# Immunolocalization of the fatty acid-binding protein Sj-FABPc within adult *Schistosoma japonicum*

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SUMMARY

This paper describes the first localization study of the 14·7 kDa fatty acid-binding protein in *Schistosoma japonicum* (Sj-FABPc) using transmission electron microscopy. A polyclonal antibody raised against recombinant Sj-FABPc was used in combination with a colloidal gold marker to determine the distribution of the protein within adult parasites. Sj-FABPc was localized within lipid droplets below the subtegumental region of the male parasite. Additionally, Sj-FABPc was present in the vitelline droplets of the vitelline glands of female parasites. There were no detectable levels of Sj-FABPc on the surface or within the tegument of male or female parasites. Possible functions of Sj-FABPc within *S. japonicum* and the relevance of these immunolocalization findings in light of the recent reports that the homologue Sm-FABPc is an important anti-*S. mansoni* vaccine target molecule are also discussed.

Key words: *Schistosoma japonicum*, FABPc, electron microscopy, immunolocalization, cryopreparation, vitelline glands.

## INTRODUCTION

Schistosomiasis *japonica* and *mansoni* are chronic parasitic diseases which cause extensive liver damage in humans. The associated pathology is caused mainly by the lodging of eggs (produced by paired schistosomes) in the wall of the hepatic portal vein of the host (Wiest *et al*. 1992). Schistosomiasis is estimated to infect 200 million people throughout the world (WHO, 1993). In an attempt to control this debilitating disease, a vaccine against schistosomiasis has been sought because the possibility of drug resistance and the need for re-administration of treatment makes chemotherapy an ineffective long-term measure (McManus *et al*. 1993).

Schistosomes cannot produce fatty acids or sterols and are presumed to be dependent on the host for these molecules (Meyer, Meyer & Bueding, 1970). Fatty acid binding proteins (FABPs) appear to be involved in the acquisition and utilization of these molecules in other organisms (Glatz & van der Vusse, 1990). FABPs have been biochemically and genetically examined in a number of parasites including *Fasciola hepatica* (see Rodriguez-Perez *et al*. 1992), *Schistosoma mansoni* (see Moser *et al*. 1991, Tendler *et al*. 1996) and *S. japonicum* (see Becker *et al*. 1994). The possibly crucial biological functions of FABPs have stimulated much interest in these molecules as vaccine targets and up to 67% protection against *S. mansoni* cercarial challenge in mice has been reported following immunization with recombinant Sm14 FABP (Tendler *et al*. 1996). Since schistosomes and other parasites use FABPs in important metabolic processes, further characterization of these proteins is needed. The aim of the work described in this paper was to localize Sj-FABPc within adult *S. japonicum*.

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## MATERIALS AND METHODS

### *Parasites*

The *S. japonicum* (Philippine and Chinese strains) life-cycles were maintained in *Oncomelania hupensis* snails and BALB/c mice. Adult worms were perfused from mice 40–45 days post-infection with cercariae. Mice were heparinized (50 units/mouse) and killed by $CO_2$ inhalation. Adult parasites were obtained by perfusing the portal veins of mice with warm phosphate-buffered saline (PBS).

### *Production of anti-FABP antibodies*

Purified 14·7 kDa functionally active recombinant cytoplasmic fatty acid-binding protein from the Chinese strain of *S. japonicum* was used to immunize rabbits (see Becker *et al*. 1994). The rabbits were injected subcutaneously 3 times at 2-weekly intervals with the recombinant protein in Freund's Complete

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Adjuvant (CFA) and injected with a final boost without adjuvant. Sera were collected 10 days after the final boost.

### *Western blot analysis*

After separation of paired parasites, 5 male or 5 female parasites were placed into 250 $\mu$l of SDS–PAGE loading buffer. The parasites were sonicated, centrifuged and heated in a 100 °C water bath for 3 min. Electrophoresis was performed on a 15 % SDS–PAGE gel, lanes were then either stained with toluidine blue or the proteins were electrophoretically transferred from the gel onto nitrocellulose membranes in 10 mM cyclohexylaminopropane sulfonic acid, pH 11, (CAPS) containing 10 % (v/v) methanol. Non-specific binding sites on the nitrocellulose membrane were blocked with 5 % (w/v) skimmed milk in PBS containing 0·05 % (v/v) Tween20. The primary anti-FABP antibody, diluted 1 : 300 in blocking solution, was applied overnight at 4 °C. Excess primary antibody was removed and a peroxidase-labelled IgG second antibody (goat anti-rabbit, Silenus) then applied for 2 h at room temperature. Excess antibody was removed and the bound secondary antibody visualized with a chemiluminescence kit (Renaissance Kit, Dupont) as described in the instructions provided by the manufacturers.

### *Conventional processing for transmission electron microscopy*

Male and female parasites were separated and dissected, the medial region removed and cut into small (1–2 mm in length) regions and processed further. Parasites were fixed overnight at 4 °C in 3 % (v/v) glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4. Parasites were washed in cacodylate buffer, and then post-fixed with 1 % (w/v) aqueous osmium tetroxide and washed in water. Samples were placed in 1 % (w/v) aqueous uranyl acetate and sequentially dehydrated through 50 % to 90 % (v/v) ethanol and then 100 % acetone. Samples subsequently were infiltrated with Spurr epoxy resin, orientated in embedding molds and the resin polymerized at 70 °C for 10 h. Ultrathin (70–90 nm) sections were cut on an LKB III ultramicrotome, and placed onto 300 mesh copper grids. Sections were contrasted with uranyl acetate and lead citrate and subsequently viewed and photographed with a JEOL 1200EX transmission electron microscope.

### *Cryo-ultramicrotomy*

Adult *S. japonicum* were either fixed in 4 % paraformaldehyde in 0·1 M cacodylate buffer, pH 7·4, for 3 h or were fixed and contrasted after the primary aldehyde fixation by a 1 h incubation with osmium tetroxide. The parasites were cut into small sections (approximately 1–2 mm in length) briefly washed in PBS and cryoprotected for a minimum of 1 h in 1·8 M sucrose and 20 % (v/v) polyvinylpyrrolidone (PVP) (Tokuyasu, 1989). Parasite tissue was orientated onto cryo-ultramicrotomy specimen pins and plunged into liquid nitrogen.

Ultrathin cryosections were cut at −120 °C using a RMC cryo-ultramicrotome. Ultrathin sections (approximately 90–100 nm thick) were collected from the knife with a wire loop containing 2·3 M sucrose and placed onto 200 mesh nickel grids coated with colloidin. Grids were then floated, sections down, on PBS in a Petri dish and stored at 4 °C prior to immunolabelling.

### *Immunolabelling for transmission electron microscopy*

All procedures were carried out at room temperature unless otherwise noted. Non-specific binding sites were blocked by incubating sections with 2 % BSA in PBS for 40 min. Cryosections were then incubated with rabbit anti-Sj-FABPc antibody (diluted 1 : 100 in PBS) for 40 min. As a negative control, immune serum was substituted with normal rabbit serum as primary antibody. Non-reacted primary antibody was removed with 2 × 10 min washes with PBS. Cryosections were then incubated on goat anti-rabbit IgG conjugated to 15 nm gold particles (diluted 1 : 100 in PBS) for 40 min.

Cryosections were washed with PBS for 2 × 5 min to remove non-complexed secondary antibody and subsequently distilled water 3 × 2 min to remove PBS. Grids were placed section down onto droplets of 1 part 1 % (v/v) aqueous uranyl acetate and 9 parts 2 % (v/v) aqueous polyvinyl alcohol (MW 10000, Sigma) solution for 10 min and air dried after removal of excess solution (Tokuyasu, 1989). Cryosections were then viewed and photographed with a JEOL 1200EX transmission electron microscope.

### *Osmium tetroxide contrasting of cryosections*

Some cryosections of tissue not previously treated with osmium tetroxide were further fixed and contrasted. Cryosections stored on PBS were floated onto distilled water (to remove PBS) and then onto 1 % osmium tetroxide for 30 min. After another distilled water wash, cryosections were dehydrated through 50 % (v/v) and 70 % ethanol to allow the deposition of electron opaque osmium. Cryosections were rehydrated, then embedded and contrasted with uranyl acetate and PVA (as described above), and viewed.

## RESULTS

A number of complementary techniques were used to fully define the immunolocalization detected in this study. The justification of these techniques is explained in the Discussion section of this paper.

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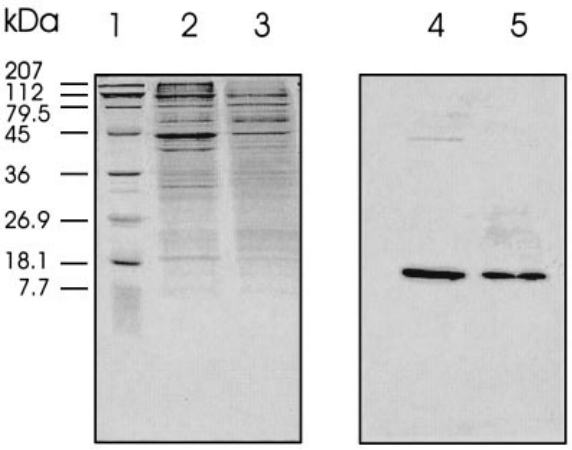


Fig. 1. Western blot analysis of whole *Schistosoma japonicum*. Lane 1, molecular mass indicators, stained with toluidine blue; lanes 2 and 3, total extract of male (2) and female (3) parasite, stained with toluidine blue; lanes 4 and 5, binding of anti-Sj-FABPc antibody to male (4) and female (5) parasite extracts, detected with chemiluminescence. Note the dominant band in lanes 4 and 5 at approximately 15 kDa.

### *Specificity of anti-Sj-FABPc antibody*

Western blot analysis demonstrated the specificity of the primary antibody used. The anti-Sj-FABPc antibody detected a single band with a molecular weight of approximately 15 kDa in male and female parasites (Fig. 1).

### *Immunolabelling of cryosections*

Negative controls (normal rabbit serum) were run concurrently for all parasite samples examined by immunocytochemistry. No immunoreactivity was detected when normal rabbit serum was substituted for anti-FABP primary antibody (data not shown). Sj-FABPc was detected in cryosections of *S. japonicum* within two structures, one type in the female and a different type in the male.

Electron micrographs of cryosections of female *S. japonicum* labelled with anti-Sj- FABPc antibody are presented in Fig. 2A and C. No immunoreactivity was seen in the tegument region. Vitelline cells were seen within vitelline glands deep in the parenchyma of the female parasite (Fig. 2A). At higher magnification, positive immunolabelling for Sj-FABPc was evident within the vitelline droplets (approximately 300 nm in diameter) of the female parasite (Fig. 2C). No other lipid droplets were seen to immunoreact with anti-Sj-FABPc (data not shown).

Cryosections of male *S. japonicum* also showed labelling for Sj-FABPc (Fig. 2B and D). As with the female worms, no immunoreactivity was noted in the tegument of male parasites. Immunolabelling was detected in regions below the muscle layer (Fig. 2D). Fig. 2B shows the relative position of the labelled region (arrowheads) in relation to the tegument in a sample not treated with osmium. A higher magnification shows immunoreactivity within filamentous structures (arrowheads), but no specific structures reacting positively with the anti-Sj-FABPc antibody could be identified (Fig. 2D). In an attempt to better visualize the structures labelling for Sj-FABPc in male parasites, osmium tetroxide contrasting of cryosections was used, but this did not result in any definable structure being visualized (data not shown).

Osmium tetroxide fixation of male *S. japonicum* tissue was also performed prior to cryoprotection and cryosectioning. Subsequent immunolabelling detected Sj-FABPc within lipid droplets which were located below the muscle layer of the parasite (Fig. 3). Immunolabelling levels were reduced, compared to conventional cryosections. No immunolabelling was detected in the tegument region. The lipid droplets that contained Sj-FABPc were spherical and approximately 500 nm in diameter.

### *Ultrastructure of resin sections*

Resin sections of male and female adult *S. japonicum* were examined for ultrastructural analysis of lipid and vitelline droplets, to confirm the identity and relative position of the structures observed in the cryosections. Fig. 4A shows the position of vitelline cells containing vitelline droplets within the female parasite. Fig. 4C shows a higher magnification of these droplets. The vitelline droplets appeared spherical and approximately 200–300 nm in diameter.

The male parasite contained lipid droplets below the muscle layer (Fig. 4B). Lipid droplets were less opaque than vitelline droplets and were only contrasted in tissue processed with osmium tetroxide. The lipid droplets within the male parasite were approximately 500–1000 nm in diameter and spherical (Fig. 4D).

## DISCUSSION

This is the first study describing the distribution of a fatty acid-binding protein (Sj-FABPc) in *S. japonicum* and also represents the first use of electron microscopy in the localization of a FABPc in any of the *Schistosoma* species.

Western blot analysis was used to confirm the specificity of the polyclonal antibody used in this study. A strong band at approximately 15 kDa corresponds to the reported size of Sj-FABPc from *S. japonicum* (see Becker *et al*. 1994). The band was detected in both male and female parasites, indicating there is no sex specificity for this molecule in *S. japonicum*.

Cryopreparative techniques for immunolabelling were used to retain the antigenicity of Sj-FABPc. The protein was detected in the parenchyma of *S. japonicum*. In the female parasite, vitelline droplets were identified as containing Sj-FABPc. The male

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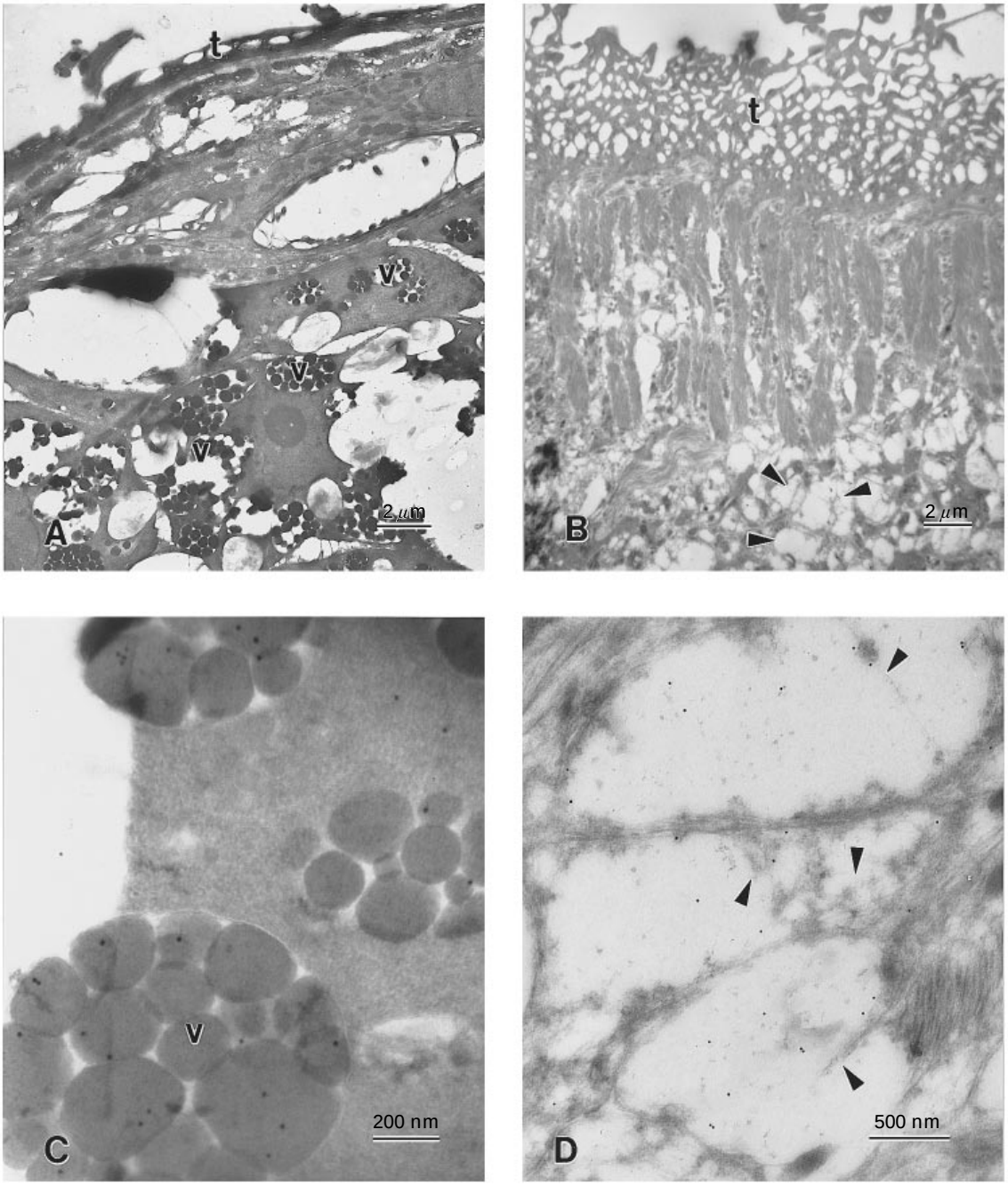


Fig. 2. Transmission electron micrographs of cryosections of adult *Schistosoma japonicum* after anti-Sj-FABPc immunogold labelling. (A) Low magnification of the female parasite tegument and parenchyma. Vitelline cells and vitelline droplets (v) are observed below the tegument (t). (B) Low magnification of the male parasite tegument and parenchyma. Extracted areas (arrowheads) are observed below the tegument (t). (C) High magnification of the female parasite vitelline droplets (v) showing FABPc immunoreactivity as indicated by colloidal gold particles. (D) High magnification of extracted regions of the male parasite parenchyma. FABPc is detected in this region which is enclosed by fibrous material (arrowheads).

parasite also contained Sj-FABPc in the parenchyma, but conventional cryosections did not reveal any identifiable structures corresponding to the immunolabelling of the region. Since it has been reported by others that Sm-FABPc (Moser *et al*. 1991) and Sj-FABPc (Becker *et al*. 1994) both have a binding affinity for fatty acids, we then postulated that the immunolabelled structure(s) within the male parasite may be lipid material. The cryopreparation of parasite tissue was modified to include osmium tetroxide, since it enhances the retention of lipids during preparation for electron microscopy (Hyatt, 1993).

Fixation with osmium tetroxide prior to sectioning and immunolabelling allowed the retention of lipid-like droplets in the parenchymal region of male *S. japonicum*. Although the efficiency of immunolabelling was decreased by the modified technique,

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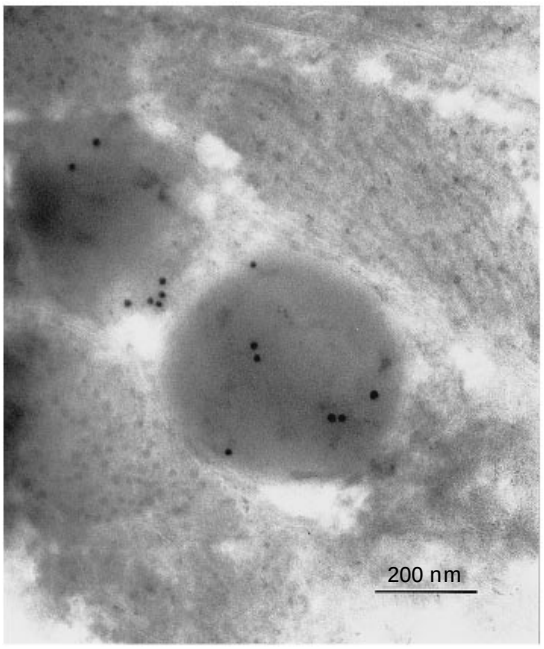


Fig. 3. Transmission electron micrograph of cryosectioned osmium-fixed male *Schistosoma japonicum*. FABP is detected within lipid droplets.

Sj-FABPc could still be detected within the lipid droplets. In the osmium tetroxide-treated cryosections of male tissue we examined, the lipid droplets were different in size to those seen in conventional resin sections. This could be due to the differing processing steps involved in producing cryosections compared with conventional techniques, which includes treatment with uranyl acetate, resin infiltration and resin polymerization by heat.

The visualization of the lipid droplet in the male *S. japonicum* appears to be dependent on its retention due to the fixation effects of osmium tetroxide rather than simply due to the contrasting by osmium. This was apparent since contrasting of cryosections with osmium tetroxide did not reveal the lipid droplets (data not shown).

The only study to examine the distribution of FABPc within schistosomes is a report by Moser *et al*. (1991). The electron microscopy study presented here both confirms and enhances observations described in the study by Moser *et al*. (1991) and further identifies differences between *S. mansoni* and *S. japonicum*. They examined *S. mansoni* with fluorescence microscopy and detected Sm-FABPc mainly in the tubercles, and to a lesser extent, in areas between the muscle and body area of the male worm. They did not present any findings of FABPc within female parasites and this omission was not explained in the text. Our study confirms that FABPc is not located within the tegument matrix or on the surface of the male or female *S. japonicum*. Male *S. japonicum* do not have tubercles, we could not confirm the presence of FABPc in these structures. A separate electron microscopy study using *S. mansoni* would need to be performed to clarify this point. However, the low fluorescence labelling of the parenchymal region of the male *S. mansoni*, seen by Moser *et al*. (1991), could correspond to FABPc detected in the lipid droplets of the male *S. japonicum* by electron microscopy in our study. While FABPc was not detected within the muscle fibres of *S. japonicum*, transmission electron microscopy allowed the observation of lipid droplets between muscle bundles which could correspond to the source of FABPc detected in *S. mansoni* by fluorescence microscopy (Moser *et al*. 1991).

The structures found to contain Sj-FABPc within the female *S. japonicum* in this study were identified ultrastructurally as vitelline droplets (Erasmus, 1975; Erasmus *et al*. 1982). Although lipid droplets were present within the female parasite, Sj-FABPc appeared not to be associated with these structures. Vitelline droplets are possibly involved in drug sequestration (Erasmus, 1975; Erasmus *et al*. 1982), and combined with the immunolocalization results of this present study, we suggest that Sj-FABPc could play a role in this important function in the female parasite.

Fatty acids act as precursors for lipid and phospholipid synthesis and are involved in a wide range of functions in schistosomes such as membrane formation, lipid anchors for proteins, sexual maturation and regulation of egg production (see Furlong (1991) for review). Fatty acids are not synthesised by schistosomes and must be acquired from the host (Meyer *et al*. 1970). In this study, Sj-FABPc was not detected on the tegument surface of the adult parasite. Although the mechanism of fatty acid uptake from host serum is not clear, this study suggests that FABPc proteins are not actively involved in this function during the adult stage of *S. japonicum*.

In this study, FABPc was detected within lipid-like structures such as the droplets within the male parasite or the vitelline droplets within the female parasite. The presence of the protein was not expected since the current opinion is that FABPc normally functions within the aqueous environment of the cell cytoplasm (see Glatz & van der Vusse, 1990). The interaction of FABPc with lipid storage structures is not understood, however, this study does indicate there may be a close association between these two components in schistosomes. Research examining the relationship of cytoplasmic supply of fatty acids and lipid storage may indicate further functions of FABPc within schistosomes and other tissues.

Although not expressed on the surface of the adult parasite, Sj-FABPc may be a target for a vaccine against other developmental stages. Stage-specific expression of schistosome proteins is well recognized (Simpson, 1990) and Sj-FABPc may be exposed on

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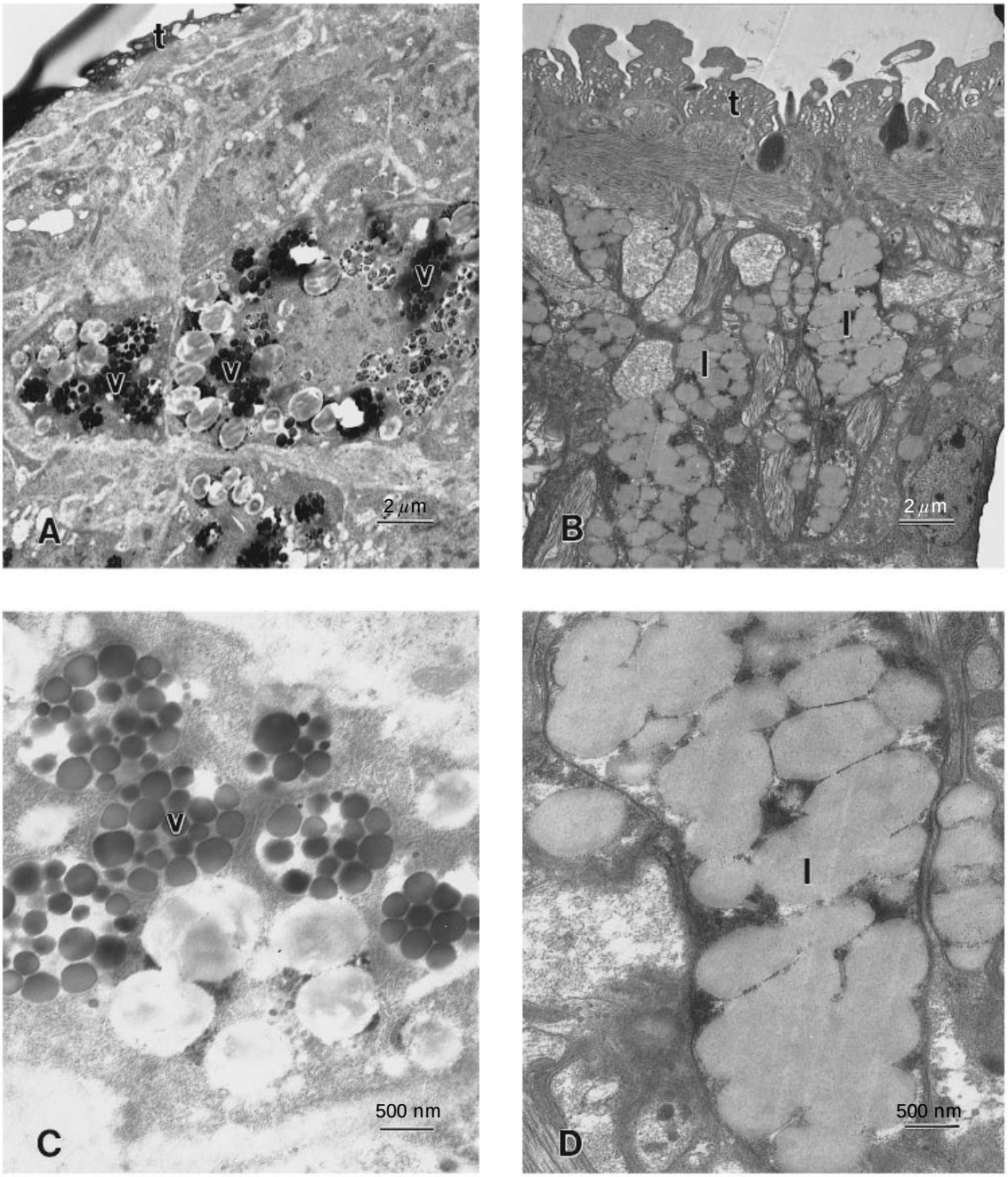


Fig. 4. Transmission electron micrographs of ultrathin sections of parasites prepared by conventional resin embedding. (A) Low magnification of female parasite tegument and parenchymal regions. Vitelline cells and vitelline droplets (v) are present below the tegument (t). (B) Low magnification of male parasite tegument and parenchymal regions. Lipid droplets (l) are observed below the tegument (t). (C) High magnification of vitelline droplets in a female parasite (v). (D) High magnification of lipid droplets (l) in a male parasite.

the surface of the parasite at other times, as has been shown for another schistosome protein, paramyosin (Gobert *et al*. 1997). Further localization of Sj-FABPc in other life-cycle stages of *S. japonicum* may provide additional information regarding the function of this protein and its involvement in vaccine-mediated protection.

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