Opisthorchis viverrini: ultrastructure and cytochemistry of the glycocalyx of the tegument

W. Apinhasmit[1], P. Sobhon[2*], C. Tarasub[2], W. Mothong[2], P. Saitongdee[2], P. Sretarugsa[2], C. Wanichanon[2] and E.S. Upatham[3]

[1]Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330, Thailand: [2]Department of Anatomy and [3]Department of Biology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand

Abstract

The ultrastructure and cytochemistry of the glycocalyx of the tegument of *Opisthorchis viverrini* during maturation from newly excysted juvenile to adult stages were investigated using colloidal iron, ruthenium red and lectin stainings. The results showed that the glycocalyx was intensely stained by the first two dyes, thus indicating the presence of relatively high amounts of negative charges. However, the thickness and intensity of the staining decreased during the fluke's maturation. Binding studies using lectin probes on the surface of adult parasites showed that binding sites for *Canavalia ensiformis* (Con A), *Triticum vulgaris* (WGA) and *Ricinus communis I* (RCA I) were present in relative large amounts on the glycocalyx of the adult tegument, whereas those for *Dolichos biflorus* (DBA) were relatively fewer in number, and those for *Ulex europaeus I* (UEA I) were absent. The binding patterns of Con A, WGA, RCA I and DBA were generally similar, and the reaction product was uniformly distributed over the dorsal and ventral surfaces of the parasite's body. These bindings, therefore, indicate the presence of D-mannose/D-glucose, N-acetyl-D-glucosamine/sialic acid, D-galactose and N-acetyl-D-galactosamine residues on the glycocalyx of the adult tegument.

Introduction

In trematode parasites, such as *Fasciola* and *Schistosoma* species, the glycocalyx, which is the external coating of the tegument's surface membrane, consists of several types of glycoproteins (Norden & Strand, 1984; Hayunga & Sumner, 1986; Nyame *et al.*, 1987; Sobhon & Upatham, 1990). Immunologically, carbohydrate moieties on the parasites' glycocalyx are some of the major epitopes of antigens that elicit immune responses in hosts (Omer-Ali *et al.*, 1986; Weiss *et al.*, 1986; Sobhon & Upatham, 1990). In the present study, we have investigated the ultrastructure, the charges and the presence of certain sugar residues in the glycocalyx of *Opisthorchis viverrini,* a human liver fluke prevalent in South-east Asia, during its development from newly excysted juvenile to adult stages.

*Author for correspondence.
Fax: 662 2479880
E-mail: scpso@mahidol.ac.th

Materials and methods

The parasites

Encysted metacercariae of *O. viverrini* were obtained from naturally infected cyprinoid fishes collected from a lake in Khon Kaen province in the north-eastern part of Thailand. They were separated from minced and partially trypsinized fish muscle as described previously (Apinhasmit *et al.*, 1993). The metacercariae were artificially excysted to obtain the newly excysted juveniles. The

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first-week and adult stages were collected from two groups of adult golden Syrian hamsters, each of which was infected by gastric intubation with 500 and 50 metacercariae for one and 12 weeks, respectively (Tuti *et al*., 1982).

Colloidal iron, ruthenium red and lectin stainings

The freshly-collected newly excysted juveniles, first-week and adult stages were immediately fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C for 2 h. Ten flukes at each stage were then either stained with positive or negative colloidal iron according to the procedure of Gasic *et al*. (1968), or stained with ruthenium red (Polyscience Co., Ltd, USA) according to Luft (1971). The positive colloid was not dialysed and used at pH 1.8 for charge stabilization, whereas the negative one was dialysed and used at pH 6.0 for the same reason.

Glutaraldehyde-fixed adult flukes were also labelled with five biotinylated lectins with different carbohydrate specificities (Vector Laboratories, USA) (table 1), by using the bridged avidin-biotin technique (Pino, 1984). After fixation, the specimens were washed in 0.1 M phosphate buffered saline (PBS), and treated sequentially with 0.15 M glycine in PBS for 30 min, in 0.3% H_2O_2 in PBS for 15 min, and 1% bovine serum albumin (BSA) in PBS for 30 min. Ten flukes were then incubated in optimal concentration of each biotinylated lectin (table 1) for 1 h at room temperature. The specimens were immersed in PBS containing 1% BSA, for 30 min and incubated in 25 $\mu g\,ml^{-1}$ avidin D for 1 h. After additional rinsing in PBS, the flukes were immersed in 20 $\mu g\,ml^{-1}$ biotinylated HRP for 1 h at room temperature. The reaction product was visualized by incubation in 10 ml of 0.05 M Tris/HCl buffer, pH 7.6, containing 15 mg of 3,3′-diaminobenzidine tetrahydrochloride (DAB), and 0.01% H_2O_2 for 15 min at room temperature. The following controls were used: (i) blocking of lectins with their specific hapten sugars at concentration of 0.2 M, for 1 h, prior to their applications to specimens; (ii) omission of biotinylated lectins in the incubation sequence; (iii) some control samples were incubated in DAB and H_2O_2 only.

All samples were subsequently processed for conventional transmission electron microscopy (TEM). Both uncounterstained and uranyl acetate-, and lead citrate-counterstained sections were examined in a Hitachi H-300 transmission electron microscope, operated at 75 kV.

Results

Colloidal iron staining

In the uncounterstained sections of newly excysted juveniles, the first-week and adult flukes, positive colloidal iron bound strongly throughout the thickness of the glycocalyx, which appeared as a thick coat of electron-dense product on the surface membrane (fig. 1A–C). The staining was distributed over the entire body surface of the parasites, and appeared as an uneven layer of iron particles ranging from small individual grains to clumps of coarse granules. The intensity of staining decreased from newly excysted juveniles to adults (fig. 1A–C). The negative colloidal iron staining on the surface of the three stages of parasites appeared very sparse, and wherever it occurred, the staining had a spotty and highly uneven appearance (fig. 1D). The uranyl acetate- and lead citrate-counterstained sections showed that the colloidal iron only stained glycocalyx but not the surface membrane.

Ruthenium red staining

The ruthenium red staining of all three stages was intense and distributed over the entire body surface of the parasites; and it decreased as the parasites became older (fig. 1E–G). The stained glycocalyx appeared as black filaments that were interwoven together, and the staining deposits were much finer and appeared more homogeneous than that of the positive colloidal iron (fig. 1E–G). Ruthenium red also stained the trilaminate surface membrane (fig. 1H–J). The thickness of glycocalyx, in the newly excysted juveniles, first-week and adult stages, was about 34, 22 and 19 nm, respectively. Generally, ruthenium red was excluded by the plasma membrane from staining the cytoplasm of the tegument; however, in some sections granules in the tegument, particularly those lying close to the surface were also stained (fig. 1E, G).

Table 1. Lectins used and their major carbohydrate specificities.

Lectins (abbreviation)	Concentrations for staining ($\mu g\,ml^{-1}$)	Major carbohydrate specificity	Binding inhibitor
Canavalia ensiformis (Con A)	20	α-D-Man, α-D-Glc	D-Man
Triticum vulgaris (WGA)	20	$(D\text{-}GlcNAc)_2$, NeuNAc	D-GlcNAc
Ricinus communis (RCA I)	20	α/β-D-Gal	D-Gal
Dolichos biflorus (DBA)	100	α-D-GalNAc	D-GalNAc
Ulex europaeus (UEA I)	100–1000	α-L-Fuc	L-Fuc

Abbreviations: Man, mannose; Glc, glucose; GlcNAc, N-acetyl-glucosamine; NeuNAc, N-acetyl-neuraminic acid (sialic acid); Gal, galactose; GalNAc, N-acetyl-galactosamine; Fuc, fucose.

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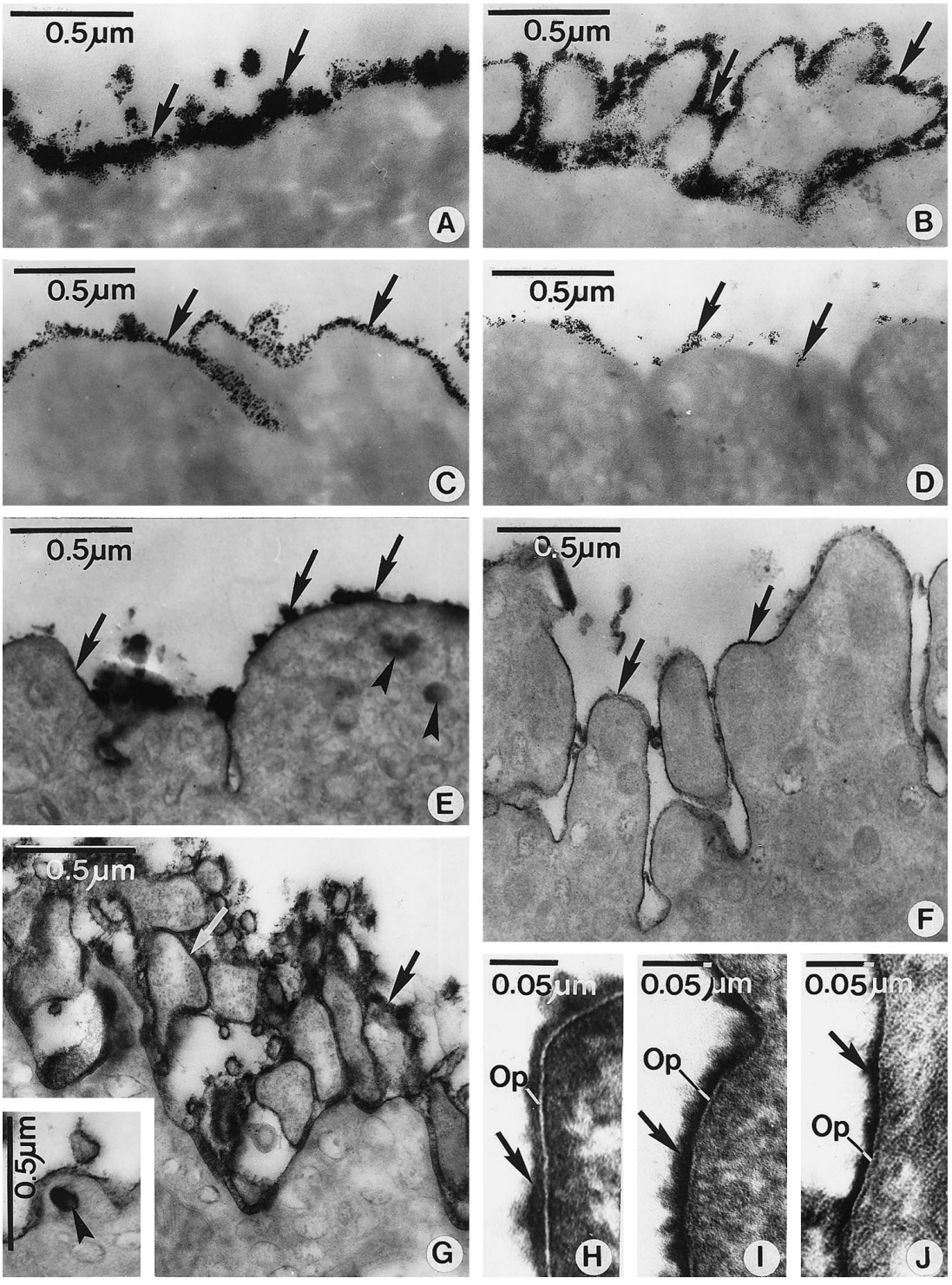

Fig. 1. Transmission electron micrographs of colloidal iron and ruthenium red staining of newly excysted juveniles (A, D, E and H), first-week (B, F and I) and adult flukes (C, G and J) of *Opisthorchis viverrini*. Both uncounterstained (A–G) and uranyl acetate- and lead citrate-counterstained (H–J) sections were exhibited. A–C. Positive colloidal iron staining showed intense and uneven deposits of iron particles (arrows) on the glycocalyx. The staining intensity and the thickness of glycocalyx decreased during development from the newly excysted juvenile to the adult stage. D. Negative colloidal iron staining illustrated sparse and irregular deposits (arrows) on the glycocalyx coating. E–G. Ruthenium red staining showed intense staining on the glycocalyx and tegumental surface membrane (arrows). Arrow heads in fig. E and inset of fig. G indicated that some tegumental granules lying close to the surface were also intensely stained. H–J. High magnification of ruthenium red staining with uranyl acetate and lead citrate counterstain, demonstrated densely stained glycocalyx (arrows) on the trilaminate surface membrane (Op).

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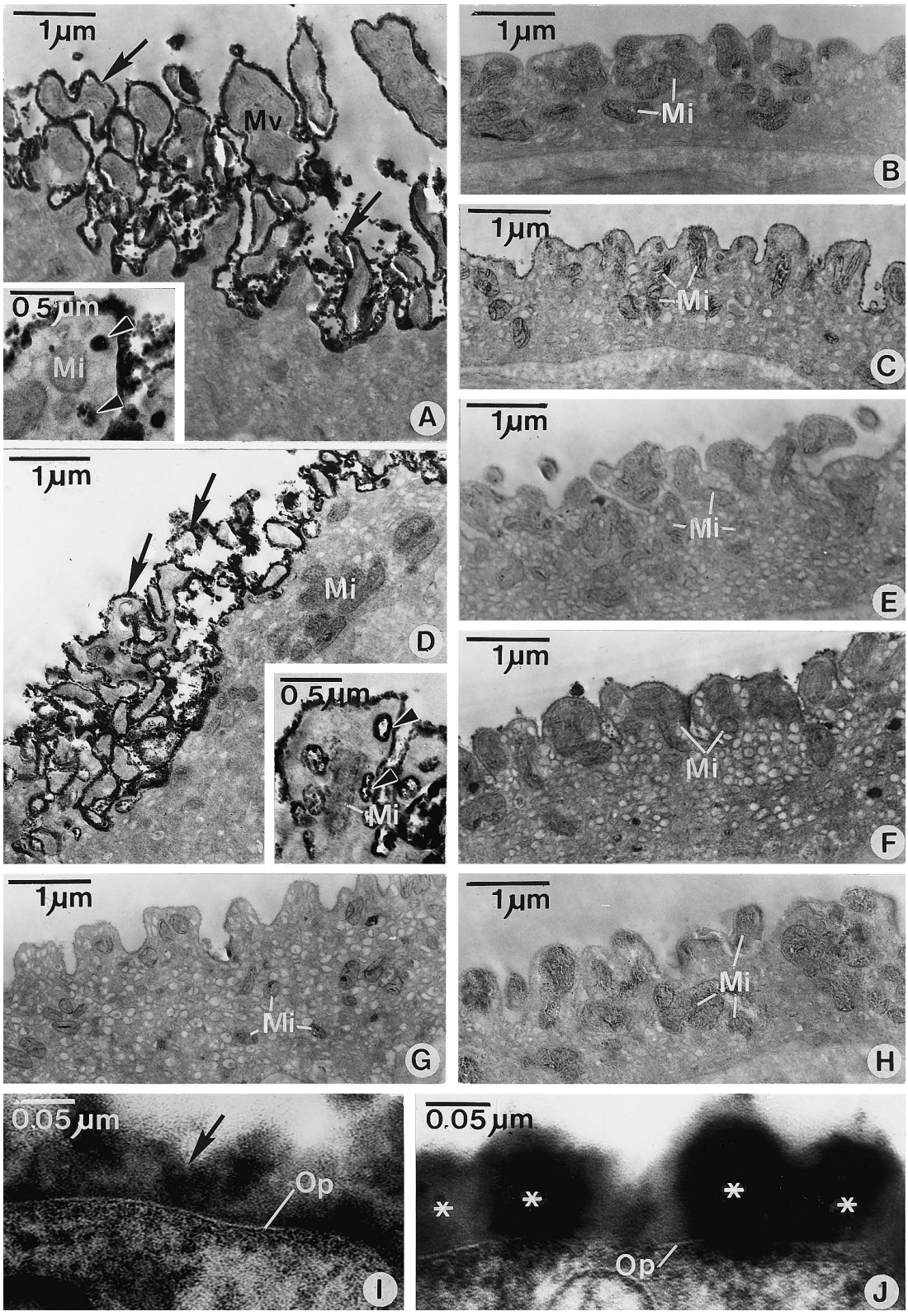

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Lectin staining

Initially, the optimal concentration of each lectin was determined by using the concentration that resulted in the first visible and fairly intense staining on the glycocalyx covering the surface of adult flukes. If the lectin concentration was lower or higher than the optimal concentration, the staining product could only be barely observed or not significantly increased. The optimal concentrations of biotinylated Con A, WGA and RCA I in this experiment were 20 μg ml^{-1}. The bindings of Con A and RCA I were completely blocked with 0.2 M D-mannose and 0.2 M D-galactose, respectively (fig. 2B), while the binding of WGA was substantially but not completely inhibited by 0.2 M N-acetyl-D-glucosamine (fig. 2C). The parasites labelled with Con A, WGA and RCA I showed similar patterns and intensities of staining (fig. 2A), and the reaction product was uniformly distributed over the dorsal and ventral surfaces of the body. The binding was intense and evenly distributed on the surface membrane (fig. 2A). Under a high magnification, the reaction product appeared as a homogeneous electron-dense layer superimposed on the glycocalyx external to the trilaminate surface membrane (fig. 2I). The thickness of the staining was even throughout the surface of the tegument, which implied that the whole width of the glycocalyx was stained. The mitochondria were also stained and stood out in contrast to the cytoplasmic matrix and tegumental granules (fig. 2A,D). In some sections, tegmental granules, particularly those lying close to the surface, were also stained (fig. 2A,D). The staining of mitochondria was believed to be non-specific and arose from endogenous peroxidase present in these organelles, since the staining also occurred in control specimens incubated without lectins (fig. 2G), or in those incubated only in DAB + H_2O_2 (fig. 2H). The optimal concentration of DBA staining was at 100 μg ml^{-1} which was relatively high when compared to that of Con A, WGA and RCA I stainings. Preincubation of this lectin with 0.2 M N-acetyl-D-galactosamine inhibited the staining completely (fig. 2E). At this higher concentration, DBA staining exhibited similar distribution and relative intensity as in Con A, WGA and RCA I stainings (fig. 2D). At high magnification, the binding appeared as an electron-dense layer lying external to the trilaminate surface membrane which may have resulted from the merging of adjacent globules of the reaction product (fig. 2J). The staining with UEA I was not seen even at relatively high concentrations ranging from 100 to 1000 μg ml^{-1} (fig. 2F). At high magnification, only a thin layer of glycocalyx was present on the surface membrane, which was similar to that in the conventionally prepared tegument.

In the control group, where lectins were omitted from the incubation sequence, the tegument showed no trace of dense product on the surface membrane and in the tegument, except in mitochondria which were also stained (fig. 2G). The other control group, which was incubated only in DAB + H_2O_2, also exhibited no observable reaction product on the glycocalyx, whereas the mitochondria were still stained (fig. 2H).

Discussion

Conventional TEM preparation could only partially reveal the glycocalyx of the trematode tegument (Threadgold, 1976; Apinhasmit *et al.*, 1994) because the major part of the glycocalyx might be removed during dehydration, or it could not be made sufficiently electron dense by osmication and conventional electron staining. In contrast, the *en bloc* staining with positive colloidal iron and ruthenium red showed a thick layer of glycocalyx coating evenly over the surface membrane of the parasite. The black deposit of ruthenium red was much finer than colloidal iron (Luft, 1976), which might explain why the stain could penetrate the entire layer of glycocalyx down to the level of the surface membrane. In some regions, the tegumental granules close to the surface were also stained implying the deep penetration of the stain, as well as suggesting that these granules contained contents with properties similar to those of the glycocalyx. In fact, we have demonstrated previously that some superficial granules fused with the surface membrane, and therefore contribute their membrane to the former (Apinhasmit *et al.*, 1994). The present observation suggested that the matrix contained within the tegumental granules contributed to the formation of glycocalyx coating. Positive colloidal iron and ruthenium red, which contained cations, bound strongly to the whole thickness of glycocalyx in contrast to the negative colloidal iron. It was, therefore, interpreted that the glycocalyx of *O. viverrini* contained many more negative charges than positive charges. According to Gasic *et al.* (1968) the chemical groups bearing the negative charges on the *O. viverrini* surface that were bound to colloidal iron could be interpreted as acidic carbohydrates, while the few positively charged groups present might be due to the amino groups of amino sugars. Ruthenium red stain might reflect the presence of negatively charged carboxyl groups of N-acetyl neuraminic (sialic) acid of

Fig. 2. Transmission electron micrographs of the adult tegument of *Opisthorchis viverrini* stained with five biotinylated lectins. Both uncounterstained (A–H) and uranyl acetate- and lead citrate-counterstained (I and J) sections were demonstrated. A and D. The bindings of Con A and DBA to the glycocalyx, respectively at low magnification. The staining product (arrows) appeared as homogeneous electron-dense deposits on the glycocalyx of the surface membrane of the microvilli (Mv). Mitochondria (Mi) were also weakly stained. Insets exhibiting some tegumental granules lying close to the surface (arrow heads) were also stained. B, C and E. In the controls with flukes incubated with lectins and their specific sugars: Con A + D-mannose (B), WGA + N-acetyl-D-glucosamine (C) and DBA + N-acetyl-D-galactosamine (E), the tegument showed no reaction product (B and E), or only slight staining (C) on its surface membrane. Mitochondria (Mi) were slightly stained. F. Slight and patchy reactions of UEA I labelling on the surface of the tegument at low magnification. Mitochondria (Mi) were also stained. G and H. The tegument of two control groups incubated with avidin, biotinylated-HRP followed with DAB + H_2O_2 (G), and with DAB + H_2O_2 only (H), showed no staining product on the surface membrane, whereas all mitochondria (Mi) were also stained. I and J. The glycocalyx heavily stained with Con A and DBA, respectively at high magnification. The former appeared as a continuous electron dense layer (arrows) coating the trilaminate surface membrane (Op). The latter also exhibited dense globules (asterisks) in the thick layer.

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sialoglycoproteins (Luft, 1976), and sulphated, or phosphate groups of polysaccharides (McDiarmid & Podesta, 1984). The present results were similar to the findings in other trematodes, such as *Haematoloechus medioplexus* (Rothman & Elder, 1970), *F. hepatica* (Threadgold, 1976), *S. japonicum* and *S. mekongi* (Sobhon & Upatham, 1990), and cestodes (Rothman & Elder, 1970; Lumsden, 1972), in which the parasite surfaces bore intense negatively charged acidic polysaccharides.

Although the physiological function of this polyanionic surface coat remained a matter for speculation, it has been implicated in a number of roles. In *S. mansoni*, the negatively charged glycocalyx was thought to confer protection against the immune responses of the host, by reducing the adhesion of immuno-effector cells, and neutralizing the effects of complement (McDiarmid & Podesta, 1984). Furthermore, in newly excysted juveniles, the thick glycocalyx coating might assist in protecting the parasite against hydrolytic actions of the intestinal juice and bile. A significant decrease in the thickness and quantity of negative charges of the glycocalyx were observed in the first-week and adult flukes when compared with newly excysted juveniles. This finding was also similar to that in other trematodes, as in *F. hepatica* (Bennett & Threadgold, 1975), *S. mansoni* (Stein & Lumsden, 1973), *S. japonicum* and *S. mekongi* (Sobhon & Upatham, 1990). This was likely to be an adaptation to the changing environment in different compartments of the host's body as the parasites matured.

By using lectins as probes, the present study demonstrates the presence of carbohydrate residues on the glycocalyx of adult *O. viverrini*. The relative abundance and distribution of lectin binding sites can be estimated by comparing the optimal concentrations of each lectin that results in a visibly strong staining intensity. Thus the receptor sites for Con A, WGA and RCA I might be present in relatively large numbers on the parasite surface because they need relatively low concentrations of these lectins (at $20\,\mu g\,ml^{-1}$) to show visible staining. On this basis, DBA receptors might be relatively fewer in number, and receptor sites for UEA I are unlikely. The staining due to Con A, WGA, RCA I and DBA was evenly distributed on the body surface of *O. viverrini*, while UEA I failed to stain. This pattern was similar to previous observations on lectin bindings on the surface of *S. mansoni* (Simpson & Smithers, 1980; Linder & Huldt, 1982; MacGregor *et al.*, 1985). The staining product was not observed in three control experiments except in WGA staining, which indicated that the binding of lectins, other than WGA, was quite specific. The reason why WGA binding could not be completely blocked might be due to non-specific binding because of electrostatic interactions between the positively charged WGA with negatively charged groups on the tegument (Simpson & Smithers, 1980). Furthermore, the tegument of *O. viverrini* incubated in $DAB + H_2O_2$ either with or without lectins appeared to show stained mitochondria, particularly in the cristae portion, while the surface membrane was unstained. This might be due to the action of the oxidoreductase enzymes embedded within the membrane of the cristae (Alberts *et al.*, 1983), which could never be blocked completely even by pretreatment with 3.0% H_2O_2 (Banerjee & Pettit, 1984).

Con A was known to bind specifically to D-mannosyl and D-glucosyl residues (Lis & Sharon, 1973), which in mammalian cells mostly serve as the core or internal sugar of oligosaccharide chains of glycoproteins, whereas WGA was known to bind specifically to both internal N-acetyl-D-glucosamine, and the terminal N-acetyl-neuraminic (sialic) acid residues (Lis & Sharon, 1973). In addition, RCA I and DBA are known to bind to D-galactose and N-acetyl-D-galactosamine, respectively, both of which are terminal sugars. The present results, therefore, imply that there are abundant core sugars in *O. viverrini* surface which are composed of D-mannose, D-glucose and/or N-acetyl-D-glucosamine, and a large amount of terminal sugars including sialic acid, and D-galactose. Another terminal sugar, N-acetyl-D-galactosamine, might also be present in a lower amount. The failure of UEA I to bind to the *O. viverrini* surface suggested that the fluke did not have L-fucose on its surface glycoproteins, a situation similar to that reported in *S. mansoni* (Simpson & Smithers, 1980).

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