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Extraction and partial characterization of surfactant-soluble antigens from adult female *Dirofilaria immitis*

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Summary

Antigens were solubilized from aqueous-insoluble material of adult female *Dirofilaria immitis* with Triton X-100 and sodium dodecyl sulfate (SDS) and partially characterized. Concentrations of 2.1–8.3 mg Triton X-100/mg total protein and 1.06–4.26 mg SDS/mg total protein were used to solubilize antigens. Triton X-100 was more efficient in solubilizing protein than SDS; Triton X-100 and SDS released 75% and 26% of the available protein, respectively. Protein mixtures solubilized with different levels of Triton X-100 appeared to be identical in immunodiffusion. Crossed immunoelectrophoresis revealed six antigen systems. Triton X-100 solubilized antigens did not react in immunodiffusion with sera from 7 *D. immitis*-infected, microfilaremic dogs, but did react with sera from 4 of 5 dogs with occult *D. immitis* infections.

Key words: *Dirofilaria immitis*; antigen.

Introduction

Antigens isolated from adult *Dirofilaria immitis* have been used frequently for serodiagnosis of canine and human filarial infections (Kagan, 1974). Aqueous soluble antigens partially-purified by chemical methods initially devised by Sawada et al. (1965) and employed by Grieve et al. (1979, 1981) and Glickman et al. (1984) and by immunochemical methods (Welch and Dobson, 1978) have demonstrated improved specificity in studies on immunologic and serologic responses to *D. immitis*. Partially-purified aqueous soluble antigens

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may still be complex (Sawada et al., 1965), however, and may not contain all potentially important antigens.

Research on antigens present in aqueous-insoluble portions of adult *Dipetalonema viteae* has revealed that antigens solubilized with surfactants (Neilson, 1975) or certain enzymes (Baschong et al., 1982) may represent a distinct group of antigens. Similarly Triton X-100-solubilized proteins from *Onchocerca volvulus* differed antigenically from proteins present in aqueous-soluble extracts (Marcoullis and Grasbeck, 1976). The purpose of the present study was to extract and partially describe, by immunochemical means, surfactant-soluble proteins from aqueous-insoluble material of adult female *D. immitis*.

Materials and Methods

Adult gravid female *Dirofilaria immitis* were obtained from dogs at necropsy, thoroughly washed in 0.01 M phosphate buffered saline (pH 7.2) and stored at -70°C . After thawing, worms were weighed, comminuted, and mixed 1:10 (w/v) with 0.05 M Tris (pH 8.3) containing 0.02% NaN_3 . This mixture was repeatedly homogenized and extracted at 4°C until no protein was evident in the supernatant solution as measured by optical density at 280 nm. This process required approximately 30 days of homogenization and extraction at 72 h intervals. After the final extraction the pellet was resuspended in the Tris buffer to give a 1:8 (w/v) ratio of original weight of worms to buffer.

Aqueous-insoluble material was treated with Triton X-100 (Bio Rad Laboratories, Richmond, CA, USA) or sodium dodecyl sulfate (SDS) (Bio Rad Laboratories, Richmond, CA, USA). Unless otherwise noted, protein concentrations of Triton X-100-solubilized antigens were determined with a colorimetric protein assay (Bio Rad Laboratories, Richmond, CA, USA) with a bovine serum albumin reference. To ascertain optimum surfactant levels, different amounts of Triton X-100 and SDS were analyzed. Two-tenths ml of the suspension of aqueous-insoluble material was added to 1.0 ml of 1.0 M NaOH, incubated for 18 h at 28°C with agitation and assayed by a colorimetric assay (Markwell et al., 1978) to determine total protein. Solubilization with Triton X-100 was performed by adding Triton X-100 in different quantities to 5.0 ml of the suspension of aqueous-insoluble material and incubating the suspension for 1 h at 28°C with continuous agitation. Triton X-100 to total protein ratios (w/w) employed were 3.1, 3.2, 4.3, 4.8 and 8.3. Two-tenths ml of each suspension was extracted in NaOH and protein assayed as before. The remainder of each suspension was centrifuged at $100,000 \times g$ for 60 min over an equal volume of buffer containing 10% glycerol with Triton X-100 at a concentration equivalent to that of the respective suspension. The resulting supernatant solutions were assayed for protein; pellets were resuspended, extracted in NaOH and protein assayed as before. Each Triton X-100 solubilized supernatant solution was dialyzed against 0.05 M Tris pH 8.3, 0.5% Triton X-100 and concentrated at 4°C using ultrafiltration with an exclusion limit of 5,000 d (Amicon Corporation, Lexington, MA, USA). Solubilization with SDS was performed by adding 0.5%, 1.0% or 2.0% SDS (w/v) to the suspension of aqueous-insoluble material and incubating for 18 h at 28°C . The corresponding detergent to total protein ratios (w/w) at the SDS concentrations listed above were 1.06, 2.13 and 4.26, respectively. A colorimetric method (Markwell et al., 1978) was used to determine protein concentrations in the presence of SDS.

Aqueous-insoluble material was lyophilized and used to immunize rabbits (Vaitukaitis et al., 1971). For the period of time antibody was evident, as indicated by immunodiffusion against homologous antigen, blood was collected every 3–4 days, sera were pooled and stored at -70°C . Immunoglobulins were isolated for use in immunodiffusion and crossed immunoelectrophoresis (CIEP) by ammonium sulfate precipitation. Precipitate was dialyzed against 0.1 M NaCl (pH 7.2) with 0.015 M NaN_3 and 1000 KIE units/ml of aprotinin (Sigma Chemical Co., St. Louis, MO, USA) was added to inhibit proteases. Prior to CIEP or immunodiffusion rabbit immunoglobulins were

absorbed with canine liver powder (Sigma Chemical Co., St. Louis, MO, USA). Sera from 4 dogs with clinically and serologically diagnosed occult (amicrofilaremic) *D. immitis* infections which showed significant antibody level increases following anthelmintic treatment (Grieve, 1981) and from 1 experimentally infected dog with occult *D. immitis* infection were used in immunodiffusion. Four of the 5 sera from occult dogs were positive for antibody to microfilaria surface antigens using an indirect fluorescent antibody test (Wong and Suter, 1979).

Antigenic activity of Triton X-100-solubilized preparations was assessed against homologous rabbit immunoglobulin and against canine sera with an immunodiffusion assay (Wadsworth, 1962). Gels were stained for 5 min with 0.1% Naphthol Blue-Black in 45% methanol/10% glacial acetic acid and destained. Crossed immunoelectrophoresis (Weeke, 1973) was used to enumerate and partially characterize antigenic constituents of the Triton X-100-solubilized preparations. Triton X-100 soluble antigens were analyzed at 6 or 12 mg/ml and rabbit immunoglobulin was used at 0.015 ml/cm². One percent low-gelling temperature agarose (Miles Laboratories, Inc., Elkhart, IN, USA) in Tris-barbital/sodium barbital buffer (pH 8.8) diluted 1:4 with 0.5% Triton X-100 was used for both dimensions. Fifteen μ l antigen samples were separated in the first dimension at 15° C for 90 min at 10 V/cm. Agarose strips containing the separated sample were cut, washed, and stained with Coomassie Blue (Weeke, 1973) and duplicate strips were used to electrophorese the separated antigens into the agarose containing immunoglobulin. Electrophoresis in the second dimension was performed at 15° C for 17 h at 2 V/cm and gels were stained for analysis (Weeke, 1973).

Results

The efficiencies of Triton X-100 and SDS in solubilizing adult female *D. immitis* aqueous-insoluble protein are illustrated in Fig. 1. Solubilization of suspensions of aqueous-insoluble material with weight ratios of 1.06, 2.13, 4.26 mg SDS/mg protein resulted in the recovery of 26%, 23% and 21% of the total protein, respectively. Triton X-100 provided increased solubilization with increased weight ratios up to a weight ratio of 4.8; a maximum recovery of 75% of the available protein was determined.

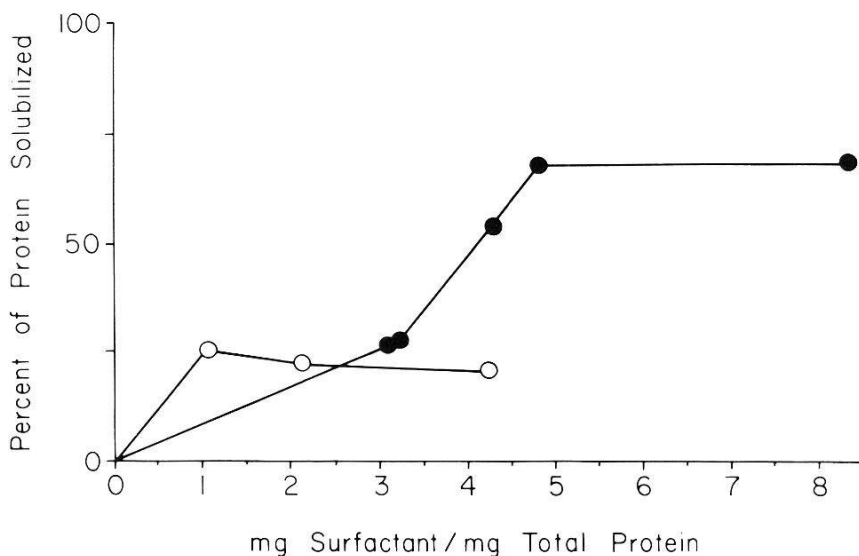


Fig. 1. Percent of total protein solubilized from aqueous-insoluble material following treatment with different concentrations of Triton X-100 (●—●) or SDS (○—○).

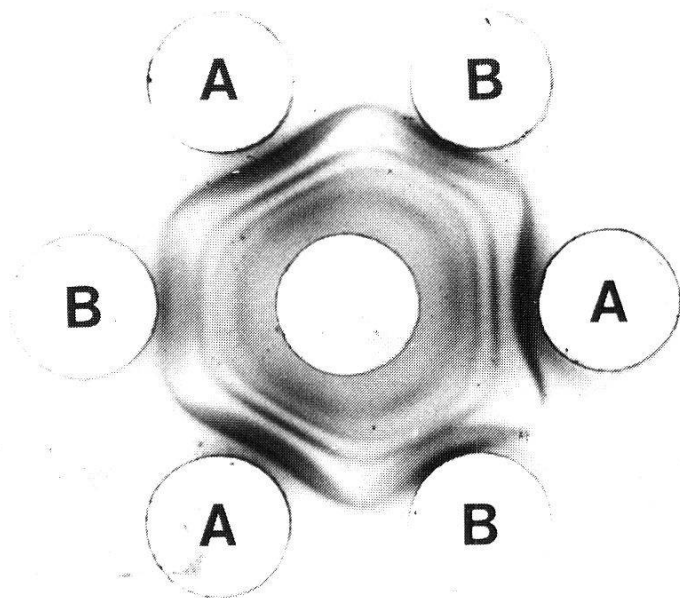


Fig. 2. Immunodiffusion of Triton X-100-solubilized antigens (3 mg/ml) against homologous rabbit immunoglobulin. A = Antigens solubilized with 4.3 mg Triton X-100/mg total protein. B = Antigens solubilized with 4.8 mg Triton X-100/mg total protein.

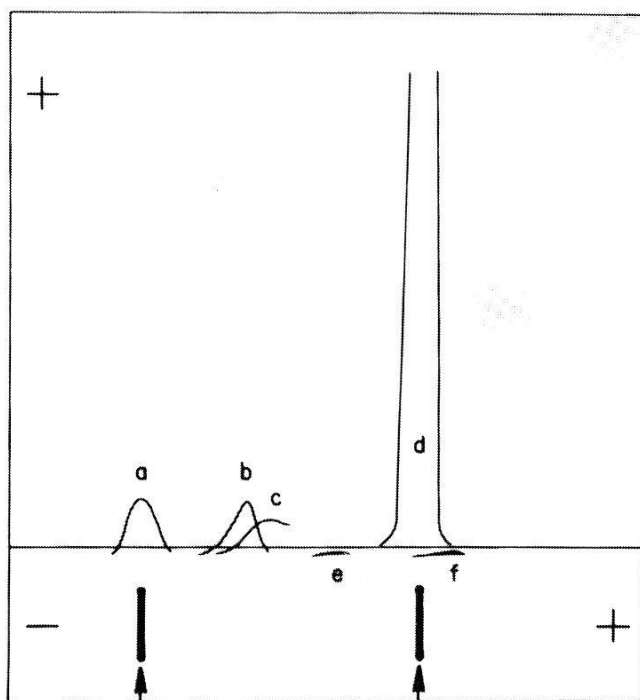
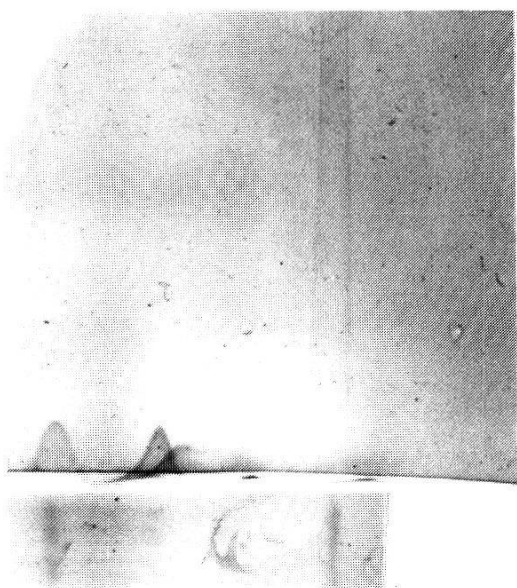


Fig. 3. Photograph and diagram of the crossed immunoelectrophoresis pattern of Triton X-100-solubilized antigens (6.0 mg/ml) electrophoresed against homologous rabbit immunoglobulin. Major peaks = a, b, c, d; minor peaks = e, f. \uparrow = protein-staining bands after electrophoresis in the first dimension.

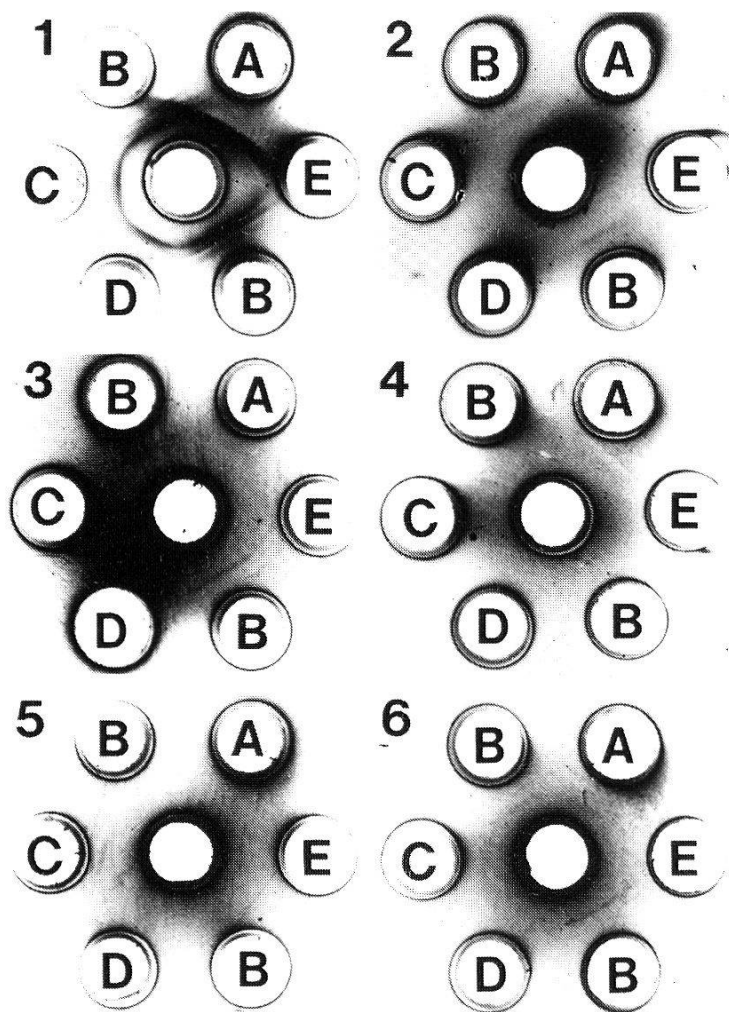


Fig. 4. Precipitin results of sera from 5 occult dogs and one microfilaremic dog diffused against Triton X-100 solubilized antigens. Sera 1, 2, 3, 4 and 6 were from occult dogs. Serum 5 was from a microfilaremic dog. A = Aqueous soluble somatic extract from female *D. immitis* (2.8 mg/ml). B = Triton X-100 solubilized antigens (8.7 mg/ml). C = Triton X-100 solubilized antigens (4.4 mg/ml). D = Triton X-100 solubilized antigens (2.2 mg/ml). E = Saline control.

Immunodiffusion of antigens solubilized with different concentrations of Triton X-100 against homologous rabbit immunoglobulin produced at least 4 lines of identity with no lines of partial or non-identity (Fig. 2).

Crossed immunoelectrophoresis of Triton X-100 solubilized antigens (6 mg/ml) against homologous rabbit immunoglobulin produced 4 major peaks (a, b, c, d) and 2 minor peaks (e, f) (Fig. 3). Only peaks a and d were associated with distinct protein staining in the first dimension (Fig. 3). When the antigen concentration was increased to 12 mg/ml the size of each peak increased, but no additional peaks appeared. Absorption of immunoglobulin with canine liver powder did not affect CIEP results.

Triton X-100-solubilized antigens did not react in the immunodiffusion assay with sera from 7 infected, microfilaremic dogs. However, these same antigens reacted with sera from 4 of 5 dogs with occult *D. immitis* infections.

Serum from 1 of 5 occult dogs reacted in immunodiffusion with aqueous soluble somatic female *D. immitis* antigen. None of the sera from microfilaremic dogs reacted with this antigen. Immunodiffusion results with sera from each occult dog and one microfilaremic dog are illustrated in Fig. 4.

Discussion

Triton X-100 was more efficient than SDS in solubilizing proteins from aqueous-insoluble *D. immitis* cuticular debris. This may indicate that the proteins solubilized by Triton X-100 were primarily proteins which were bound to lipids by hydrophobic interactions (Helenius and Simons, 1975). This finding was encouraging in view of the relative non-denaturing effects of Triton X-100 as compared to SDS (Helenius and Simons, 1975).

Solubilized protein was recovered using the Triton X-100 at a level which could not be increased by addition of more detergent (Fig. 1). Immunological identity between the protein preparations produced with different levels of Triton X-100 was confirmed with immunodiffusion (Fig. 2). A minimum of 4 antigen systems was evident in immunodiffusion. Crossed immunoelectrophoresis, a more sensitive technique, revealed a minimum of 6 antigen systems. Reactions of partial identity or identity were not obvious among the constituent crossed immunoelectrophoresis peaks indicating that each peak represented a unique antigen (Fig. 3). Only 2 of the crossed immunoelectrophoresis peaks were associated with protein-staining bands in the first dimension (Fig. 3). It is likely that any protein in antigens represented by the other peaks was present in quantities below the sensitivity of the protein stain.

Each canine serum sample employed in immunodiffusion was serologically positive in an enzyme-linked immunosorbent assay employing adult *D. immitis* antigen (Grieve et al., 1981). It is interesting, therefore, that serum from only 1 occult dog was reactive with soluble somatic female *D. immitis* antigens. This finding underscores the relative insensitivity of immunodiffusion; however, some interesting conclusions are possible with the immunodiffusion data. Sera from different dogs, demonstrated quantitative differences in reactivity to Triton X-100-solubilized antigens (Fig. 4). Serum 1 demonstrated reactivity against all concentrations of Triton X-100-solubilized antigens with multiple precipitin lines evident. Serum 6 was reactive against all concentrations of Triton X-100 solubilized antigens; however, only a single precipitin line can be visualized. Sera 2 and 3 were only reactive against the highest concentration of antigen. This type of individual variation may be related to host- and/or parasite-dependent factors.

There was an apparent pattern of Triton X-100-solubilized antigen reactivity in infected dogs. Serum from each of seven microfilaremic dogs was negative. However, sera from 4 of 5 occult dogs was positive. Furthermore, the occult dog which was negative in immunodiffusion was the only occult dog which was

negative for antibody to microfilaria surface antigens. It has been established that occult *D. immitis* infections exist for several reasons (Rawlings et al., 1982). In the present investigation it is possible that the occult dog which did not react with Triton X-100 solubilized antigens may have had an infection of a single sex. A relationship may exist between Triton X-100 solubilized antigens from female *D. immitis* and the surface antigens of *D. immitis* microfilariae which appear to be involved in immune-mediated clearance of microfilariae. It remains to be determined if the Triton X-100-solubilized antigens obtained in this study were derived from the female only, the microfilariae present in the female, or both.

In summary, immunodiffusion and crossed immunoelectrophoresis indicated there were a discrete number of antigens present in the Triton X-100-solubilized preparations. Furthermore, these antigens appear to have unique reactivity with sera from occult dogs. Since these antigens were reactive with only those sera containing anti-microfilaria surface antibody, they may be of value in the specific diagnosis of occult infection which results from immune-mediated clearance of microfilariae.

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