

Onchocerca volvulus: Ultrastructural Localization of Two Glutathione S-Transferases

Gabriele Wildenburg,^{*,1} Eva Liebau,[†] and Kimberly Henkle-Dührsen[†]

^{*}Department of Helminthology and Entomology and [†]Department of Biochemical Parasitology, Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany

Wildenburg, G., Liebau, E., and Henkle-Dührsen, K. 1998. *Onchocerca volvulus*: Ultrastructural localization of two glutathione S-transferases. *Experimental Parasitology* **88**, 34–42. Glutathione S-transferases (GSTs) are essential detoxification enzymes for virtually all cells and may additionally aid in parasite survival by counteracting host-induced damage. GSTs from parasitic nematodes have been identified as potential targets for both immuno- and chemotherapy. To more closely characterize a 31-kDa (OvGST1) and a 24.5-kDa (OvGST2) GST from the pathogenic human filarial parasite *Onchocerca volvulus*, immunolocalization by electron microscopy was performed using two distinct affinity-purified polyclonal antisera raised against the recombinant OvGST1 and OvGST2. The strongest immunogold staining for OvGST1 was identified in the body wall of adult worms, especially in protuberances of the cuticle which lie in pouches of the hypodermis and in the outer zone of the syncytial hypodermis, where the external plasma membrane forms series of lamellae. Gold particles were also observed on the epicuticle of the adults and in the region of the border between the cuticle and hypodermis of microfilariae. The larval stages L1, L2, and infective L3 were also immunopositive for OvGST1. There was no specific labeling in the longitudinal musculature, the intestine, or the uterine wall of the adult worm. In contrast to the results for OvGST1, immunogold labeling for OvGST2 was observed throughout the whole hypodermal cytoplasm. The epithelial cells of the uterine wall showed moderate labeling. These ultrastructural immunolocalization results are consistent with the molecular characterization of both enzymes, indicating that OvGST1 is secreted out of the hypodermis into the cuticle and is acting at the host-parasite interface, while OvGST2 functions as an intracellular cytosolic housekeeping enzyme. © 1998

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Index Descriptors and Abbreviations: Glutathione S-transferase; nematode; *Onchocerca volvulus*; ultrastructural localization; vaccine candidate; L1, L2, L3, larval stages 1–3; OvGST, *Onchocerca volvulus*

glutathione S-transferase (EC 2.5.1.18); rOvGST, recombinant *Onchocerca volvulus* glutathione S-transferase.

INTRODUCTION

The filarial nematode *Onchocerca volvulus* is a blackfly-transmitted human parasite, causing skin and eye diseases. The adult worms are located in subcutaneous tissues, where they induce the formation of the fibrous onchocercal nodule. During their development and persistence in humans, these parasites encounter an immune response directed against them (Butterworth 1984; Ottesen 1995; Wildenburg *et al.* 1996). However, they can survive up to 15 years, probably due to a variety of defense mechanisms.

Glutathione S-transferases (GSTs) are multifunctional proteins encoded by a multigene family and are ubiquitous among eukaryotes. These isoenzymes have a broad range of substrates, enhancing their detoxification capabilities. Their functions range from the detoxification of electrophilic compounds by their conjugation to reduced glutathione (Armstrong 1991) to the protection against peroxidative membrane damage induced by secondary products of lipid peroxidation, such as lipid hydroperoxides and reactive carbonyls generated by host immune cell effector molecules (Brophy and Pritchard 1992; Mitchell 1989). GSTs have been intensively studied in helminths (Brophy and Barrett 1990), where it is suggested that their detoxifying activity

¹To whom correspondence should be addressed. Fax: (49) 40-31182-309.

may play an important role in parasite survival by counter-acting host-induced damage.

The structure and activity of two *O. volvulus* GSTs (OvGST1 and OvGST2) have been characterized. Both of these enzymes were localized in adult and larval stages of the parasite at the light microscopic level (Liebau *et al.* 1994a, b, c, 1996; Salinas *et al.* 1994). OvGST1 was the first description of a secreted form of a GST (Liebau *et al.* 1994c). This novel enzyme is glycosylated and possesses a hydrophobic signal peptide sequence, which is processed to produce the mature enzyme subunit. Furthermore, the localization profile placed the enzyme in the vicinity of the cuticle. However, OvGST1 could not be localized directly in the cuticle nor on the surface of the worm with this method.

To more precisely localize both of these GSTs, with particular interest in the OvGST1, immunogold electron microscopy was performed. The ultrastructural detection of OvGST1 in parts of the cuticle and in the outer zone of the hypodermis is consistent with the fact that it is a secretory protein and supports the hypothesis that it plays an important role at the host-parasite interface. In contrast, OvGST2 is detected throughout the cytoplasm in the hypodermis and the uterus epithelium, indicating its function as a housekeeping enzyme.

MATERIALS AND METHODS

Reagents. The LR White acrylic resin was purchased from London Resin Co. Ltd. (Basingstoke, UK). The bovine serum albumin (BSA) was from Behring (Marburg, Germany). The biotinylated sheep anti-rabbit IgG (H+L) and the streptavidin-colloidal gold complex (15 nm) were obtained from Amersham International (Amersham, UK). The nitrocellulose filter was from Schleicher & Schuell (Dassel, Germany). All other chemicals were reagent grade and were purchased from Sigma-Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

Parasites. Subcutaneous nodules (onchocercosomata), which contained adult *O. volvulus* as well as microfilariae, were removed by nodulectomy. Nodulectomies were performed in several African countries on untreated patients with generalized onchocerciasis, as described previously (Albiez *et al.* 1988). Nodulectomies for research purposes were approved by the Ethics Commission of the Medical Board Hamburg. Blackflies (*Simulium yahense*) containing the L1 (sausage stage), L2, and L3 larvae of *O. volvulus* in the thoracic musculature and/or infective L3 larvae in the head were collected in Guinea and Liberia.

Production of OvGST1 and OvGST2 rabbit polyclonal antibodies. The recombinant antigens rOvGST1 and rOvGST2 were expressed in *Escherichia coli* and purified by affinity chromatography. Rabbit polyclonal antisera were raised by subcutaneous immunization as previously described by Liebau *et al.* (1994c, 1996).

Affinity purification of antibodies (anti-rOvGST1, anti-rOvGST2). One milligram of the purified recombinant OvGST1 or

OvGST2 (resuspended in 0.1% sodium dodecyl sulfate solution) were boiled for 5 min. The rOvGST1 or rOvGST2 was immobilized on a nitrocellulose filter and left to dry. The filter was incubated in blocking solution (2% BSA in phosphate-buffered saline (PBS)) for 1 h at room temperature. After addition of anti-rOvGST1 or anti-rOvGST2 (1:500 dilution in blocking buffer), the filter was incubated for 20 h at 4°C. The nitrocellulose filter was then washed for 2 × 30 min using 0.2 M NaCl solution followed by PBS. The bound antibody was then released using 0.2 M glycine, 1 mM ethylenediaminetetraacetic acid (EDTA; pH 4.0). The elution buffer was neutralized immediately by the addition of 0.1 vol of 1 M Tris-base, followed by the addition of 0.1 vol of 10× PBS. The affinity-purified antibodies were used for immunoelectron microscopy within 2 days.

Immunoelectron microscopy. Onchocercosomata, dissected into pieces not larger than 2 mm³, were fixed for 24 h (4°C) in a solution containing 1% paraformaldehyde and 0.025% glutaraldehyde in 0.2 M sodium cacodylate-HCl buffer (pH 7.2) and rinsed three times (15 min) in cacodylate buffer with sucrose.

Specimens were dehydrated via a graded ethanol series and embedded in medium-hardness LR White acrylic resin after infiltration with a 1:1 mixture of LR resin:100% ethanol for 1 h, followed by several changes of pure resin for a total of 20–24 h. Polymerization was performed in covered rubber molds on ice for 24 h. Ultrathin sections were collected on clean hexagonal nickel grids (300 mesh).

Immunolabeling was performed at room temperature. After quenching in 1% NH₄Cl (30 min) and 0.1 M glycine (15 min), sections were washed with 0.25% (v/v) Tween 20 in 50 mM Tris buffer (0.5% BSA, pH 7.6), incubated with the affinity-purified anti-rOvGST1 or anti-rOvGST2 antibodies (1 h, 1:2), washed three times (see above), and then incubated with biotinylated sheep anti-rabbit IgG at a dilution of 1:250 (30 min). After three washes in Tris buffer, the sections were reacted with a streptavidin-colloidal gold (15 nm) arm at a dilution of 1:150 for 30 min. Sections were washed three times in Tris buffer, rinsed in distilled water, and air-dried.

For negative controls, the primary antibodies were replaced by the corresponding preimmune rabbit sera (1:2).

After labeling, sections were counterstained with 2% aqueous uranyl acetate (10 min) and Reynold's lead citrate (5 min) followed by examination with a Philips TM 300 transmission electron microscope operating at 80 kV.

RESULTS

Immunolocalization of OvGST1

For a detailed analysis of the tissue distribution of the OvGST1 antigen, electron microscopical analysis using immunogold staining of adult *O. volvulus* and microfilariae in the nodular tissue was performed. GST1 was present in the body wall of the adult worms. A strong labeling for OvGST1, as indicated by the presence of numerous gold particles, was detected in the margin between the cuticle and the hypodermis, especially in the inner basal cuticular layer

where protuberances of the cuticle lie in pouches of the hypodermis (Fig. 1A). Moderate labeling was observed in the outer zone of the syncytial hypodermis where the external plasma membrane forms a series of lamellae (Fig. 1A). The longitudinal oriented stacks of lamellae insert at the inner basal layer of the cuticle, where sometimes a moderate labeling was found (Figs. 2A and 2B). In young female worms, where no protuberances of the basal cuticle layer are present, gold particles were arranged in a line just below the cuticle (Fig. 2B); some were also present in the basal layer of the cuticle. No binding of anti-rOvGST1 to any organelle in the hypodermal cytoplasm was found.

Moderate immunogold labeling occurred on the outer covering of the cuticle, the epicuticle, which forms a thick layer of regular folds and protrusions (Fig. 2C). Significant numbers of gold particles could not be detected in the median and cortical cuticular layers. No labeling occurred in the musculature, the intestine, or the uterine wall of the adult worms.

Microfilariae in the nodular tissue also revealed a moderate labeling in the region of the border between the cuticle and hypodermis (Figs. 3A and 3B). No gold particles were detected in their musculature (Fig. 3B). Incubation with preimmune serum revealed no labeling, either in the adult worms (Fig. 1B) or in the microfilariae.

At the light microscopic level, the anti-rOvGST1 antibodies positively immunostained L1 and L2 larvae embedded in the thorax musculature of *S. yahense*, although less intensely than the moderately labeled L3 in the musculature and the infective L3 larvae in the head of a blackfly (data not shown).

Immunolocalization of OvGST2

The ultrastructural analysis of the distribution of the OvGST2 protein revealed a strong labeling throughout the entire hypodermis of adult *O. volvulus*. Gold particles were evenly distributed in the hypodermal cytoplasm and the labeling was not associated with nuclei, mitochondria, endoplasmic reticulum, or other cellular organelles (Fig. 4A). The epithelial cells of the uterine wall showed a moderate staining. The cuticle, the musculature, and the intestine were devoid of gold particles. Microfilariae in the nodular tissue were immunopositive. Incubation with the corresponding preimmune serum yielded no labeling in any part of the adult worms (Fig. 4B) or the microfilariae.

The results of the OvGST1 and OvGST2 localization studies in various tissues and organelles of adult *O. volvulus* and in the larval stages are summarized in Table I.

DISCUSSION

In this study, affinity-purified antibodies were used to elucidate the ultrastructural localization of the native *O. volvulus* proteins GST1 and GST2. It was shown that both enzymes are present in high concentrations in adult *O. volvulus* as well as in all larval stages, especially in L3 larvae. These results both confirm and extend our previous results describing the light microscopical immunolocalization of these enzymes with rabbit antisera (Liebau *et al.* 1994c, 1996).

Immunoelectron microscopy allows a more precise localization of the antigens in *O. volvulus* (Lustigman *et al.* 1991). This method has revealed, for the first time, the presence of OvGST1 not only in discrete areas of the hypodermis, but also in the overlying cuticle of adult *O. volvulus* and their microfilariae. Within the cuticle of the adults, labeling was observed in the basal zones, especially in so-called protuberances of the inner basal layer, which lie in pouches of the hypodermis. OvGST1 was also found on the epicuticle. The intriguing localization of the native OvGST1 protein to these distinct areas, considered together with the reported detection of this protein in the supernatant of *in vitro* cultured adult female *O. volvulus* (Liebau *et al.* 1994c), suggests that OvGST1 is a secreted protein. These localizations imply that the syncytial hypodermal tissue represents the site of synthesis of the protein and that turnover occurs via a transcuticular route. Similar routes of secretion have been noted for other hypodermally synthesized antigens (Cookson *et al.* 1992; Poole *et al.* 1992).

The surface of filarial worms, as in all other nematodes, is entirely covered by a complex, proteinaceous exoskeleton, the cuticle, which is permeable to a wide range of molecules of low molecular weight. Transport mechanisms are associated with the plasma membrane of the hypodermis, and the cuticle may be considered an "unstirred layer" distal to the transport loci. The outermost layer of the cuticle, the epicuticle, consists of a lipid bilayer (a trilaminar membrane-like structure) which differs from a typical plasma membrane. The function of this tough "shell" with a cross-linked insoluble structure may simply be to enclose the extracellular cuticular matrix (Howells 1987). The cuticle-hypodermis complex represents the major interface between the nematode and its environment, along with the gut. The apparent lack or reduction of function of the gut in some adult filariae strongly suggests that transcuticular permeation is the route of entry and export of solutes in these worms. In *O. volvulus*, the outer portion of the hypodermis is probably responsible for the metabolism of materials which are incorporated from

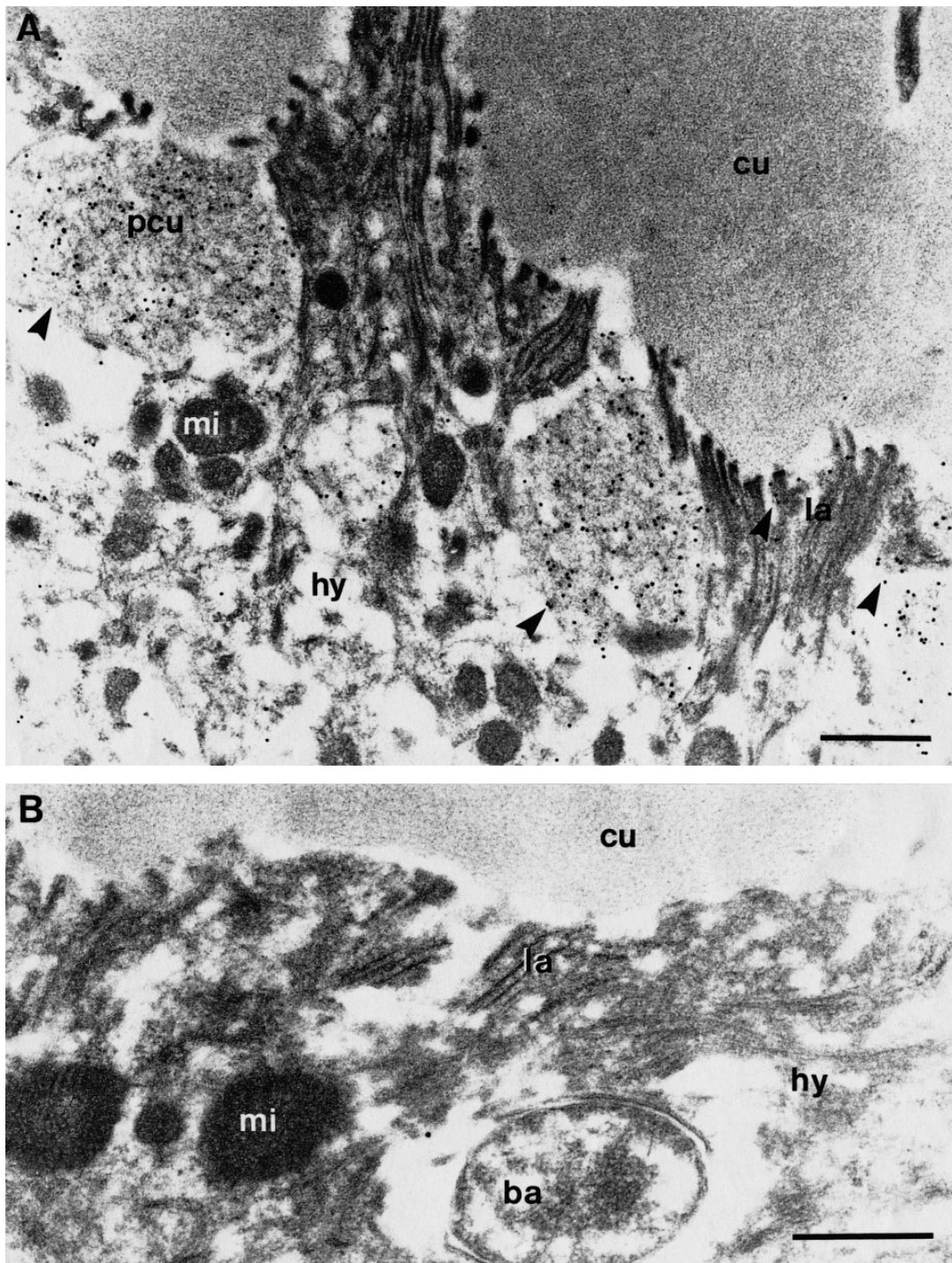


FIG. 1. Ultrastructural localization of OvGST1 by immunoelectron microscopy. Ultrathin sections of adult *O. volvulus* were incubated with the affinity-purified anti-OvGST1 antibodies and streptavidin-colloidal gold (15 nm) for indirect antigen localization in adult *O. volvulus*. ba, bacterium; cu, cuticle; hy, hypodermis; la, lamellae of the outer hypodermal membrane; mi, mitochondria; pcu, protuberances of the inner basal cuticular layer. Bars, 0.5 μ m. (A) Detail of the cuticle/hypodermis region with gold particles (arrowheads) in the protuberances of the cuticle and in the lamellae of the outer hypodermal membrane. (B) Ultrathin section of the cuticle/hypodermis region incubated with the preimmune serum for OvGST1. No labeling was detected.

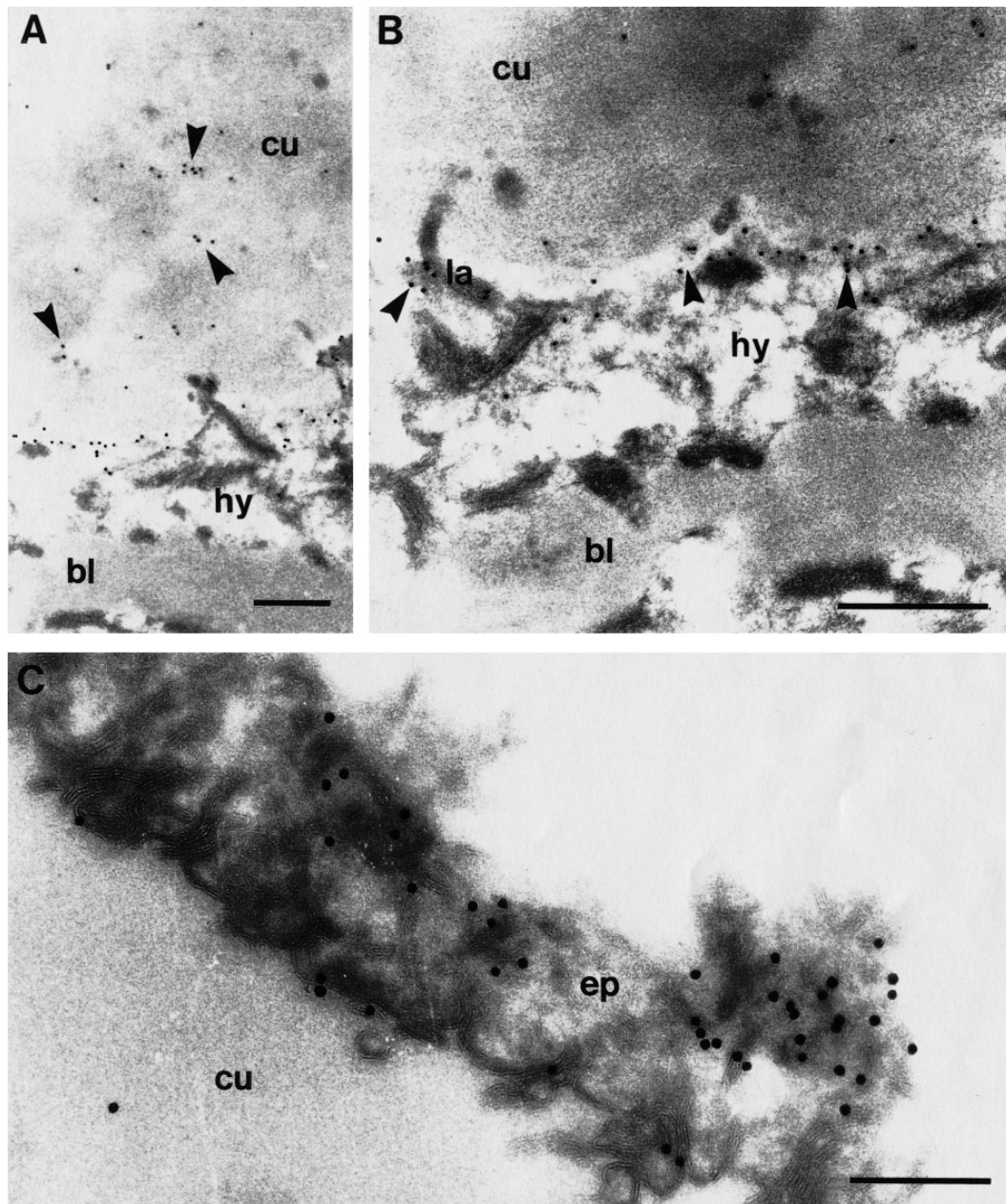


FIG. 2. Ultrastructural localization of OvGST1 by immunogold electron microscopy in adult *O. volvulus*. bl, basal lamina; cu, cuticle; ep, epicuticle; hy, hypodermis; la, lamellae of the outer hypodermal membrane. (A) Moderate labeling for OvGST1 (arrowheads) occurred sometimes in the inner basal cuticular layer. Bar, 0.5 μm . (B) Ultrathin section of a young female worm with gold particles (arrowheads) situated in the hypodermis forming a line just below the cuticle. No labeling occurred in the basal lamina of the hypodermis. Bar, 0.5 μm . (C) Moderate immunogold labeling was found in the thick layer of the epicuticular foldings of a female *O. volvulus*. Bar, 0.2 μm .

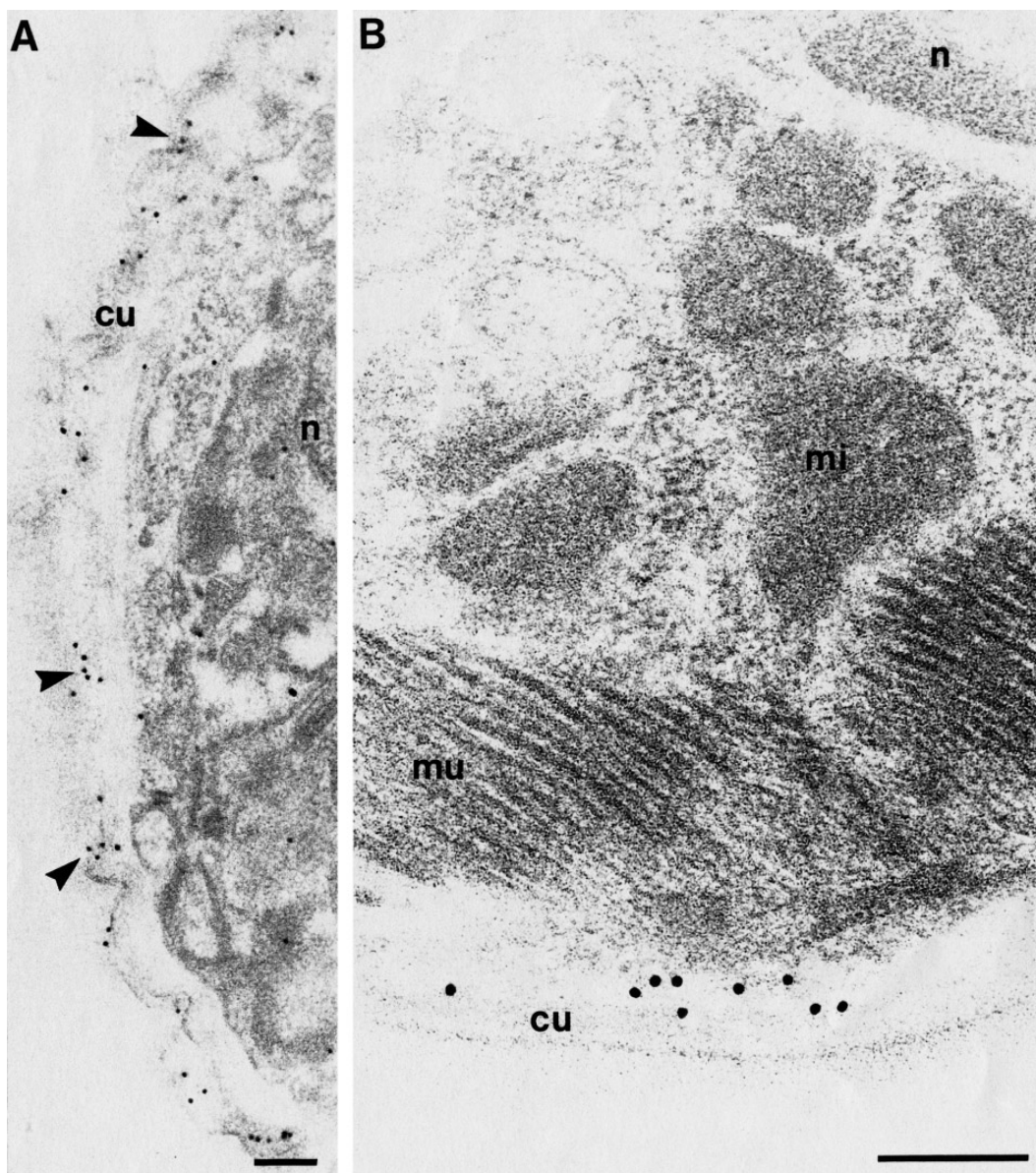


FIG. 3. Localization of the OvGST1 protein by immunogold electron microscopy in ultrathin sections of *O. volvulus* microfilariae. cu, cuticle; mi, mitochondria; mu, musculature; n, nucleus. Bars, 0.2 μ m. (A) Cross section of a microfilaria in the nodular tissue. Gold particles (arrowheads), indicating a localization site for OvGST1, were mainly present in the region of the border between cuticle and hypodermis of the larva. (B) Detail of a microfilaria. Labeling for OvGST1 was observed in the region of the border between cuticle and hypodermis. There were no gold particles in the musculature, in mitochondria, or nuclei.

the environment, as well as nutrient and waste product transport. It appears that at least some nematodes (Forsyth *et al.* 1981; Howells 1987) release surface proteins into the environment, suggesting that cuticle components are synthesized and replaced as they are lost. The pathway through which newly synthesized cuticle antigens reach the surface has not been conclusively identified (Geary *et al.* 1995). It

has also been proposed that the epicuticle is continuously formed and shed throughout the lifespan of the worm (Franz 1988). Since the OvGST1 is found on the epicuticle, this may explain its discharge into the environment. GST1 may be either physically anchored in or only transiently associated with the epicuticle.

The fact that OvGST1 is on the epicuticle means that it

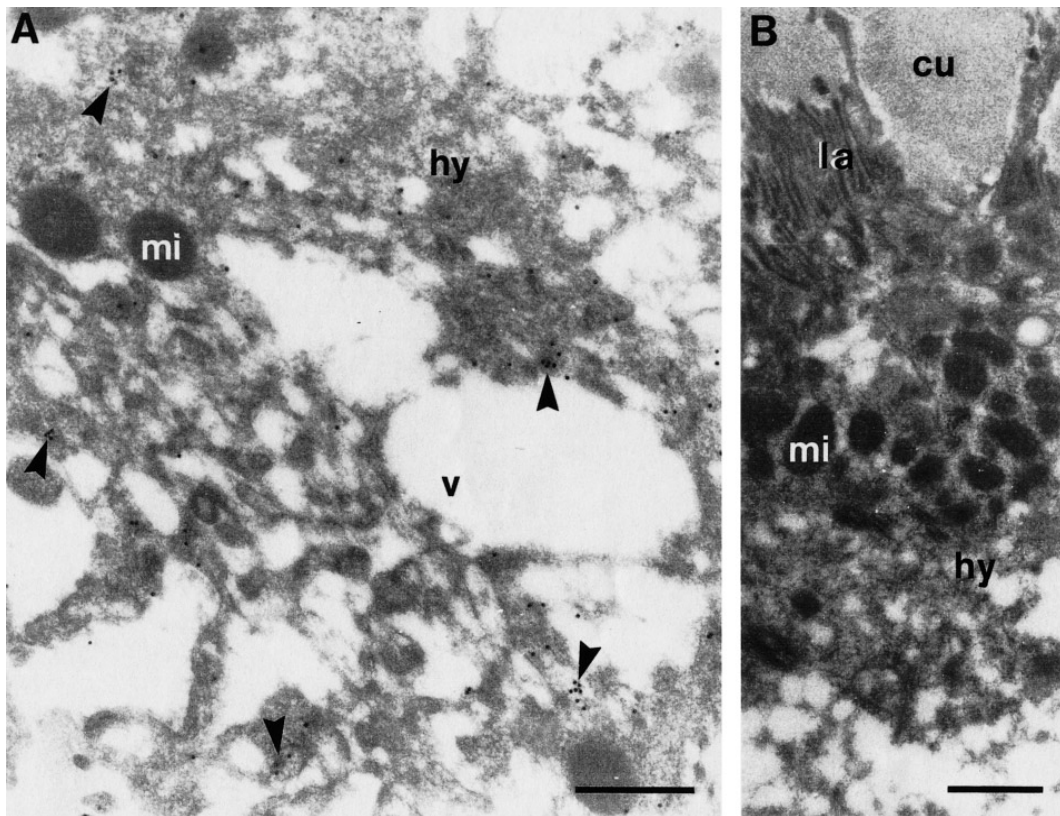


FIG. 4. Ultrastructural localization by immunoelectron microscopy of the recombinant OvGST2 protein in adult *O. volvulus*. cu, cuticle; hy, hypodermis; la, lamellae of the outer hypodermal membrane; mi, mitochondria; v, vacuole. Bars, 0.5 μ m. (A) Cross section of a female *O. volvulus* incubated with the affinity-purified anti-OvGST2 antibodies and streptavidin-colloidal gold (15 nm). The cytoplasm of the hypodermis showed evenly distributed gold particles (arrowheads). (B) Section of a similar region of the body wall incubated with the preimmune serum for OvGST2. The hypodermis divulged no labeling.

might be accessible to antibody even in intact worms and could therefore serve as a target for antibody-dependent cellular cytotoxicity (ADCC). Antibody specific for the exposed determinants on the nematode surface could direct the adherence of cytotoxic leukocytes and lead to the death of the parasite. In addition, it is very likely that OvGST1 is involved in counteracting the damage caused by the host immune effector cells, by detoxifying the resulting secondary products of lipid peroxidation. Toxic products of host effector cells may pass through the outer layers of the cuticle. The strong accumulation of GST1 in the protuberances of the inner basal layer may be an additional zone of detoxification, acting as a second front, before any toxic products can reach the vulnerable hypodermis. These protuberances may also represent a reservoir of GST1 for the epicuticle. Therefore, OvGST1 is an important part of the resistance mechanism which enhances the survival of *O. volvulus*.

The localization profile of OvGST1 differs from those

described for the GSTs of other helminths like *Ascaris* and *Schistosoma* species. For example, we have shown a high incidence of GST in the intestinal epithelium of *Ascaris suum* (Liebau *et al.* 1997). The Sm26GST of *Schistosoma mansoni* is restricted to the tegument, subtegumental parenchymal cells and digitations in the apical chamber (Trottein *et al.* 1990). The Sm28GST is predominant in the tegument and subtegumental parenchyma (Liu *et al.* 1996). However, all of these examples in other helminths localize enzymes that are more closely related to OvGST2. A localization profile for a GST similar to that of OvGST1 has not been previously described in other organisms, consistent with its novel structure.

The localization pattern of OvGST2 by immunoelectron microscopy confirmed the localization profile at the optical level (Liebau *et al.* 1996; Salinas *et al.* 1994). Using affinity-purified antibodies against the 24.5-kDa OvGST2, the native glutathione-binding protein was primarily localized in the

TABLE I

Tissue Distribution of OvGST1 and OvGST2 Protein in Various Developmental Stages of *O. volvulus* Based on Light (Liebau *et al.* 1994c, 1996) and Electron Microscopic Analyses

Localization	OvGST1	OvGST2
Adult worms		
Cuticle		
Epicuticle	++	—
Cortical layer	—	—
Middle layer	—	—
Basal layer	+++	—
Hypodermis		
Lamellae of outer membrane	++	—
Cytoplasm	—	+++
Nuclei	—	—
Mitochondria	—	—
Rough ER	—	—
Vacuoles	—	—
Golgi complex	—	—
Multivesicular bodies	—	—
Basal labyrinth	—	—
Musculature		
Fibrillar part	—	—
A-fibrillar part	—	—
Uterus		
Epithelial cells	—	++
Muscle cells	—	—
Intestine	—	—
Microfilariae		
Intrauterine	—	—
In nodular tissue	+	++
L1, L2 larvae	+	n.d.
Infective L3 larvae	++	+++

Note. n.d., not determined; —no, + weak, ++ moderate, +++ strong labeling.

hypodermis and the uterus epithelium of adult worms. The pattern of distribution of gold marker particles over the entire hypodermis suggested that the antigen is intracellular and that it is generally distributed throughout the cytoplasm. The colloidal gold label does not appear to be localized to any identifiable subcellular structure. Taken together, these observations indicate striking differences in the localization and function of these two GSTs in *O. volvulus*, in agreement with their structural and biochemical characterizations.

Finally, because both antigens are present in adult parasites, in microfilariae, and in the infective L3, any protective immune response associated with recognition of OvGST1 or OvGST2 would be continually boosted during an active infection, perhaps contributing to the development of concomitant immunity. Antigens from the infective stage of the parasite may be especially important (Abraham 1992;

Dopheide *et al.* 1991; Lucius *et al.* 1991; Nutman *et al.* 1991; Yates *et al.* 1985). Further investigation of recombinant antigens such as OvGST1 and OvGST2, together with the reported induction of acquired immunity in animal models of onchocerciasis (Lange *et al.* 1993), may lead to a better understanding of the complex process involved in the development of protective immunity to *O. volvulus* infection. The clarification of function and immunogenicity of these antigens, especially OvGST1, may shed some light on their actual potency for chemotherapy and vaccine development for onchocerciasis.

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