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Demonstration of anti-cuticular antibodies by immuno-electron microscopy in sera of mice immunized with cuticular extracts and isolated cuticles of adult *Dipetalonema viteae* (Filarioidea)

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Summary

The immunogold technique was used for the ultrastructural localization of antibody-binding sites on thin sections of Lowicryl K4M embedded adult females, infective larvae and pieces of adult cuticles of *Dipetalonema viteae* insoluble in SDS-2-ME. The antisera used were either produced against SDS-2-ME extracts of cuticles or the insoluble pellet after SDS-2-ME extraction. With both types of antisera a labelling of epitopes on fibers was achieved in intact cuticles. In isolated cuticles the corresponding structures were absent. The same sera crossreacted with the larval and microfilarial cuticle as well as with somatic structures of all three stages. The only serum against isolated cuticle, which did not recognize cuticle fibers also did not crossreact with somatic structures. The recognition of the electron dense cortical layer insoluble in SDS-2-ME depended on the number of immunizations. A labelling of the filarial surface was never achieved. – A dense labelling of the apical membrane enfoldings of the hypodermis pointed to an involvement in the synthesis of the nematode cuticle. The rough endoplasmic reticulum in the apex of the epithelial cells of the uterus, the content of the nutrient channels, and the substance between eggshell and mirofilariae crossreacted with most of the antisera. This led to the conclusion that these substances are partly produced by the uterus epithelium.

Key words: *Dipetalonema viteae;* cuticle; immuno-EM; low temperature embedding.

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Introduction

Several groups have attempted to isolate cuticular proteins of different parasitic nematodes with the aim to identify protective antigens. For that purpose iodination experiments were performed followed by extraction of the labelled proteins with detergents (reviewed by Maizels et al., 1982; Philipp and Rumjanek, 1984) or their enrichment by enzymatic digestion (Baschong et al., 1982). The results of the iodination were visualized by electron-microscopic autoradiography (Philipp et al., 1984; Baschong and Rudin, 1982).

Another approach to isolate cuticular antigens was performed by the isolation of cuticles of *D. viteae* using sonication and SDS-2-mercaptoethanol solubilization steps (Betschart et al., 1985). If SDS purified cuticle extracts were used for the immunization of mice, antibodies directed against all organs of the worm were found in an indirect fluorescence antibody test (IFAT). The insoluble residue of the cuticle stimulated the production of antibodies which reacted with the cuticle. As the optical resolution of the IFAT is limited, it was not possible to decide if any surface reaction was present. To localize in detail antibody-binding sites in or on the cuticle of *D. viteae,* electron-microscopic immunocytochemistry was used in the present investigation. Antigen-antibody reactions were carried out on thin sections of low temperature embedded (Kellenberger et al., 1980) filarial material, and the reaction sites were visualized by immunogold technique (De Mey, 1983).

Material and Methods

Antigens

The filarial parasite *Dipetalonema viteae* was maintained in the jird, *Meriones unguiculatus,* and in the soft tick, *Ornithodorus moubata* (Worms et al., 1961). Adult females were harvested from the connective tissue of the subcutis of infected hamsters. Third-stage larvae (L3) were collected from infected ticks (Gass et al., 1979). Cuticles of adult females were isolated and extracted by sequential sonication in SDS and SDS-2-ME (Betschart et al., 1985). Briefly, the cut worms were sonicated twice in a Tris-buffer pH 7.4 or 6.8 containing 1% SDS to remove internal organs. A 3–4 h incubation in SDS buffer containing 5% 2ME removed the remaining somatic material. Three more incubation steps with 2-Mercaptoethanol solubilized the basal and parts of the median zone of the cuticle. The pooled SDS-2-ME extracts and the insoluble pellet were used as antigens.

Antisera production

Antisera were produced in C57Bl/6 mice, 4 to 6 weeks old. The animals were either immunized with the SDS-2-ME adult cuticle extracts (sera code AE I and II) or with the remaining cuticle pellets after SDS-2-ME treatment (sera code AP I to IV). Two to five injections were given in two-week intervals. Mice were bled 4 to 6 days after the last injection. Details of the immunization schedules are given in Table 1.

Electron microscopy

Female worms, L3 and SDS-2-ME purified cuticles were prepared for immuno-electron microscopy. Adult worms were cut into about 2 mm long pieces and immediately fixed in 0.5% glutaraldehyde in 0.1 M PBS overnight, dehydrated in graded ethanol and embedded in Lowicryl K4M according to Kellenberger et al. (1980). Clumps formed by living L3 in RPMI 1640 medium (Flow) at

Table 1. Summary of immunization schedules

	Sera			
	AE I and II	AP I	AP II	AP III and IV
1st injection				
antigen	100 µg (protein) of SDS-2-ME cuticle extract[a]	cuticle pellet from 2 ♀♀	cuticle pellet from 10 ♀♀	cuticle pellet from 10 ♀♀
adjuvant	Freund's complete	–	–	Freund's complete
route	intraperitoneal	intraperitoneal	intraperitoneal	subcutaneous
Booster injection(s)				
number	1	1	4	4
antigen	100 µg (protein) of SDS-2-ME cuticle extract	cuticle pellet from 2 ♀♀	cuticle pellet from 5 ♀♀	cuticle pellet from 5 ♀♀
adjuvant	Freund's incomplete	–	–	Freund's incomplete
route	intraperitoneal	intraperitoneal	intraperitoneal	subcutaneous
Serum harvested (after)[c]	1st booster	1st booster	4th booster	4th booster

[a] pool of 2nd and 3rd SDS-2-ME extracts
[b] two weeks interval between injections
[c] serum harvested 4 to 6 days after last injection

37° C and pellets of purified cuticles of female worms (Betschart et al., 1985) were covered with 2.5% Noble Agar (Difco Lab.) in PBS (40° C). After cooling in the refrigerator the part of the agar-pellet containing the parasite material was cut into pieces of about 1 mm^3 and processed for electron microscopy as described above.

IFAT

The antisera, harvested after different boosters by retroorbital bleeding, were screened for cuticle fluorescence in an indirect fluorescent antibody test (IFAT) on frozen sections of adult female worms (Weiss, 1978) or on fixed larvae and microfilariae in a suspension IFAT (Weiss and Tanner, 1981). A FITC conjugated rabbit anti-mouse IgG (whole molecule) antiserum (Miles-Yeda Ltd.) was used at a dilution of 1 in 80 in PBS containing Evan's blue (1 in 10,000) as a counterstain.

Immunocytochemistry

Colloidal gold particles were prepared by the sodium ascorbate method (Stathis and Fabrikanos, 1958). The gold particles, with an average particle diameter of 12 nm, were coated with rabbit anti-mouse immunoglobulin antibodies, IgG-Fraction (Dakopatts, Denmark) according to the method for citrate gold (De Mey, 1983). The pH of the gold sol was adjusted to 9.0 by mixing one part 10 mM borax buffer with 9 parts gold sol. It was found by titration that for coating of 1 ml gold sol (≙ 0.1 mg $HAuCl_4$) 22 µg immunoglobulins were necessary. After two centrifugations (80,000 *g* for 1 h and 70,000 *g* for 50 min) the pellet was resuspended in 1/40 of the original volume of 20 mM Tris-buffered-saline (TBS, pH 8.2) which contained 0.5% bovine serum albumin and 20 mM sodium azide.

For the antigen localization thin sections were mounted on parlodion-carbon-coated copper grids. In order to reduce unspecific bindings, grids were pretreated with 10% normal rabbit serum in PBS for 10 min at room temperature and 100% relative humidity (r.h.) prior to incubation on the antiserum diluted in 20 mM PBS (pH 7.2). The optimum dilution for each antiserum was estimated in the first experiment (1 in 10 to 1 in 10,000). After 2 h of incubation on 20 μl drops of the mouse antiserum, the grids were first washed on a drop of PBS followed by two drops of TBS (pH 8.2) that contained 0.1% BSA. The gold labelled immunoglobulins were diluted to an OD_{525} of 0.5 with TBS before incubation of the grids on 20 μl drops for 1 h at 100% r.h. As a final wash the grids were floated two times for 5 min on drops containing 0.1% BSA prior to rinsing with triple distilled water from a squeeze bottle. As controls, thin sections were incubated a) on normal mouse serum prior to the application of immunogold and b) on immunogold only. The thin sections were stained with 5% (w/v) uranylacetate for 10 min followed by 0.4% (w/v) lead citrate for 2 min. Electron-microscopic photography was performed with a Philips EM 300.

Results

In the IFAT all antisera showed a fluorescence of the cuticle on frozen sections of adult female worms (Table 2). In addition with AE I and II (sera against SDS-2-ME soluble material) and AP II–IV (sera against SDS-2-ME purified cuticles) a fluorescence of the underlaying hypodermis, the gut epithelium and the uterus content surrounding the microfilariae was observed. For AP I no somatic fluorescence could be detected. In the suspension IFAT on infective larvae (L3) or microfilariae (Mf) no fluorescence resulted with antisera against SDS-2-ME purified cuticles (AP I–IV). With antisera against SDS-2-ME-extracts (AE I and II) a fluorescence was attributed to the surface of infective larvae (Table 2). These results were in agreement with those described for analogous sera (Betschart et al., 1985).

Immunocytochemical application of the same sera on thin sections of female worms revealed a good correlation between IFAT and immuno-EM results. As compared to IFAT a similar distribution of the marker could be

Table 2. Reactivity of antisera raised against SDS-2-ME cuticle extracts or isolated cuticles as demonstrated by indirect fluorescent antibody tests (IFAT) on frozen sections of female *D. viteae* or with the surface of infective larvae or microfilariae. Positive results given as reciprocal titers of highest serum dilution

Structure	Sera					
	AE I	AE II	AP I	AP II	AP III	AP IV
Cuticle of adult worm[a]	80	160	160	1280	2560	5120
Uterus (epithelium/content)[a]	80	160	neg[b]	40	160	160
Surface of microfilariae[c]	neg	neg	neg	neg	neg	neg
Surface of infective larvae[c]	40	40	neg	neg	neg	neg

[a] tested on frozen sections of female *D. viteae*

[b] neg = no fluorescence at 1 in 40

[c] tested on formalin-fixed intact instars (suspension test)

Table 3. Binding of immunogold to cuticle related antigens of *D. viteae* with sera directed against SDS-2-ME soluble extracts or SDS-2-ME purified cuticles

Antigen		Serum			
Stage	Structure	AE I/II	AE I	AP II	AP III/IV
Adult cuticle (♀♀)	surface layer	–	–	–	–
	inner cortical layer (e – dense)	–	–	+	+
	cuticle fibers	+	–	+	+
	cuticle matrix	+	+	+	+
	hypodermal folds	+	–	+	+
Isolated cuticles	surface layer	–	–	–	–
	inner cortical layer (e – dense)	–	–	+	+
	cortical matrix	+	+	+	+
	median matrix	+	+	+	+
Infective larvae (L3)	surface layer	–	–	–	–
	cortical	+	+	+	+
	median/basal	+	+	+	+
	hypodermis	+	–	+	+
Microfilariae (Mf)	surface layer	–	–	–	–
	inner cuticle	+	+	+	+

shown but more accurately localized using gold labelled rabbit anti-mouse immunoglobulins for the detection of the antibody-binding sites (Table 3, Figs. 1–10). On thin sectioned female worms antisera against soluble extracts (AE I and II) produced a dense labelling of the fibrous structures interweaving the central part of the cuticle. The electron dense layer within the cortical zone and surface layer were unlabelled (Fig. 1). Fibrous material was also labelled in the median zone, where it was mainly tangentially orientated with respect to the worm surface as well as in the basal zone, where it appeared to be arranged in net-like fashion (Figs. 1 and 4).

The same sera applied on thin sections of isolated cuticles resulted in a labelling of the cortical and median matrix (Fig. 2). Fibers were absent in isolated cuticles after SDS-2-ME treatment, just as the basal part and parts of the median zone of the cuticle were removed. The surface and the electron dense cortical layer remained unlabelled. A very faint background labelling on thin sections of female worms (Fig. 3), SDS-2-ME isolated cuticles and infective larvae was observed in controls with normal mouse sera. Numerous gold particles were seen on somatic structures with AE I and II on the apical part of the hypodermis consisting of membrane enfoldings (Fig. 4). The fibrous material in the uterus surrounding the microfilariae and the nutrient channels were densely labelled (Fig. 5). The basal part of the microfilarial cuticle was labelled but not its surface. The gold particles which could be seen on the surface of the microfi-

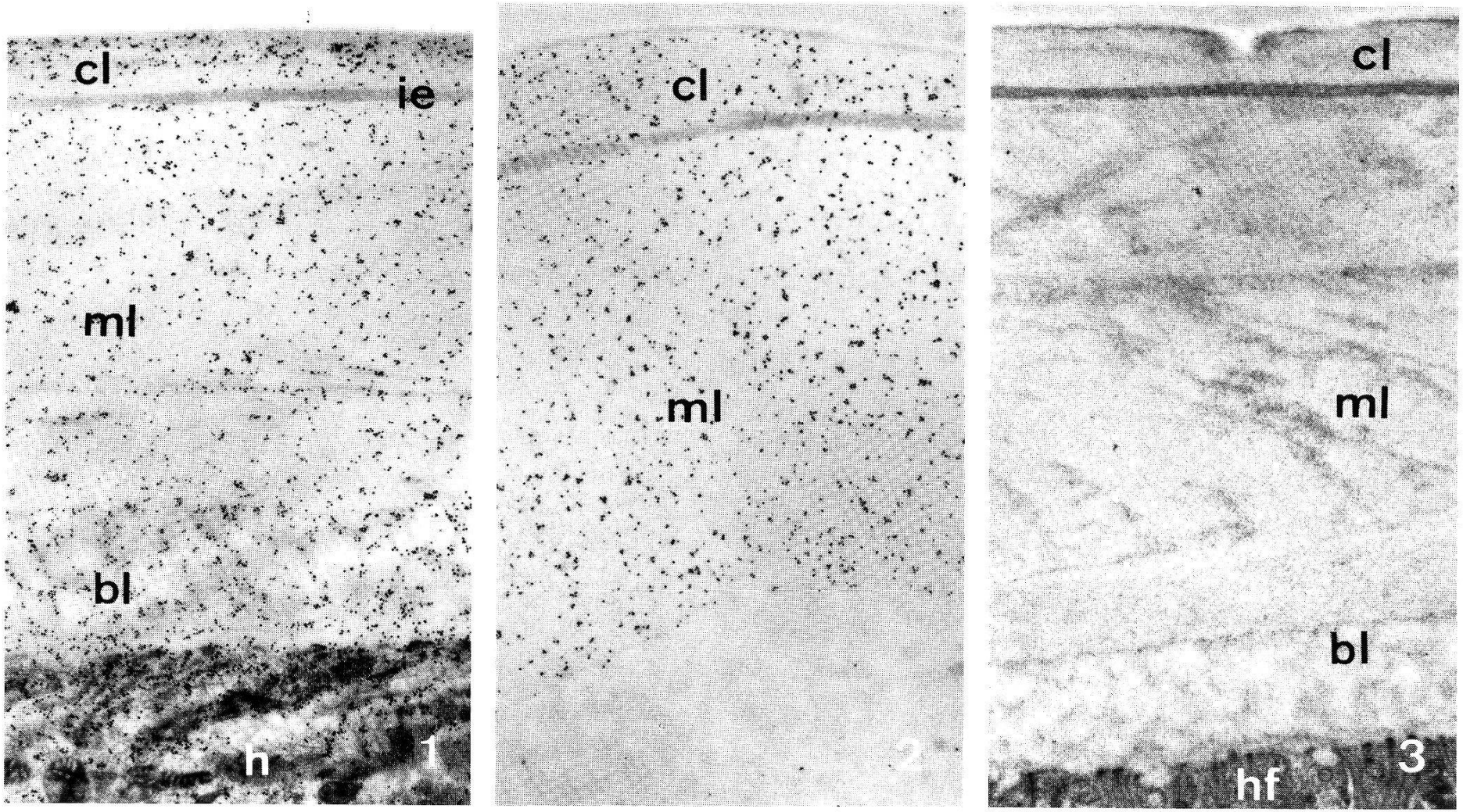


Fig. 1. *Dipetalonema viteae,* female, cuticle and hypodermis, serum AE I (1:1000). ×17,000.

Fig. 2. *Dipetalonema viteae,* female, SDS-2-ME purified cuticle, serum AE I (1:1000). ×14,000.

Fig. 3. *Dipetalonema viteae,* female, cuticle, normal mouse serum (1:10), control. ×13,000.

bl = basal layer, cl = cortical layer, h = hypodermis, hf = hypodermal folds, ie = inner electron dense cortical layer, ml = median layer.

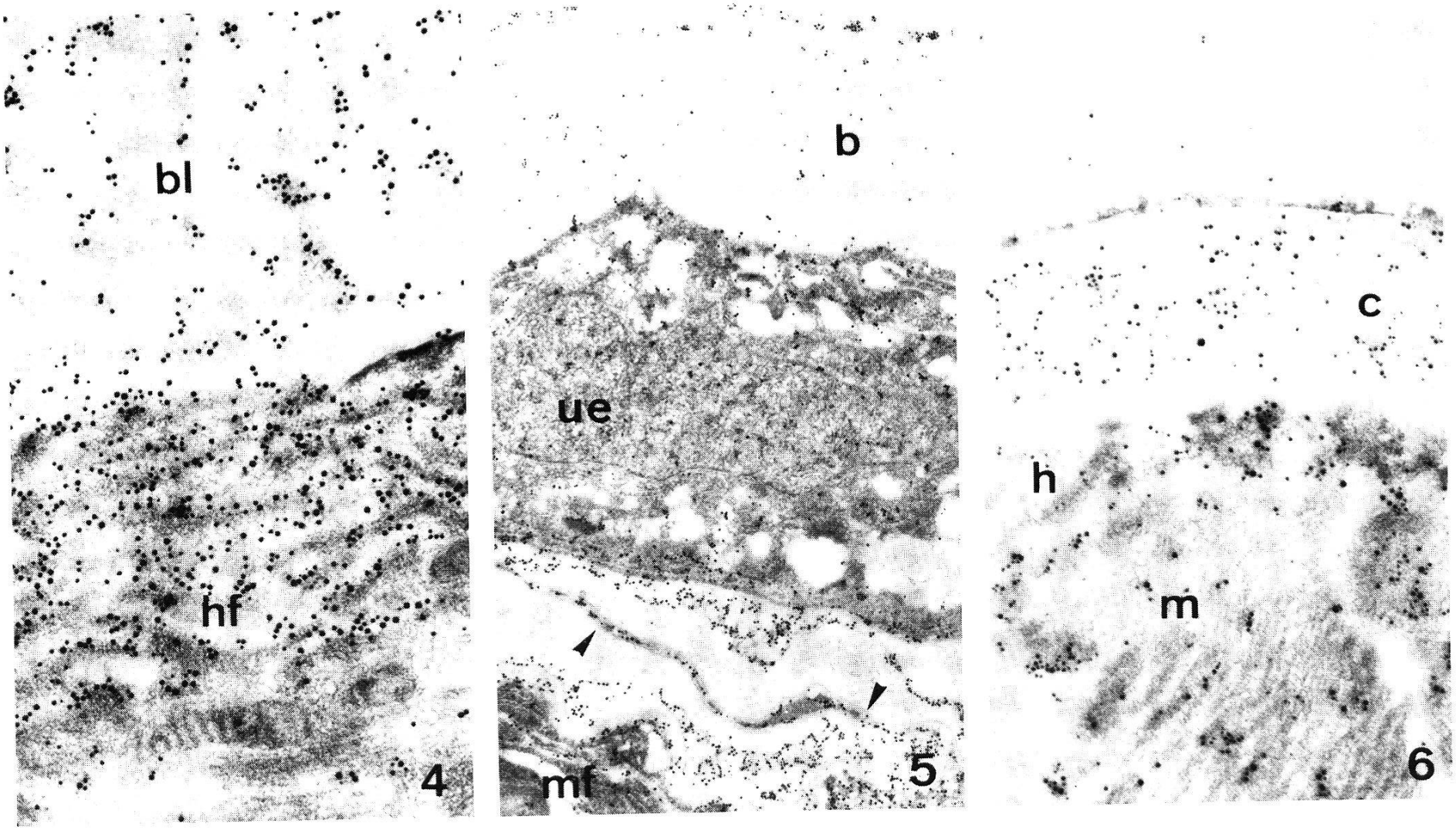
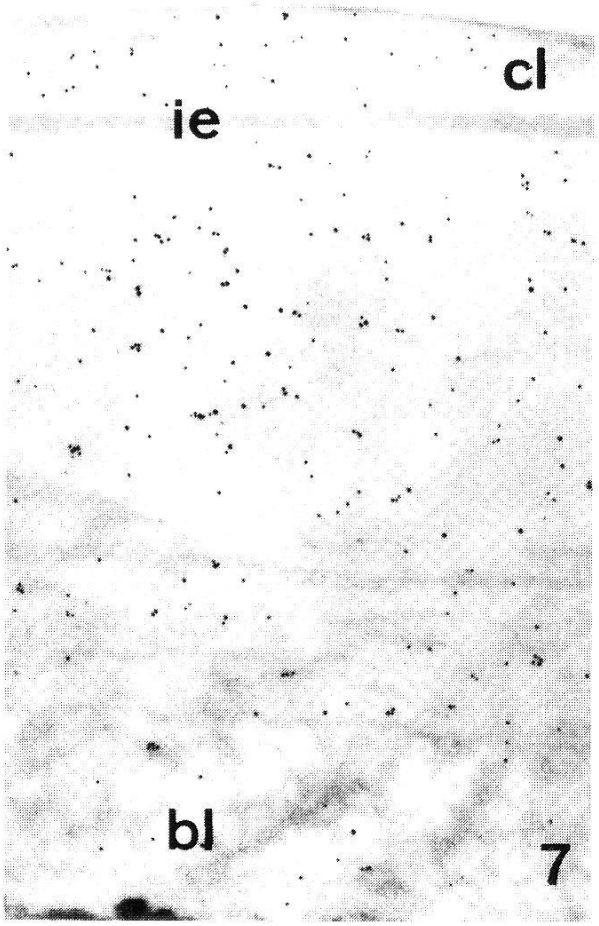


Fig. 4. *Dipetalonema viteae,* female, basal cuticle layer and hypodermal folds, serum AE I (1:1000). ×36,000.

Fig. 5. *Dipetalonema viteae,* female, uterus epithelium, eggshell and microfilariae, serum AE I (1:1000). ×19,000.

Fig. 6. Larva 3, serum AE I (1:1000). ×56,000.

b = basal lamina, bl = basal layer, c = cuticle, h = hypodermis, hf = hypodermal folds, m = muscle filaments, mf = microfilariae, ue = uterus epithelium, ▸ = eggshell.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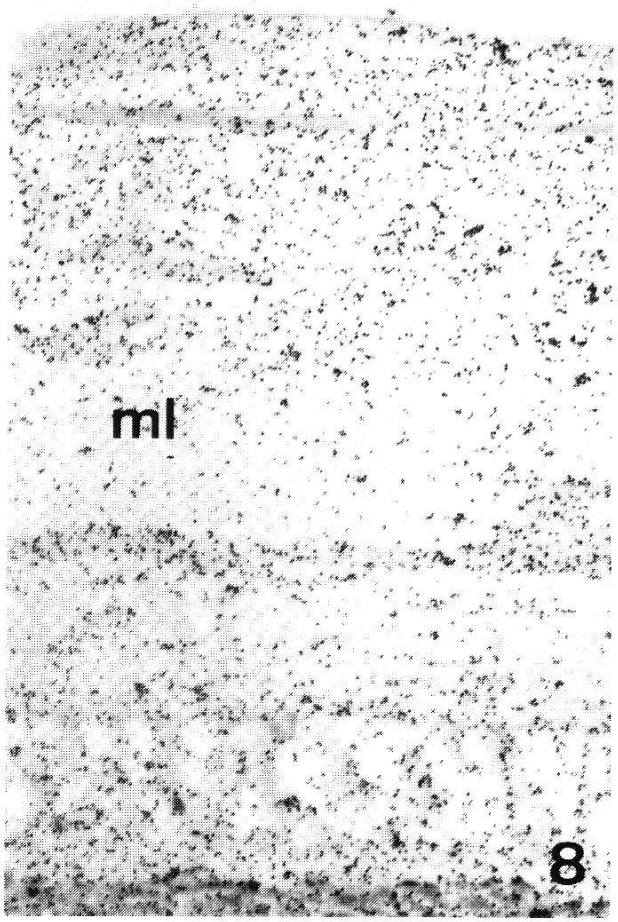
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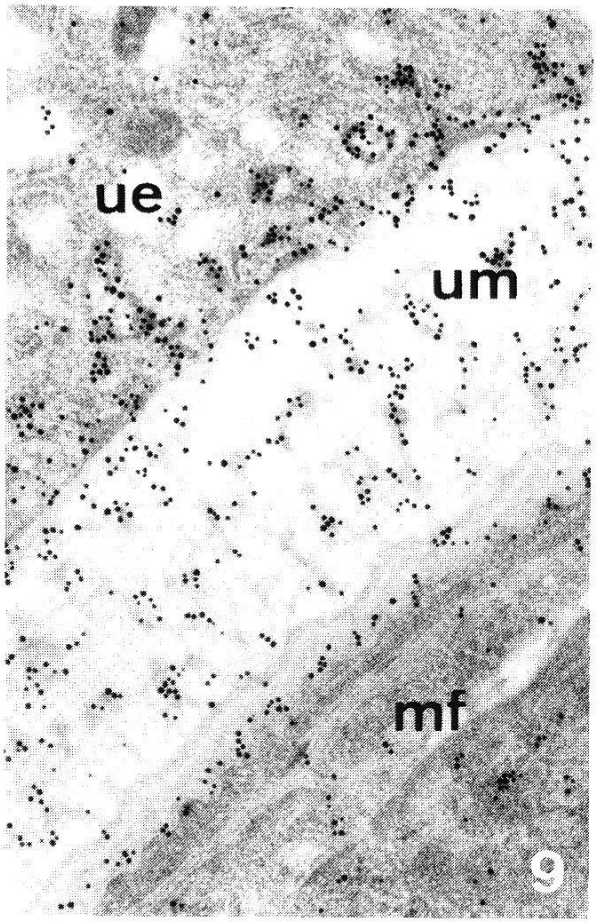
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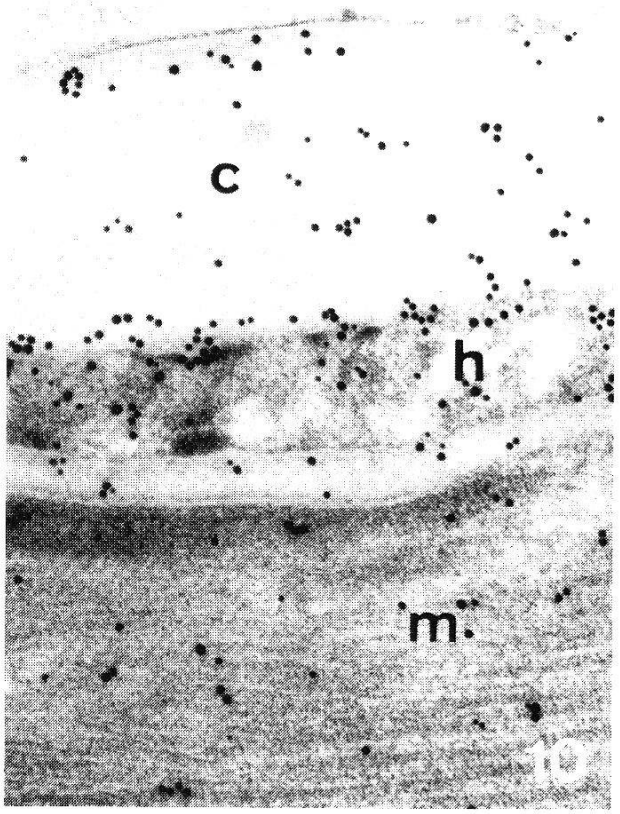
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lariae were attributed to the fibrous uterus material adhering to the cuticle. This was most obvious with antiserum AP IV (Fig. 9). As concerning the uterus epithelium there was a conspicuous labelling of the rough endoplasmic reticulum in the apical part of the cells and of the outermost part of the basal lamina. The epithelium and the basal lamina showed only a faint labelling. In addition mainly the basal lamina of the gut epithelium was labelled. Incubation of thin sections of infective larvae on sera AE I and II resulted in labelling of the cuticle (Table 3, Fig. 6) and somatic tissues. The surface layer of the L3 was devoid of marker. Using antisera against SDS-2-ME purified cuticles (AP I–IV) different labelling patterns resulted on thin sectioned adult worms. With serum AP I, harvested after the second immunization with purified cuticles, a labelling of the cuticle matrix was achieved. Fibers and hypodermal folds were unlabelled (Fig. 7). In agreement with IFAT results only a very faint background labelling resulted on somatic structures and the microfilarial cuticle which was considered as negative (Table 3). In contrast to AP I sera AP II–IV yielded a labelling of the cuticle fibers and the hypodermal folds. In addition, with AP II–IV a similar distribution of the gold particles resulted on somatic tissue as it was described for sera against SDS-2-ME extracts. AE I and II showed a preference for the cortical zone of the cuticle (Fig. 8).

Long-term immunization and the use of Freund's adjuvant resulted in a drastic enhancement of the number of gold particles. These findings were in agreement with the high antibody titer found for these sera in the IFAT (Table 2). An additional labelling of the inner cortical electron dense layer was achieved with sera harvested after the fifth injection (AP III and IV). The same sera screened after the third immunization with cuticle pellets, yielded no labelling of this structure. Thin sections of SDS-2-ME purified cuticles incubated on AP I serum showed the same distribution of the marker as it was mentioned above for AE I and II. For AP II–IV an additional labelling of the electron dense cortical layer was observed with sera harvested after five immunizations (Table 2).

AP I–II applied on thin sections of L3 yielded a labelling of the larval cuticle. With sera AP II–IV also different somatic structures carried gold particles. The surface layer was again free of marker (Table 3, Fig. 10).

Fig. 7. *Dipetalonema viteae*, female, cuticle, serum AP I (1:10). ×21,000.

Fig. 8. *Dipetalonema viteae*, female, cuticle and hypodermis, serum AP IV (1:1000). ×16,000.

Fig. 9. *Dipetalonema viteae*, female, uterus and microfilariae, serum AP IV (1:1000). ×46,000.

Fig. 10. Larva 3, serum AP IV (1:1000). ×58,000.

bl = basal layer, c = cuticle, cl = cortical layer, h = hypodermis, ie = inner electron dense cortical layer, m = muscle, mf = microfilariae, ml = median layer, ue = uterus epithelium, um = uterus matrix.

Discussion

This study has shown that the injection of SDS-2-ME extracts and SDS-2-ME purified cuticles in C57Bl/6 mice evoked a humoral immune response against different cuticular layers of *D. viteae* female worms. Most of the sera used showed cross-reactions with somatic epitopes of different *D. viteae* stages. In addition to the IFAT, immuno-electron microscopy was performed in the present investigation, because the low resolution power of the IFAT did not allow to correlate a cuticle fluorescence to fine structures, such as fibers or surface layers. In order to achieve a better preservation of the antigens than in classical EM-preparations, a mild fixation followed by low temperature embedding in Lowicryl K4M (Kellenberger et al., 1980; Roth et al. 1981; Bendayan, 1984) was selected. To be independent from the immunoglobulin isotype and the species specific affinities of protein A (Romano and Romano, 1984) antibody-binding sites were revealed ultrastructurally with the well detectable colloidal gold particles bound to a secondary antibody (De Mey, 1983).

A good correlation between IFAT and immuno-EM results was generally found. In some cases only the exact localization of the antibody binding sites by the immuno-EM technique made a detailed interpretation of the IFAT results possible. In agreement to the IFAT, the use of Freund's adjuvant yielded sera with high antibody titers which were suitable to get an intensive labelling with the immunogold technique.

In the IFAT a cuticle fluorescence was observed on frozen sections of female worms with all sera used in this study. In the immuno-EM experiments, carried out with the same sera a labelling of the cuticle could also be demonstrated with the exception of the surface layer (external cortical layer) where an appropriate labelling could never be seen. Only repeated injections with SDS-2-ME purified cuticles evoked an antibody response against the inner electron dense cortical layer, which is always present in EM-preparations of SDS-2-ME purified cuticles of *D. viteae*. The matrix of the isolated cuticles seems to be more immunogenic, because antibodies against this structure could already be detected after the second injection with SDS-2-ME purified cuticles without any adjuvant.

Insoluble electron dense layers of worm cuticles were demonstrated in several cases (Fujimoto and Kanaya, 1973; Cox et al., 1981a, b; Betschart et al., 1985). Their resistance against different detergents and enzymatic digestion could be an explanation for the absent or late humoral immune response in C57Bl/6 mice. The present results might indicate that these layers have first to be processed by the immune system of the mouse. It may be possible that only breakdown products induce an appropriate antibody production. Whether a long-term immunization would also produce antibodies against the surface layer has still to be explored.

Antisera against SDS-2-ME cuticle extracts reacted with fibers, mainly present in the median and basal zone of intact cuticles. The same was found for

antisera directed against SDS-2-ME purified cuticles (except AP I), although electron-microscopically visible fibers were absent in SDS-2-ME purified cuticles. The absence of fibers in SDS-2-ME purified cuticles was supported by the fact that no appropriate labelling was seen on thin sections of SDS-2-ME purified cuticles embedded in Lowicryl K4M. The SDS-2-ME cuticle extracts containing the solubilised fibrous material evoked an antibody response in the mouse against fibers which can be shown on intact cuticles only. It is likely that in SDS-2-ME purified cuticles antigenic determinants of fibers were still present, which were responsible for the cross-reaction of the AP-antisera with fiber epitopes. The morphological and biochemical properties of the fibers in the nematode cuticle were considered to be collagens (Lee, 1965; Bird, 1971). Cox et al. (1981a, b) demonstrated for the cuticle of *Caenorhabditis elegans* the solubility of the structural elements in the basal cuticle by SDS-2-ME treatment for collagenase digestion. McBride and Harrington (1967) have shown that the cortical, median and basal zones of *Ascaris lumbricoides* are composed of collagens crosslinked by disulfide bridges. The collagen chains were solubilised by 2-ME treatment. Winkfein et al. (1985) reported for *Ascaris lumbricoides,* that all cuticular proteins solubilised by 2-ME contained collagenous domains with the exception of a 173 kD component.

In all cases where an antibody response to fibers was found, an additional labelling of the apical part of the hypodermis, consisting of membrane enfoldings, was observed. The labelling of the hypodermal membrane enfoldings may reflect the secretion of components of the cuticle. The present findings suggest that the cuticle of *D. viteae* is a specialized extracellular matrix, secreted by the hypodermis and consisting of collagens and other still unknown components.

AP I showed no antibody binding to cuticle fibers, enfoldings of the hypodermis membrane and to somatic tissue in contrast to all other sera used in this study. Obviously the antibodies in AP I only recognized cuticle specific antigenic determinants. Whether the discrepancy between AP I and AP II–IV is due to the short immunization scheme used for AP I serum production or due to a better purified cuticle preparation, is not known. A broad specificity of the sera (except AP I) was observed concerning somatic tissue, although SDS-2-ME extracts and purified cuticles were used for antiserum production in C57Bl/6 mice. The complex composition of the used antigen preparations was already shown by SDS polyacrylamide gel electrophoresis where about 14 major bands could be identified (Betschart et al., 1985).

For the basal lamina of the uterus epithelium and the gut epithelium it was found, that mainly fibrous components were labelled. The basal lamina and embryonic connective tissue of vertebrate epithelia have been shown to contain type IV collagen (Fawcett, 1981; Timpl et al., 1982; Chen and Little, 1985). For invertebrates the insect basal lamina is described as a complex mixture of collagen, laminin, mucopolysaccharides and numerous unidentified fibrous components (François et al., 1980; Levinson and Bradley, 1984).

In previous IFAT observations (Betschart et al., 1985) the fluorescence within the proximal uterus parts with differentiated microfilariae was attributed to the cuticle of the microfilariae. On the other hand in the suspension IFAT no fluorescence could be detected on fixed microfilariae. This discrepancy could not be explained. Immuno-EM experiments revealed that the electron opaque, labelled material surrounding the microfilariae is partly responsible for the uterus-fluorescence in the IFAT. Due to the low resolution power of the IFAT this material could not be discriminated from the cuticle of the microfilariae. The rough endoplasmatic reticulum in the apical part of the uterus epithelium, as well as the electron dense material within the nutrient channels were also labelled. A similar immunoreactive staining has been shown by Pruesse et al. (1983) with antisera from naturally infected *Meriones unguiculatus* and *Mastomys natalensis* using the peroxidase-antiperoxidase technique on Epon embedded female worms. The results support the presumption of Ellis et al. (1978) that nutrient products from apocrine cells of the uterus wall are secreted into the nutrient channels formed by adjacent eggshells of microfilariae. The labelled fibrous material surrounding the microfilariae within the eggshell seems to contain components of the same origin.

Besides the cross-reactions with somatic epitopes we also found stage unspecific epitopes within the cuticles of microfilariae and infective larvae. The results indicate, that at least the median and basal cuticle zone contain common epitopes in the different stages. Common cuticular antigens among different parasite stages have already been found in *Brugia* sp. (Maizels et al., 1982) and *Litomosoides carinii* (Philipp et al., 1984) in addition to stage specific cuticular antigens (Philipp et al., 1981; Parkhouse et al., 1981).

A labelling of the surface layer of the microfilarial, the L3 and the adult cuticle could never be detected by immuno-EM methods with all sera produced in C57Bl/6 mice with the immunization scheme used. In agreement to EM-results, no fluorescence could be demonstrated with AP I–IV sera on the cuticle surface of infective larvae in the suspension IFAT. On the other hand in the suspension IFAT sera against SDS-2-ME extracts (AE I and II) yielded a cuticle fluorescence. This discrepancy to the EM-results cannot yet be explained and is under investigation.

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