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The isolation and immunogenicity of the cuticle of *Dipetalonema viteae* (Filarioidea)

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Abstract. The cuticle of the filaria *Dipetalonema viteae* was isolated by sonication in 1% sodiumdodecylsulphate (SDS) and in a mixture of 1% SDS and 5% B-mercaptoethanol (BME). Sonication in SDS removed all internal parts and left the cuticle intact; this was verified by light- and electronmicroscopy. Sonication and incubation of the cuticle in the mixture of SDS-BME at pH 6.8 dissolved the basal and part of the median zone of the cuticle. The epicuticle and the cortical zone remained intact. The extracts were investigated using SDS-polyacrylamide gel electrophoresis; the early extracts contained a wide variety of proteins, whereas the later steps showed a consistent pattern with a smaller number of bands. Cuticles after SDS-purification, the extract of cuticular material in SDS-BME, and the cuticles insoluble in SDS-BME were used to immunize mice; the antibodies produced were visualized by an indirect fluorescent antibody test on cryostat sections of female worms. When SDS-purified cuticles were used for immunization, antibodies directed against all organs in the filariae were found. The SDS-BME extract and the insoluble cuticular pellet stimulated the production of antibodies restricted to the cuticle of adult worms and microfilariae. The purification method opens up the possibility of further isolation and characterization of antigens from the cuticle.

Introduction

The surface of parasitic nematodes is recognized as the target for antibody-dependent cell-mediated cytotoxic reactions (for review see Ogilvie et al. 1980). Several groups have identified and characterized surface antigens using radiolabelling techniques, followed by extraction with a variety of detergents (for review see Philipp and Rumjaneck 1984; Maizels et al. 1982).

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In our laboratory a similar approach was used involving iodination with chloroglycoluryl, followed by digestion and extraction of a total homogenate (Baschong et al. 1982). A proteinase K digest injected into hamsters produced antibodies against the whole cuticle of adult *Dipetalonema viteae*. The exact characterization and localization of the antigen(s) inducing the immunological response was difficult, since they represented enzymatic breakdown products of the whole worm. In the present investigation, the method described by Cox et al. (1981 a) for the isolation of cuticles of the soil nematode *Caenorhabditis elegans* was modified and used to isolate cuticles from *D. viteae* and prepare defined protein extracts from them. The immunogenicity of cuticles, cuticle fragments, and extracts was then studied.

Materials and methods

Dipetalonema viteae was maintained as described earlier (Baschong et al. 1982). Adult worms were dissected out 12–15 weeks post infection, rinsed with 0.15 M phosphate-buffered saline (PBS) pH 7.2 and kept frozen at −70° C. For the purification of the cuticles 25 adult females were thawed, aligned on a glass plate, frozen on dry ice, and cut into 0.5–1 mm pieces using a razor blade. The pieces were transferred into a plastic tube with 15 ml SDS_1 buffer (0.01 M Tris-HCl, 1 mM EDTA, 1 mM PMSF, 1% SDS), pH 7.4. The solution containing the worm fragments was stirred overnight at room temperature, then sonicated on ice with a 100-watt ultrasonic disintegrator (MSE, London, GB) at maximum output, using one cycle (i.e., 10 × 15 s with 5 s-intervals). The supernatant, which contained mainly fragments of internal organs was carefully decanted. The cuticles were resuspended in 15 ml SDS_2-buffer (1% SDS, 0.125 M Tris-HCl, pH 6.8) and sonicated with one cycle, the supernatant removed again, and the cuticles resuspended in 15 ml SDS_2-buffer containing 5% B-mercaptoethanol (SDS-BME_1). After 3–4 h incubation at 4° C the suspension was sonicated with 1–2 cycles. Samples were checked under the microscope, and sonication continued until all internal organs had disappeared. The cuticle pieces were sedimented by centrifugation at 1,000 *g* for 15 min (Heraeus Minifuge II), washed once in SDS_2-buffer, resuspended in 15 ml SDS-BME buffer, kept overnight at 4° C, and then sonicated using two cycles (SDS-BME_2). Two more extraction cycles with SDS-BME were performed (SDS-$BME_{3/4}$). All supernatants and the insoluble cuticle pieces were frozen. Before electrophoretic analysis the supernatants were dialyzed at 4° C against 3 × 5 l of water for 3 days, and the protein concentrations determined (Peterson et al. 1977). Samples of the cuticle extracts were lyophilized and electrophoretically analysed using the Laemmli system (Laemmli 1970) with T=15% and C=0.5%. The proteins were stained with Serva blue R in 40% methanol, 10% acetic acid.

For electron microscopy, the isolated cuticles were washed in H_2O and centrifuged at 1,500 *g* for 10 min and the pellets prefixed with 4% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) for 3 h at room temperature. After rinsing in 0.2 M Na-cacodylate buffer (supplemented with 7.3% sucrose, pH 7.4) overnight at 4° C, the worms were postfixed in 2% OsO_4 in 0.2 M Na-cacodylate buffer for 2 h. After a first dehydration step for 15 min in 70% acetone the blocks were stained with 2% uranyl acetate in 70% acetone for 1 h at room temperature in the dark and further dehydrated in graded acetone. Fixation, dehydration, and embedding were performed as already described (Rudin et al. 1980). Ultrathin sections (about 50 nm) were cut on a LKB Ultratome III and transferred to grids coated with collodium and reinforced with a carbon layer. For examination in a Philipps EM 300 electron microscope the grids were poststained with lead citrate for 20 s (Reynolds 1963).

For immunizations, C57/BL mice were injected intraperitoneally either with cuticles isolated only with SDS or those purified with SDS-BME (cuticles from three female worms in 0.9% NaCl per injection). Two and four weeks later the mice were given booster immunizations using the same amount of material. In addition, four mice were injected with BME-soluble material from SDS purified cuticles (SDS-BME_2 and SDS-BME_3 pooled, dialysed and lyophilized). This antigen preparation (100 µg protein per injection) was emulsified in Freund's com-

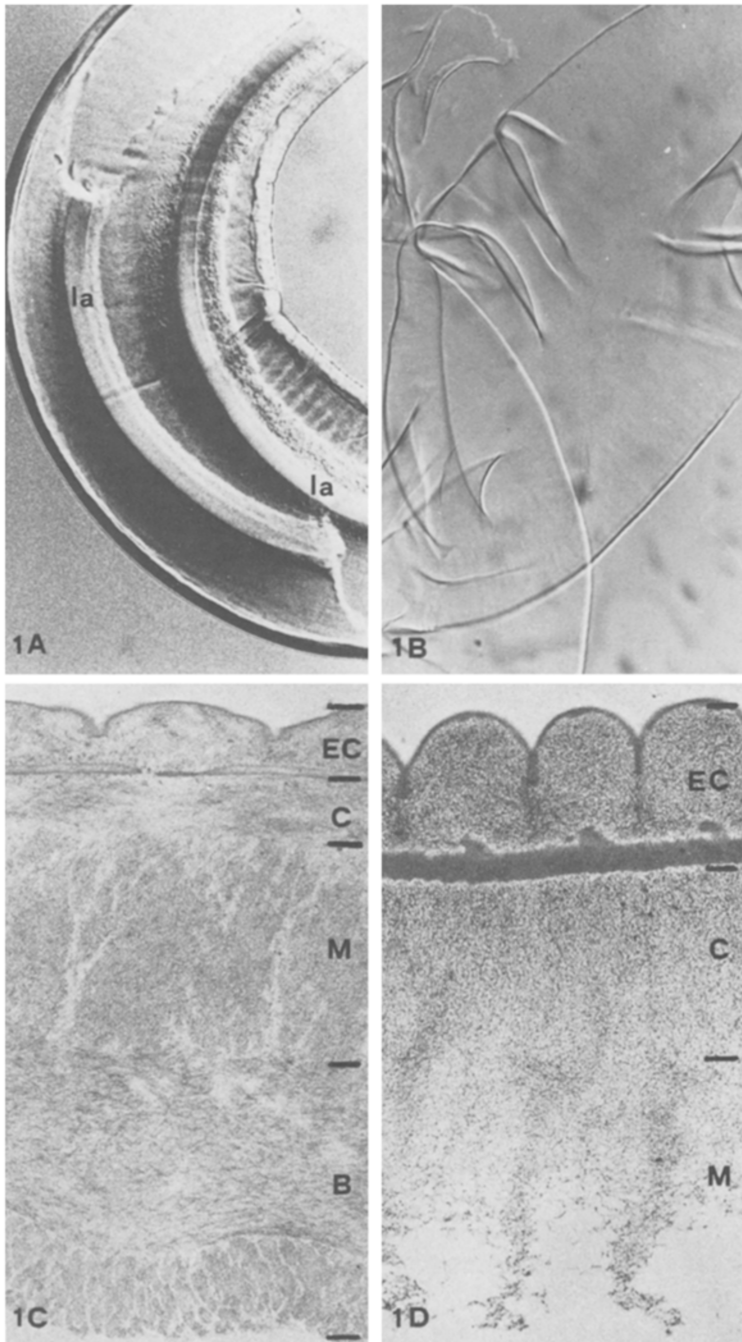

Fig. 1 A–D. Light and transmission electron micrographs of female *Dipetalonema viteae* cuticles. **A** SDS purified cuticle with lateral alae (=La). Interference contrast micrograph, ×568. **B** SDS-BME purified cuticle. The alae have disappeared, ×600. **C** Ultrastructure of SDS-purified cuticle, ×12,000. **D** SDS-BME insoluble cuticle, ×23,000. *B*, basal zone; *M*, median zone; *C*, cortical zone; *EC*, epicuticle

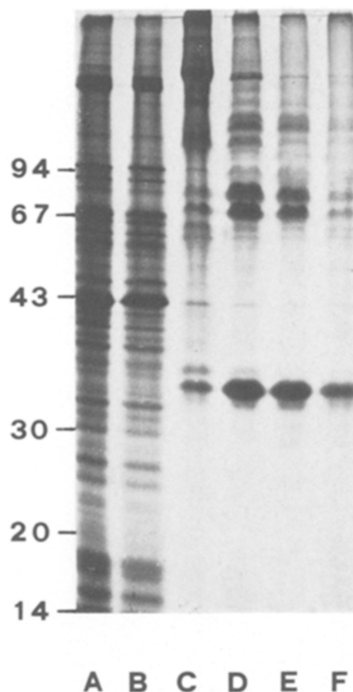

Fig. 2. 15% SDS-polyacrylamide gel electrophoresis of the extracts obtained during the purification of *Dipetalonema viteae* cuticles. $A = SDS_1$, $B = SDS_2$, $C = SDS\text{-}BME_1$, $D = SDS\text{-}BME_2$, $E = SDS\text{-}BME_3$, $F = SDS\text{-}BME_4$. Molecular weights in K.

plete adjuvant and injected intraperitoneally. Two weeks later the same amount of antigen was injected in Freund's incomplete adjuvant. All mice were bled 4 days after the last injection and the sera stored at $-70°$ C.

Sera were tested in two-fold dilutions starting at 1:40, using an indirect fluorescent antibody test (IFAT) on frozen sections of adult female worms. A FITC-conjugated rabbit anti-mouse immunoglobulin G (whole molecule) antiserum (Miles – Yeda Ltd) was used at a dilution of 1:80 in phosphate-buffered saline containing Evan's blue (1 in 10,000) as a counterstain. Photomicrographs were taken using a Leitz Dialux-20 microscope equipped with fluorescence and interference optics. The exposure time was automatically controlled by a photoautomat MPS 55a (Wild, Heerbrugg, Switzerland).

Results

Incubation and sonication in SDS resulted in the separation of internal organs from the cuticle (Fig. 1 A). The resulting suspension contained a mixture of cuticles and somatic material. The cuticle fragments sedimented faster than the somatic parts so that most of the somatic material was removed by careful decantation. Electron microscopic preparations of cuticles after SDS-treatment revealed that the cuticle was completely separated from the hypodermis. The hypodermal membrane was not found (Fig. 1 C). The nomenclature proposed by Bird (1984) is used throughout the paper to define the different cuticular layers. In SDS gels the extracts after the first two incubations in SDS yielded a complex pattern of proteins over the whole molecular weight range. The Serva blue R-patterns of a total

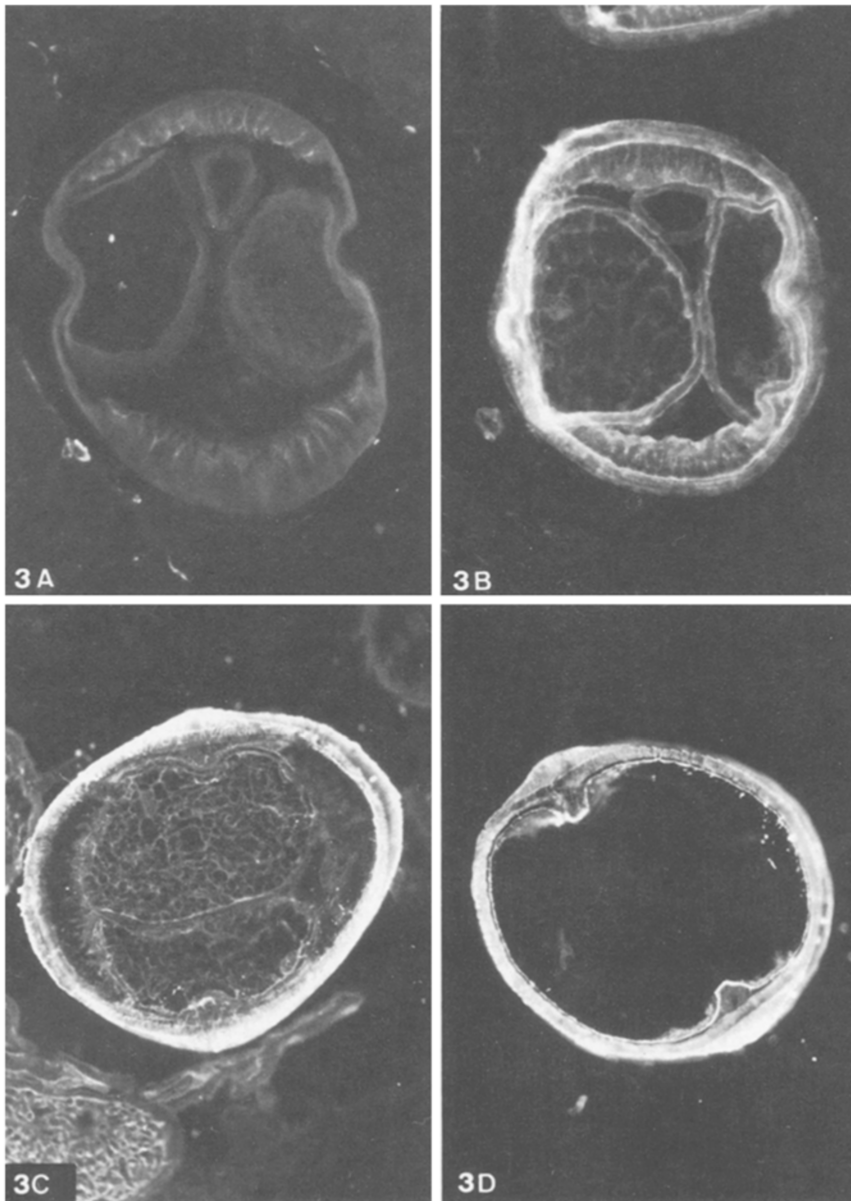

Fig. 3A–D. Reactivity of mouse sera (tested at 1:40) in the indirect fluorescent antibody test on frozen sections of adult female *Dipetalonema viteae*. **A** Normal mouse serum as control, showing slight background fluorescence. ×830. **B** Immunization with SDS purified cuticles. Serum reacted with cuticular and somatic antigens. ×740. **C** Immunization with SDS-BME_{2+3} extract. Serum reacted with adult and microfilarial cuticles. ×640. **D** Immunization with SDS-BME insoluble cuticles. Serum reacted with the cuticle of the adult worm. Some sera showed slight fluorescence on microfilariae. ×800

homogenate and the SDS extract were comparable (data not shown). A short incubation in SDS-BME was necessary to get rid of the last fragments of somatic material. The protein pattern changed between the steps in SDS alone (Fig. 2A, B) and the first step with $SDS\text{-}BME_1$ (Fig. 2C).

Further incubation in SDS-BME resulted in the selective removal of the basal and part of the median zone of the cuticle. When observed by interference microscopy the remaining sheet was translucent and the lateral alae had disappeared completely (Fig. 1B). Ultrastructurally, no basal and no clear median zones were detected. The cortical zone and the epicuticle remained intact (Fig. 1D).

In SDS gels, the number of stained proteins was reduced (Fig. 2D–F). A prominent band was present at 34 K. Below this molecular weight no bands were detectable, in contrast to the SDS extracts (Fig. 2A, B). Between 34 and 60 K only a few faint bands could be detected. Strongly staining proteins were again present at molecular weights higher than 60 K. About 14 bands could be observed in the $SDS\text{-}BME_2$ extract (Fig. 2D). In the subsequent extraction steps the total amount of proteins dissolved decreased steadily but the pattern remained similar. After the last extraction, very thin cuticles were left (Fig. 1B).

Sera of mice immunized with cuticular preparations (after SDS or SDS-BME treatment), or with material solubilized with SDS-BME, were tested for antibodies by an indirect fluorescent antibody test (IFAT) on frozen sections of female worms. A faint background staining was observed with normal mouse sera at low titres (Fig. 3A). Sera from mice, immunized with cuticles isolated using SDS only, contained antibodies against the cuticle but also against the hypodermis, muscle, mesenterial, uterine, and egg membranes (Fig. 3B). After the injection of SDS-BME soluble extract, sera reacted exclusively with the cuticles of adult worms and microfilariae (Fig. 3C). This was also the case for some mice injected with the SDS-BME insoluble pellets, while others produced antibodies reacting with the cuticle of the adult worm only (Fig. 3D).

Discussion

The treatment of adult female *D. viteae* with SDS resulted in the isolation of the whole cuticle. Sonication with SDS-BME removed the basal zone and, to an unknown degree, the median zone. Similar results have been described for the soil nematode *Caenorhabditis elegans* (Cox et al. 1981a; Ouazana and Herbage 1981) and *Panagrellus silusiae* (Leushner et al. 1979). All isolation procedures involved the use of the anionic detergent SDS to separate the cellular parts from the extracellular cuticle.

Studies with *Ascaris lumbricoides* have shown that the basal, median, and cortical zones are composed of collagens crosslinked by disulphide bridges (McBride and Harrington 1967); incubation of the cuticles with BME reduced the disulphide bridges and the collagen chains were solubilized. In adult *Caenorhabditis elegans* and *Ascaris lumbricoides* (Cox et al. 1981a, b; Fujimoto and Kanaya 1973) treatment with SDS-BME resulted

in the solubilization of all zones of the adult cuticle except the epicuticle. SDS-BME had a less pronounced effect on the cuticle of *D. viteae* (Fig. 1D). The cortical zone as well as the epicuticle usually remained intact. Since there is no clear line of demarcation between the cortical and the median zones it was not clear whether all of the median zone was removed. The "fibre-like" structures (Fig. 1D) emanating from the cortical zone have been described by Bird (1971, Fig. 8b) in *Ascaris lumbricoides* as part of the cortical zone reaching into the median zone. The function of these fibres is not known. It is possible that they, and the thickenings in the electron-dense layer of the epicuticle (Fig. 1D) have a role in the anchorage of the epicuticle and cortical zone to the basal parts of the cuticle (Bird 1971).

The electrophoretic analysis of the extracts revealed a reduction in the number of different proteins isolated during the purification process. In the first SDS-extracts a complex pattern of proteins (at least 48 bands) over the whole molecular weight range from 14 K to more than 100 K represented proteins derived mainly from the inner parts of the worm. The possibility exists that during this purification step some cuticular proteins also were solubilized. The ultrastructural preservation of the cuticle and the ability of isolated cuticles to induce antibodies directed against the cuticle is taken as evidence that at least major structural proteins were not solubilized. Experiments using radioactively labelled cuticles are now being carried out to determine the degree of solubilization of cuticular proteins in the SDS purification step.

The SDS-BME_1 step (Fig. 1C) was performed to remove the last residues of non-cuticular material. The SDS-PAGE pattern is less complex with this extract than with SDS alone, but still represents a mixture of cuticular and somatic proteins. The final steps with BME, dissolving the basal and median zones (see Figs. 1B and D) produced the simplified protein pattern described on SDS-gels (Fig. 2). It was not possible to attribute the different protein bands to a specific region within the cuticle; further work is needed to clarify this.

The epicuticle was resistant to SDS-BME extraction. The resistance of the epicuticle to solubilization in a variety of detergents, in organic solvents and with enzymes has been shown for *Strongyloides ratti* larvae (Murrell and Graham 1982).

A comparison of the SDS-gel patterns with those from *Caenorhabditis elegans* revealed some similarities. In *C. elegans* most SDS-BME soluble proteins were also resolved in the molecular weight range of 55 K and higher (Cox et al. 1981b). However, a clear difference from *C. elegans* is the presence of a strong 34 K band in *D. viteae*. Cox demonstrated the collagenous nature of the proteins in his preparation by successful digestion with collagenase, but it was not possible to digest the protein bands (Fig. 2D) from *D. viteae* with similar concentrations of enzyme (data not shown); this discrepancy may be due to differences in the enzymes used, or to differences in the type of collagen present.

The goal of this work was to isolate the cuticle and prepare cuticular

antigens by a combination of mechanical and chemical solubilization steps. All steps were performed either at room temperature or on ice; boiling as described for *C. elegans* (Cox et al. 1981 a) was avoided to prevent possible heat denaturation of cuticular antigens. The immunization experiments showed that cuticular preparations isolated by this method are immunogenic for mice. Isolated whole cuticles should theoretically have elicited only antibodies against the cuticle. Serum antibodies against somatic antigens were, however, detected after the injection of cuticles purified by SDS alone; this phenomenon is explained by the fact that SDS alone did not remove all somatic material. The remaining somatic antigens were successfully removed by the first SDS-BME_1 incubation (Fig. 2C). Its omission in one of our early experiments led to an antibody response which was partially directed against somatic organs.

We have started experiments combining surface iodination methods with the purification steps described in order to analyse in more detail the surface antigens relevant to the immune response.

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