a difference in host responses to the products of live and dead eggs, with obvious implications for granuloma formation. The ability to label the subset of released proteins biosynthetically confirms that they are actively secreted molecules, not simply the leakage products of dead eggs. In contrast to the disparity between ESP and SEA, there were similarities in composition between the ^{35}S-labelled proteins in the 2 fractions from cultured eggs. We interpret this to mean that the synthetic activity of mature eggs is largely centred on the secreted subset of proteins. This accords with the putative function of these proteins in the process of egg migration through the tissues and our identification of 2 proteases secreted by the mature egg is particularly pertinent in this context. Their presence supports the possibility that secreted proteins are partially degraded during the culture period. It is difficult to relate our observations to previous studies which have characterized major egg antigens from SEA. When Western blots of ESP and SEA were probed with monoclonal antibodies against p40 (Nene *et al.* 1986), kindly donated by Dr M. Stadecker, a single band was recognized in SEA, but no reactivity was seen in ESP, suggesting that p40 is not present. Probing with polyclonal antisera raised against other native SEA antigens (e.g. α_1 and ω_1, Dunne *et al.* 1981) resulted in the recognition of multiple bands in both ESP and SEA, suggesting that the antibodies recognized common glycan epitopes present on several proteins.

Immunocytochemistry with an antiserum directed against the secretory proteins strongly suggests that they localize within the envelope, not the miracidium or its surrounding milieu. Thus, the presence of protein synthetic machinery in the mature envelope, the likelihood that it is a barrier to passive diffusion, the coincidence of its differentiation with this onset of ESP secretion and the immunolocalization of ESP all lead us to conclude that the envelope is the source of the macromolecules which mediate egg transit through the gut tissues. We are currently undertaking the detailed characterization of the egg secretions, especially the 2 proteases, and their roles in egg excretion or as initiators of hepatic pathology.

This work was funded by grants from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and European Commission's BIOTEC programme. B.S. was funded by a BBSRC CASE Studentship with the Natural History Museum, London. We would like to acknowledge the technical assistance of Mr Srdjan Ljubojevic with the recovery and culture of the eggs.

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https://doi.org/10.1017/S0031182001007351 Published online by Cambridge University Press