A STUDY OF THE ANTIGENS OF ADULT SWINE LUNGWORMS

(METASTRONGYLUS SPECIES)

by

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INTRODUCTION

Swine lungworm infection is part of the complex of chronic swine pneumonias. The disease is caused by nematodes belonging to the genus Metastrongylus Molin, 1861. Three species, M. apri, M. pudendotectus and M. salmi, characteristically occur as mixed infections in field cases. The former two species are found together in 76 percent of natural infections, with the latter species occurring only rarely (Ewing and Todd 1961).

Unresolved Problems

Respiratory diseases cause a significant economic loss to the swine industry annually. The loss is incurred, not only by death of swine, but perhaps more importantly by inestimable loss from inefficient feed conversion and lack of weight gain caused by various chronic respiratory infections. Swine lungworms, which contribute to these losses, remain inadequately controlled even though our knowledgeof the disease has substantially increased over the years. To illustrate, Ledet and Greve (1966) found 48.2 percent of market-weight hogs from Iowa harboring lungworms and Iowa is a state with an annual production exceeding 20 million head of swine.

Swine lungworms are known to be primary agents in causing pneumonia, but they are also secondarily involved with the pathogenesis of other respiratory diseases. Shope (1941a) (1941b) demonstrated that lungworms could be a reservoir for swine influenza virus. Mackenzie (1963) found that pigs infected concurrently with swine lungworms and virus pig pneumonia developed more extensive and severe lesions than pigs infected with either agent alone. In addition, certain histological lung lesions of swine lungworm disease and virus pig pneumonia are remarkably similar, making

differentiation difficult on microscopic examination. The relationship between swine lungworms and other respiratory disease agents in swine remains to be investigated in depth.

Other deficiencies exist in our knowledge of metastrongyliasis. At the present time fecal egg recovery provides the only direct evidence of lungworm infection. The prepatent period, importance of juvenile stages in pathogenesis of the disease and the element of chance in fecal examination preclude the use of fecal egg recovery in efficient drug evaluation studies, epidemiological surveys and assessment of control measures.

Finally, the nature of the immune response in swine lungworm disease and its role in the pathogenesis of the disease remains to be studied in detail. A review of the literature has shown that our knowledge in this direction has begun, but it is incomplete.

Objectives of this Study

It is believed that the techniques and methods of immunology and serology could be used to study some of the problems mentioned herein. Before detailed immunologic studies can be done, however, basic characterization and definition of the antigenic composition of Metastrongylus species is necessary. This study was designed therefore, to establish a method by which Metastrongylus antigens could be extracted and remain in stable solution. Another objective was to compare Metastrongylus antigen extract with a similarly prepared extract of Ascaris suum, another common parasite of swine, for the purpose of identifying possible shared antigens between the two parasites. A final objective was to test lungworm-infected swine by intradermal injection of extracted antigens for the presence of hypersensitivity to those antigens.

LITERATURE REVIEW

Characteristics of the Swine-*Metastrongylus* Relationship

Life cycle

Hobmaier and Hobmaier (1929) described the role of the earthworm as intermediate host for *Metastrongylus elongatus* (= *M. apri*). They identified the earthworms as *Lumbricus terrestris major* and *L. t. minor*, names with no zoological standing, and therefore it is not clear which earthworms they used. Schwartz and Alicata (1931) (1934) defined the role of the earthworm and identified three species, *Helodrilus foetidus* (= *Eisenia foetida*), *H. caliginosus* (= *Allolobophora caliginosa*), and *Lumbricus terrestris* as suitable intermediate hosts. Shope (1941b) and Dunn (1955) added *Allolobophora terrestris*, *A. chlorotica*, and *Lumbricus rubellus* to the list of intermediate hosts for *Metastrongylus* species.

The fully embryonated eggs of *Metastrongylus* were found to hatch in the esophagus and crop of the intermediate host, with first-stage juveniles accumulating in the wall of the alimentary tract, dorsal blood vessel and hearts (Schwartz and Alicata 1931, 1934). Two molts were suspected to occur in the intermediate host prior to the stage of infectivity.

Earthworms are ingested by swine and digested in the stomach, freeing the infective third-stage *Metastrongylus* juveniles. The juveniles were found to penetrate the wall of the cecum and colon, entering the lymphatics (Hobmaier and Hobmaier 1929) (Schwartz and Alicata 1934) (Hobmaier 1934). According to these investigators, the third molt occurred in the mesenteric lymph nodes. Kersten and Becht (1960) found most fourth-stage juveniles in various layers of the large intestine and stroma of the

mesentery, with no significant accumulation occurring in the mesenteric lymph nodes. They suggested that migration through mesenteric lymph nodes was not obligatory and that the third molt occurred at a significant point in time, regardless of the tissue in which the juveniles were lying.

Hobmaier (1934) observed that the liver was not involved in the migration pattern. In only a few instances were dead juveniles found in the periphery of liver lobules.

Hobmaier and Hobmaier (1929) and Schwartz and Alicata (1934) found that fourth-stage juveniles ultimately reached the venous system via the thoracic duct and hence migrated to the bronchioles by penetrating the capillaries and alveolar walls of the lungs.

Using experimentally infected pigs Mackenzie (1959) related the sequence of migration events to time. He found fourth-stage juveniles in the wall of the cecum and colon at 24 hours post infection. At three to five days, fourth- and fifth-stage juveniles were recovered from the ileocecal and colic lymph nodes, and at seven days fifth-stage juveniles were recovered from the lungs. By 40 days, most female adults were found to bear fully embryonated eggs.

Mackenzie (1959) began seeing lungworm eggs in the swine feces 30 days post infection. Egg counts appeared to peak at 50 to 75 days and then began to decrease, but were not evaluated past 80 days post infection.

Localization of adult lungworms in the posterior portions of the diaphragmatic lobes has been noted in natural infections by Dunn et al. (1955), Whittlestone (1957), and Mackenzie (1958). Dunn et al. (1955) and White (1955) attributed this localization to a failure of the host to expel lungworms from those areas. They further suggested that persistence of

lungworms in those areas of the lungs represented a residual portion of a previously heavy infection.

Tissue response

Kersten and Becht (1960) found neutrophils and eosinophils in those areas of the intestinal wall and mesentery where migrating juveniles had been present. Where juvenile molts were presumed to have occurred, histiocytic nodules formed. These nodules had regressed in those individuals examined at a later stage in the migration phase of the infection.

Hobmaier (1934) made observations on the cellular response to migrating juveniles in mesenteric lymph nodes. During invasion the nodes were found to enlarge due to fluid accumulation. Later, eosinophilic infiltration and lymphoid hyperplasia occurred, resulting in loss of normal lymph node architecture.

Response to migrating juveniles in the lung parenchyma was reported by Mackenzie (1959) to be relatively mild. Thickening of alveolar walls, mononuclear infiltration, petechial hemorrhage, erythrophagia and presence of a few giant cells were noted at five to nine days post infection when juveniles were arriving in the lungs.

Microscopic lesions in the lungs attributed to adult Metastrongylus have been recorded by Hung (1926), Dunn et al. (1955), Dunn (1955), Mackenzie (1958) and Ewing and Todd (1961). Alveolar emphysema occurs along the ventral border of the diaphragmatic lobe near, and at, its caudal end. Smooth muscle hypertrophy and peribronchiolar lymphoid hyperplasia occur in those bronchioles in which lungworms are present or have been present. In addition, chronic bronchitis and bronchial lymph node hyperplasia

have been described.

In a more detailed study Mackenzie (1959) described the pathogenesis of experimental lungworm infection from 1 to 80 days post infection. Circulating eosinophilia commenced at 10 to 15 days, peaked at 25 days and returned to normal at 35 days. As lungworms began appearing in small bronchioles from 10 to 25 days, they were accompanied by severe eosinophilic infiltration of bronchiolar mucosa, smooth muscle hypertrophy and emphysema in dependent portions of the lung. At this time peribronchiolar lymphoid hyperplasia was observed and became more pronounced as the age of the lungworms increased. Lymphoid hyperplasia and eosinophilic infiltration of the bronchial lymph nodes commenced at 10 days post infection and persisted during the 80 day trial period.

Studies Relating to the Immune Response

Natural and acquired immunity

Hobmaier (1934) found young swine to be more severely affected by lungworms than older swine. The observation led him to postulate, "the normal anatomic and pathologic changes in the tissues of the host during life may be at least partially responsible for the higher resistance of older animals rather than the biologic process of immunity". The modern concept of immunity, according to Raffel (1961), suggests "anatomic and pathologic" tissue changes are intimately related to immunity, be it natural or acquired. Regardless of this aphorism, Hobmaier's observation is of interest as it relates the possibility of age resistance in lungworm-infected swine.

Dunn (1956) added the first experimental evidence supporting natural

age immunity. Two 8-week-old pigs were infected with 4,000 and 5,000 infective juveniles, respectively. At the peak of the infections 3,100 and 7,750 eggs per gram of feces (e.p.g.) were found respectively in the pigs. Each pig had a heavy worm burden at necropsy. Conversely, two older pigs, 13 weeks old, which had been given 3,100 and 20,000 juveniles, had 7 and 31 e.p.g. respectively. Worm burdens at necropsy were low.

Schwartz and Lucker (1935), Sen (1960), Jaggers (1965) and Dixon (1968) suggested that immunity to lungworm infection is acquired as a result of previous infection. Schwartz and Lucker (1935) infected four 10-week-old pigs with 2,500, 1,500, 1,000 and 500 juveniles respectively. At apparent termination of patent periods, each pig was given 500 more juveniles, and necropsy was done 45 days later. The two pigs that had received the largest initial doses harbored only immature worms. Conversely, the two pigs which had received the smallest initial doses had sizable lungworm infections, with fully mature parasites present as well.

Jaggers (1965) gave 17,000 Metastrongylus juveniles to each of two pigs. Fifty-seven days later both pigs were refractory to superinfection, whereas two uninfected litter-mates were susceptible at that time. Dixon (1968) reported similar results using smaller initial doses of juveniles. Each of three pigs were given 540 juveniles in small increments over a 40 day period and challenged, along with three uninfected control pigs, with 2,000 juveniles 56 days after the initial dose had been given. Fecal e.p.g. counts in the "immunized" pigs were 100, 600 and 50 on day 100. At necropsy 97, 229 and 2 lungworms were found. Control pigs had 1,700, 2,400 and 1,150 e.p.g. and 608, 1,013 and 884 lungworms respectively.

Serological studies

Formation of precipitates around juvenile Metastrongylus placed in immune serum was reported by Becht (1960), Zukovic and Wikerhauser (1964) and Jaggers (1965). Zukovic and Wikerhauser (1964) used sera from guinea pigs previously infected with M. apri. Third-stage juveniles developed a precipitate around exsheathed individuals when incubated in the serum. Becht (1960) found clumpy, homogeneous masses at the anterior end and excretory pore of ensheathed juveniles incubated in immune swine serum. Jaggers (1965) manually punctured the juvenile sheath and found a precipitate completely filled the space between the juvenile body and its sheath when incubated with immune swine serum.

Becht (1960) showed by the indirect hemagglutination technique that an antibody response occurred 15 days after initial infection with M. apri in swine. Antigen used for the test was prepared by a saline extraction procedure and boiled to remove insoluble constituents.

Jaggers (1965) used the indirect hemagglutination test with a Metastrongylus antigen prepared by saline extraction. He indicated that the test may have been detecting antibody to lungworms, but cross-reactions were apparently occurring with Oesophagostomum dentatum, which occurred in some pigs used for test serum. Jaggers also attempted to use the complement-fixation test to demonstrate swine antibodies to lungworms, but none were detected by this method. Agar-gel diffusion tests were likewise unsuccessful.

Intradermal tests in lungworm-infected swine were done by Jaggers (1965). Positive reactions characterized by immediate wheal and flare which peaked 15 minutes post injection were observed. The reaction

occurred regardless of the size of the lungworm burden and first became positive four weeks post infection.

METHODS

Extraction Procedures

Extraction procedure I

The first Metastrongylus antigen extract (hereafter referred to as M. Ag I) was prepared using modifications from the methods of Sawada et al. (1965) and Mantovani and Kagan (1967). Living lungworms were collected from market hogs freshly slaughtered at an abattoir[1]. The parasites were washed to remove bronchial mucus by serial transfer through several dishes filled with 0.9 percent NaCl while held at 3°C. After the final wash they were shell frozen in a convenient amount of physiological saline solution (PSS) and dried under vacuum at -70°C.

The dried worms were pulverized with a mortar and pestle, added to cold anhydrous ether (3 grams per 40 milliliters) for the purpose of lipid extraction. To facilitate extraction the suspension was ground for 15 minutes in a Ten Broeke grinder, centrifuged at 500 g (0°C), collected as a sediment, and the process was repeated.

The sediment was resuspended in 200 milliliters of phosphate-buffered saline (0.01M phosphate, pH 7.2) and maintained at 3°C with constant agitation for 72 hours. The material was centrifuged at 12,100 g at 0°C for 10 minutes, and the supernatant collected. Concentration was done by shell freezing the solution and drying under vacuum at -70°C. The dried material was reconstituted in a suitable amount of distilled water (to achieve approximately 10 milligrams protein per milliliter) and labeled M. Ag I.

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[1]Oscar Mayer Packing Plant, Perry, Iowa.

M. Ag I and all subsequent antigen extracts were stored frozen at -18°C.

Extraction procedure II

Metastrongylus antigen extract II (M. Ag II) was prepared in a manner similar to M. Ag I, with a change only in the washing procedure to prevent loss of eggs and uterine debris from disintegrating worms. The lungworms were washed by placing them in PSS and centrifuging at 500g for 10 minutes at 0°C, repeating the step five times. After each centrifugation, blood-tinged mucus was removed from the surface of the lungworm pellet with a spatula. After the last centrifugation no extraneous debris was present, and the lungworm pellet consisted of an upper white buffy layer and a lower layer containing whole lungworms. The upper layer consisted of eggs, first-stage juveniles and uterine debris, material presumably lost in extraction procedure I.

The lungworm material was frozen and dried under vacuum at -70°C; the remainder of the extraction was the same as procedure I. The final solution was labeled M. Ag II and stored frozen at -18°C.

Extraction procedure III

Collection and washing weredone as in procedure II. The lungworm material collected from the final wash was frozen, but not dried. With minor modification the remaining steps in the extraction procedure followed that of Jeska (1967).

The frozen worm material was added to cold absolute ethanol (4 grams per 25 milliliters) for delipidization. The mixture was homogenized for

30 minutes in a Sorvall-Omnimixer[1] while held in a dry ice-alcohol bath and held for an additional 30 minutes without mixing. An equal volume of anhydrous ether was added to the alcohol-worm mixture and mixed periodically during a 3-hour period while holding in the dry ice-alcohol bath. The suspension was centrifuged at 3000g at 0°C for 30 minutes and the supernate was discarded. The sediment was frozen and dried under vacuum at -70°C.

The dry worm powder was suspended in trishydroxychloride-buffered saline (0.1M tris, pH 8.0)[2] and held with constant stirring at 3°C for 48 hours (2.5 grams per 30 milliliters). The solution was centrifuged at 34,800g for 30 minutes at 0°C, and the supernate was collected and stored frozen at -18°C (M. Ag III).

Ascaris suum adults, collected at the same abattoir as the lungworms, were washed, frozen and extracted using procedure III. The resultant solution was labeled A. Ag and stored frozen at -18°C.

Preparation of Rabbit Antisera

Water-in-oil emulsions were prepared with M. Ag I and M. Ag II in Freund's complete adjuvant. Albino rabbits were given intradermal injections twice at 14-day intervals (approximately 20 milligrams protein per dose). Two weeks after the second dose the rabbits were given an additional one-half dose of the antigens respectively by intraperitoneal injection. Five days later the rabbits were bled by intracardiac puncture and the sera

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[1]Ivan Sorvall, Inc., Norwalk, Connecticut.

[2]The buffer was made up as follows: 12.1 grams trishydroxyaminomethane (tris), 50 milliliters 1N HCl, 58.45 grams NaCl and q.s. to 1000 milliliters distilled water.

were collected.

Antisera against M. Ag III and A. Ag were prepared with four intramuscular injections at 10-day intervals in separate rabbits (approximately 15 milligrams protein per dose in Freund's complete adjuvant). Two weeks after the fourth dose an additional one-half dose was given intraperitoneally without adjuvant, and sera were harvested five days later.

Characterization of Extracts

Chemical determinations

Protein concentration of the antigen extracts was measured with the Folin phenol method (Lowry et al. 1951) and with the biuret method (Coles 1967). Carbohydrate content was estimated using the anthrone reagent method by Yemm and Willis (1954).

Serological procedures

Electrophoresis and immunoelectrophoresis were carried out on cellulose acetate membranes.[1] Prior to electrophoresis, antigen extracts were dialyzed for 24 hours against the electrophoresis buffer (0.04M barbital, pH 8.0). One microliter of extract was applied to the cellulose acetate membrane and subjected to 150 volts for 40 minutes. Immunoelectrophoretic precipitin bands were allowed to develop for 72 hours at room temperature while the membrane was held under mineral oil.

The agar-gel diffusion method described by Ouchterlony (1958) was

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[1] Beckman Microzone System, Beckman Instruments, Fullerton, California.

used with 0.8 percent agar-gel[1] on glass slides. Precipitin bands were allowed to form for 48 hours at room temperature. Results were recorded by sketches and photographs of unstained preparations.

Skin Sensitization

Procurement of swine

Fecal samples from feeder swine (60 to 130 pounds) were collected on an Iowa farm known to have had lungworm-infected swine in the past. The samples were examined for the presence of Metastrongylus eggs, using the sugar flotation method of Benbrook and Sloss (1961). Three swine with lungworm eggs present in their feces were purchased (hereafter referred to as swine 1, 2 and 3).

Two noninfected control swine (approximately 50 pounds) were purchased from the Iowa State University swine nutrition farm (swine 4 and 5). These swine had been raised in a confinement system known to have an unusually low parasite incidence associated with it.

Skin test procedure

Antigen solutions were prepared as follows. M. Ag III and A. Ag were dialyzed against PSS for 24 hours to remove the trishydroxychloride. An aliquot of M. Ag III (not dialyzed) was adsorbed for 24 hours with an equal volume of anti-A. Ag rabbit serum and, conversely, an aliquot of nondialyzed A. Ag was adsorbed with anti-M. Ag III rabbit serum. Therefore,

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[1]The agar-gel was made up as follows: 0.6 grams agarose (Nutritional Biochemicals Corp., Cleveland, Ohio), 0.2 grams Noble agar (Difco Laboratories, Detroit, Michigan), q.s. to 100 milliliters with barbital buffer (0.04M barbital, pH 8.0).

four skin test antigen solutions were used: dialyzed M. Ag III; dialyzed A. Ag; nondialyzed, adsorbed M. Ag III and nondialyzed, adsorbed A. Ag.

Three control solutions were used: PSS, trishydroxychloride-buffered saline and rabbit serum.

All injections were given intradermally on white areas of the pectoral thorax, 0.1 milliliter in volume. Observations and photographs were recorded at 5, 15, 30 and 60 minutes after injection. Swine 2, 3, 4 and 5 were observed at 24 hours after injection. The dialyzed antigen injection sites were observed also at 48 hours after injection in swine 2.

Necropsy procedure

All five swine were necropsied following completion of the skin test procedure. Lungs were examined grossly for verminous pneumonia and adult lungworms were counted. Livers were examined grossly for presence of focal interstitial hepatitis ("white spots"), which is attributed to ascarid migration. Gastrointestinal tracts were examined by sieving the ingesta and mucosal scrapings for presence of parasites.

Skin sections for histological examination were taken from the site on swine 3 and 5 where dialyzed M. Ag III had been injected. The tissues were fixed in 10 percent formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

RESULTS

Comparison of *Metastrongylus* Extracts

Chemical and physical characteristics

M. Ag I and M. Ag II formed a heavy precipitate when they were thawed after storage. They also appeared unstable when held at refrigerator temperature for several days, since a flocculant turbidity would usually occur in the solutions. M. Ag III did not show these characteristics. The solution could be held for several days in the refrigerator, or frozen and thawed several times, with no visible changes occurring.

Protein and carbohydrate determinations were made on each *Metastrongylus* antigen extract. The initial dry weights were obtained when the lungworm material had been dried and pulverized in each respective extraction procedure, and final volumes of the antigen solutions were recorded. The results are shown in Table 1.

Table 1. Chemical analyses on *Metastrongylus* antigen extracts (before storage)

	M. Ag I	M. Ag II	M. Ag III
Total initial dry weight	3.0 gm[a]	1.25 gm[a]	2.56 gm[b]
Gms. initial dry weight per ml. final extract volume	1 gm/21.6 ml	1 gm/20.0 ml	1 gm/5.85 ml
Folin phenol (protein)[c]	8.32 mg/ml	8.44 mg/ml	11.18 mg/ml
Biuret (protein)[c]	--	6.7 mg/ml	16.5 mg/ml
Anthrone (carbohydrate)[c]	2.35 mg/ml	3.6 mg/ml	9.1 mg/ml

[a]Measured prior to lipid extraction.

[b]Measured after lipid extraction.

[c]Measured on final antigen extract.

Electrophoretic similarity was observed between the three Metastrongylus antigen extracts, even though the extraction procedures were different (Figure 1). Two fast-migrating fractions moved toward the anode in each extract. Three more anode-directed fractions were observed with M. Ag I and M. Ag II, and two more were observed with M. Ag III. Each extract had two cathode-directed fractions.

Agar-gel diffusion studies

The Metastrongylus antigen extracts were placed in wells arranged around a central antiserum well to facilitate observation of individual antigens common to each extract. Three precipitin bands common to all extracts were observed with anti-M. Ag I rabbit serum (Figure 2). Two additional common bands were present between M. Ag I and M. Ag III and one additional common band formed between M. Ag I and M. Ag II. With anti-M. Ag III serum (Figure 3) two bands of common identity occurred between all three extracts and one additional band formed between M. Ag II and M. Ag III. Three bands common to M. Ag I and M. Ag III occurred with anti-M. Ag III serum (Figure 4) and only one occurred between M. Ag II and the other two extracts.

Comparison of M. Ag III and A. Ag

M. Ag III and A. Ag, prepared by the same extraction procedure, were found to have dissimilar electrophoretic patterns (Figure 5). Four fractions migrated toward the cathode in A. Ag, whereas M. Ag III had only two cathode-directed fractions. The fast, anode-directed fractions evident in M. Ag III were absent in A. Ag and the remaining anode-directed fractions migrated at different rates.

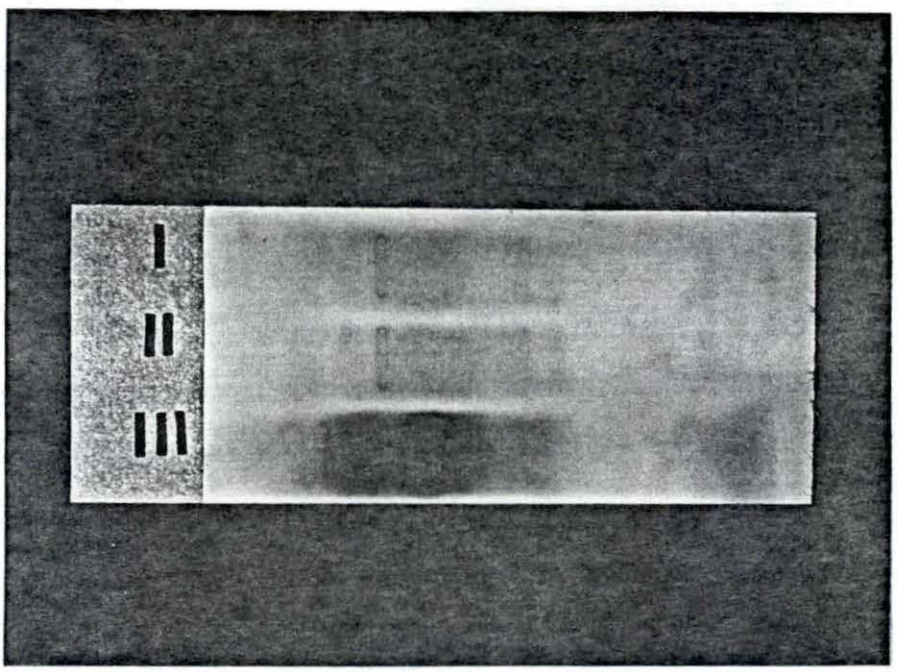
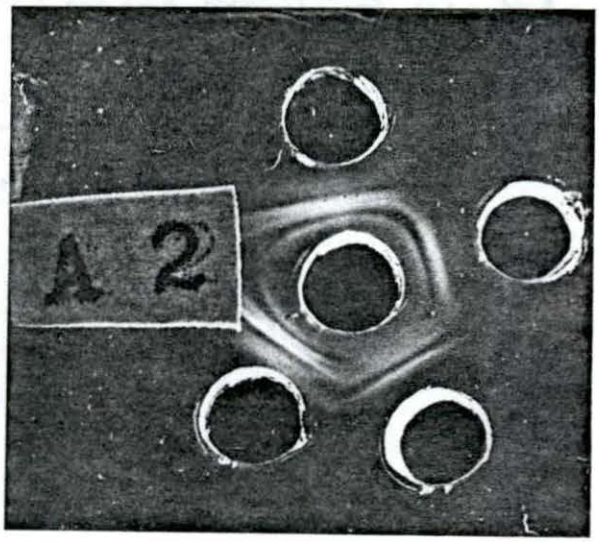


Figure 1. Electrophoretic comparison of M. Ag I, M. Ag II and M. Ag III
I = M. Ag I, II = M. Ag II, III = M. Ag III



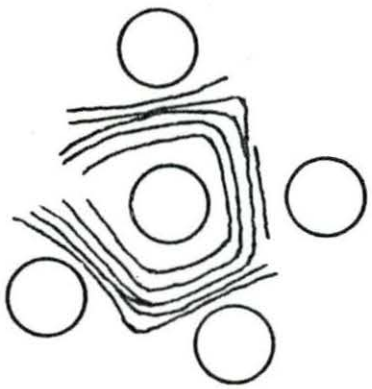
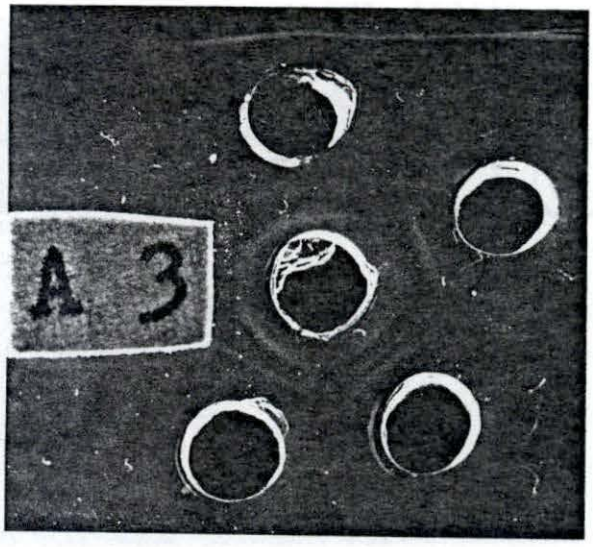


Figure 2. Agar-gel diffusion of M. Ag I, M. Ag II and M. Ag III with anti-M. Ag I rabbit serum (photograph and sketch). Center well = anti-M. Ag I rabbit serum. Clockwise, starting with the top well, M. Ag I (5 bands), M. Ag II (4 bands), M. Ag III (5 bands) and M. Ag I (5 bands)



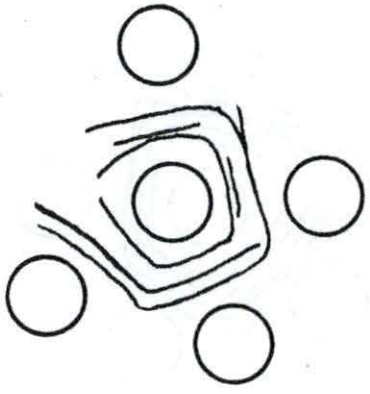
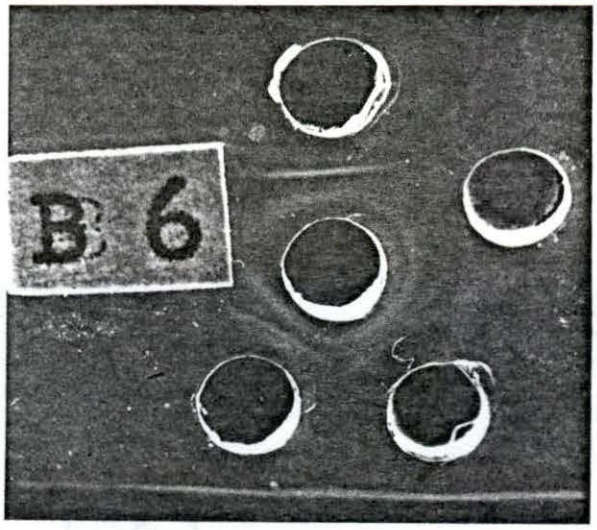


Figure 3. Agar-gel diffusion of M. Ag I, M. Ag II and M. Ag III with anti-M. Ag II rabbit serum (photograph and sketch). Center well = anti-M. Ag II rabbit serum. Clockwise starting with the top well, M. Ag I (3 bands), M. Ag II (3 bands), M. Ag III (3 bands), M. Ag I (3 bands)



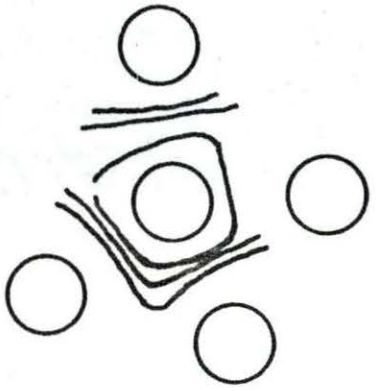


Figure 4. Agar-gel diffusion of M. Ag I, M. Ag II and M. Ag III with anti-M. Ag III rabbit serum (photograph and sketch). Center well = anti-M. Ag III rabbit serum. Clockwise starting with the top well, M. Ag I (3 bands) M. Ag II (1 band), M. Ag III (3 bands), M. Ag I (3 bands)

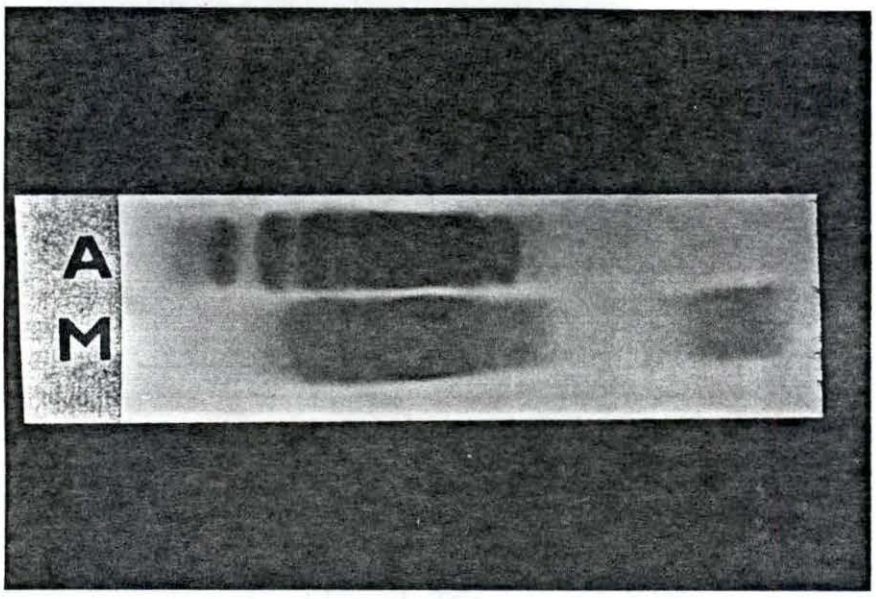


Figure 5. Electrophoretic comparison of M. Ag III and A. Ag. A = A. Ag, M = M. Ag III

Agar-gel diffusion preparations with anti-A. Ag rabbit serum (Figure 6) and with anti-M. Ag III rabbit serum (Figure 7) failed to show precipitin bands resulting from antigens shared by the two antigen extracts. At least six bands with the Ascaris-homologous system and five bands with the Metastrongylus-homologous system were observed.

At least one shared antigen between M. Ag III and A. Ag was found to exist using immunoelectrophoresis (Figure 8). The precipitin band occurred in the same relative position in both heterologous systems. In addition, more precipitin bands were observed in the Ascaris-homologous system with immunoelectrophoresis than with the agar-gel diffusion technique.

Skin Sensitization Study

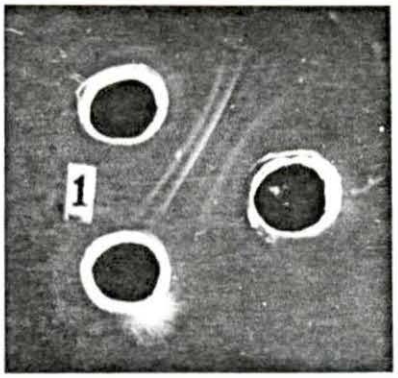
Control reactions

Injection of PSS caused a raised, white, 5 mm bleb that disappeared in 10 to 15 minutes in all cases. Rabbit serum produced a 5 mm, raised, red bleb that waned in 15 minutes and was nearly undetectable in 60 minutes. Trishydroxychloride-buffered saline caused a 10 mm flare that disappeared in 5 minutes. In addition, a 5 to 7 mm white plaque, that became smaller in time, but still visible at 60 minutes post injection, occurred. Solution-control reactions at 5 minutes post injection are shown in Figure 9.

Reaction to dialyzed M. Ag III in swine 4 and 5, later found to be free of lungworms and other adult nematodes, was typified by a 10 mm flare and slightly red injection site at 5 minutes. At 60 minutes the reaction had waned until only a slight discoloration remained. Reaction to dialyzed A. Ag was not appreciably different from dialyzed M. Ag III in the control swine.

Figure 6. Agar-gel diffusion of M. Ag III and A. Ag with anti-A. Ag rabbit serum (photograph and sketch). Top left well = A. Ag (6 bands), bottom left well = M. Ag III, right well = anti-A. Ag rabbit serum.

Figure 7. Agar-gel diffusion of M. Ag III and A. Ag with anti-M. Ag III rabbit serum (photograph and sketch). Top left well = A. Ag, bottom left well = M. Ag III (5 bands), right well = anti-M. Ag III rabbit serum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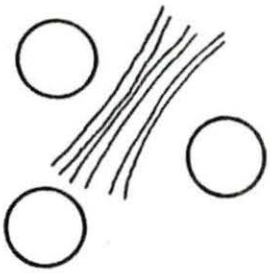

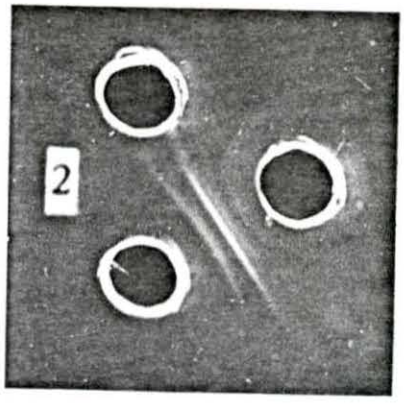
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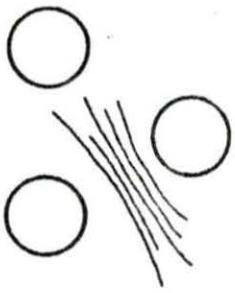


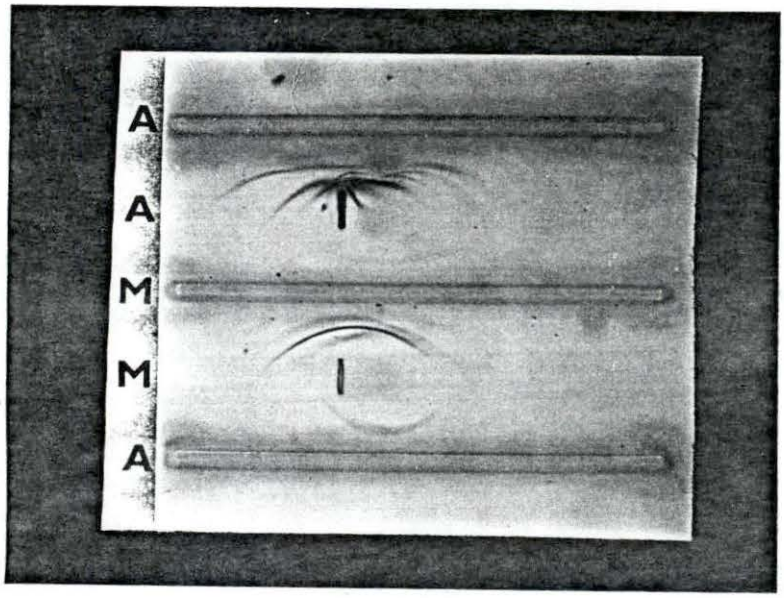
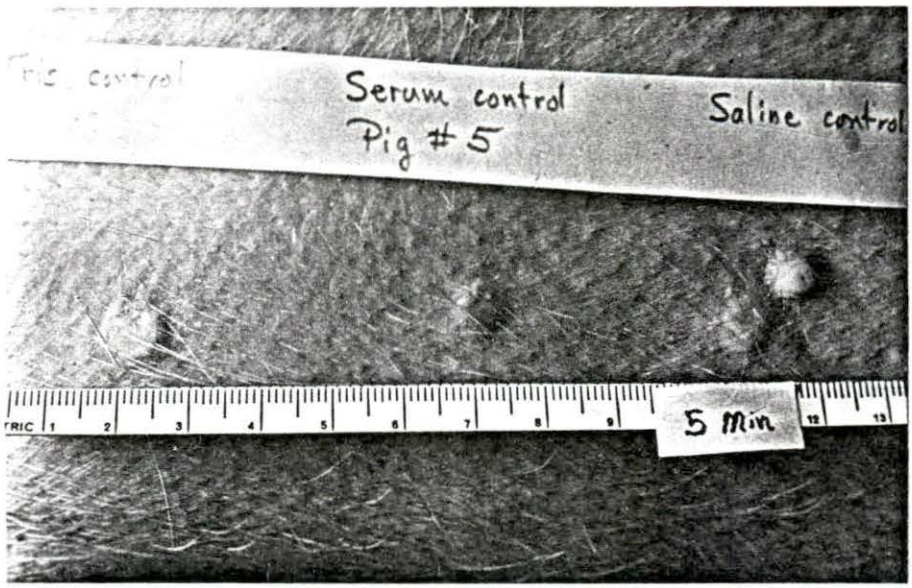
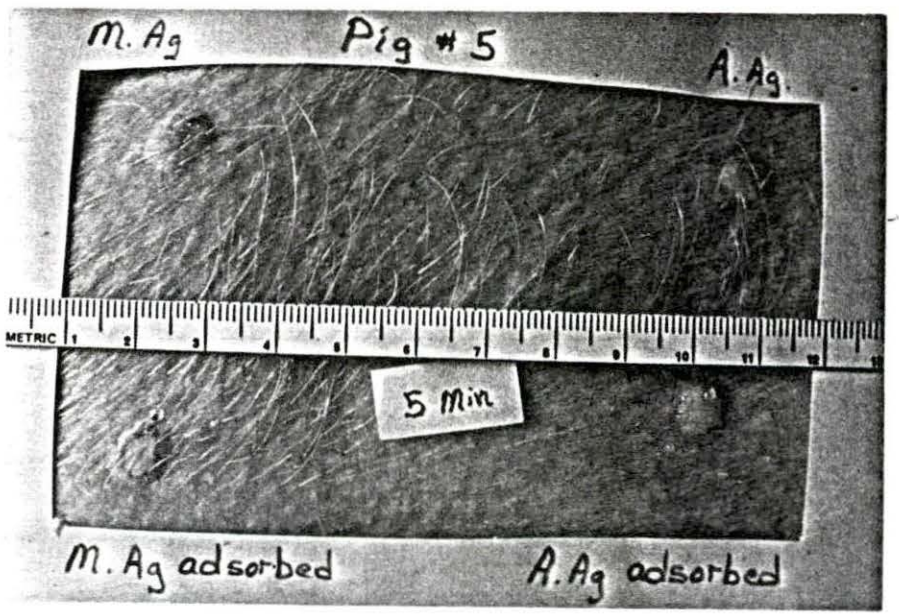


Figure 8. Immunoelectrophoresis preparation with M. Ag III and A. Ag, and respective rabbit antisera
A (top) = anti-A. Ag rabbit serum well
M (center) = anti-M. Ag III rabbit serum well
A (bottom) = anti-A. Ag rabbit serum well
A (second from top) = electrophoresed A. Ag
M (fourth from top) = electrophoresed M. Ag III

Figure 9. Solution-control reactions in swine 5 at 5 minutes post injection

Figure 10. Antigen reactions in uninfected swine 5 at 5 minutes post injection


Serum control
Pig # 5
Saline control
5 Min


M. Ag
Pig # 5
A. Ag
METRIC
5 Min
M. Ag adsorbed
A. Ag adsorbed

Adsorbed M. Ag III and adsorbed A. Ag, which had not been dialyzed to remove trishydroxychloride, produced reactions similar to the dialyzed antigens, with exception of the small white plaque apparently caused by the trishydroxychloride.

Reactions to all four antigens in swine 5 are shown at 5 minutes (Figure 10) and 60 minutes (Figure 11) post injection.

Reactions in infected swine

The reactions described below are shown in swine 3 at 5 minutes (Figure 12) and 60 minutes (Figure 13) post injection.

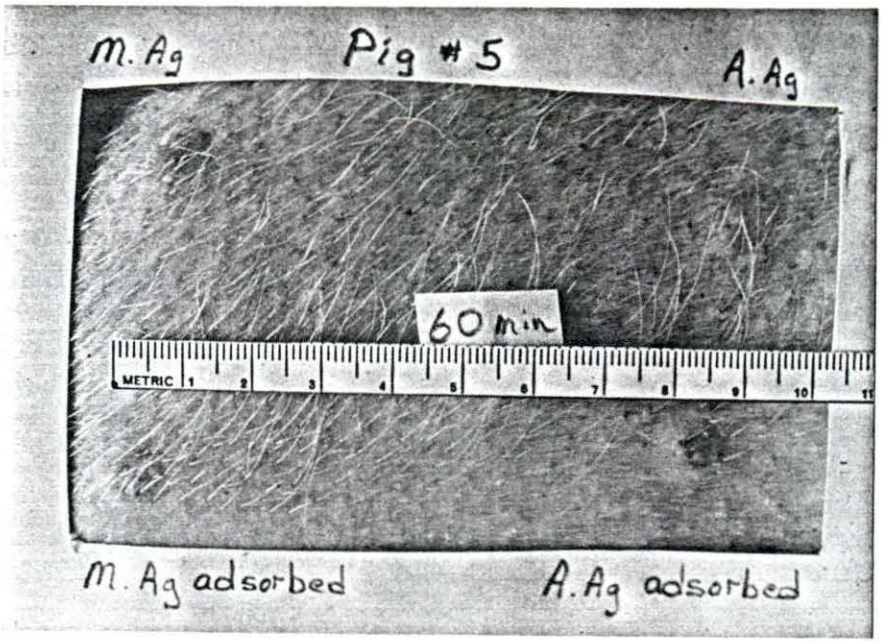
Reactions to dialyzed M. Ag III and A. Ag. Swine 1, 2 and 3 had reactions of the immediate type to both antigens. The two antigens could not be distinguished on the basis of their respective reactivities. The typical reaction sequence was as follows: 5 minutes post injection-peripheral flare 10 to 15 mm, raised red injection site 10 to 12 mm; 15 minutes -- the flare had disappeared, darkening red wheal with occasional appearance of petechiae 10 to 15 mm; 30 minutes -- the only detectable change was a darkening reddish discoloration; 60 minutes -- the wheal had disappeared, but a dark red-brown discoloration remained; 24 hours -- brown discoloration and palpable induration (swine 2 had a wide area of edema surrounding both antigen sites as well).

Reaction to adsorbed M. Ag III Adsorbed M. Ag III, in swine 2 and 3, caused a reaction similar to that described above, with the exception of the centralized white plaque caused by trishydroxychloride.

Reaction to adsorbed A. Ag Adsorbed A. Ag, in swine 2 and 3, caused a reaction similar to adsorbed M. Ag III. However, no detectable

Figure 11. Antigen reactions in uninfected swine 5 at 60 minutes post injection

Figure 12. Antigen reactions in infected swine 3 at 5 minutes post injection


M. Ag
Pig # 5
A. Ag
60 min
METRIC
M. Ag adsorbed
A. Ag adsorbed

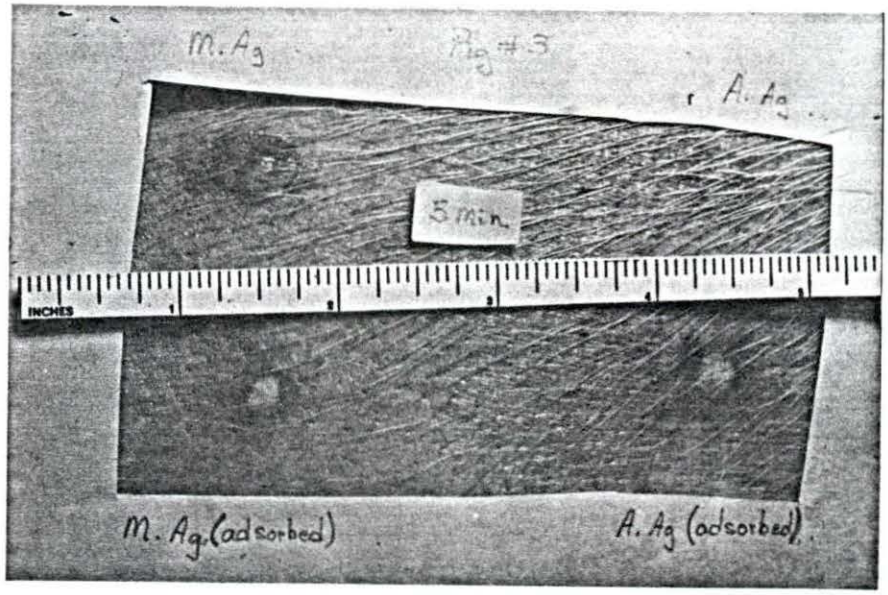
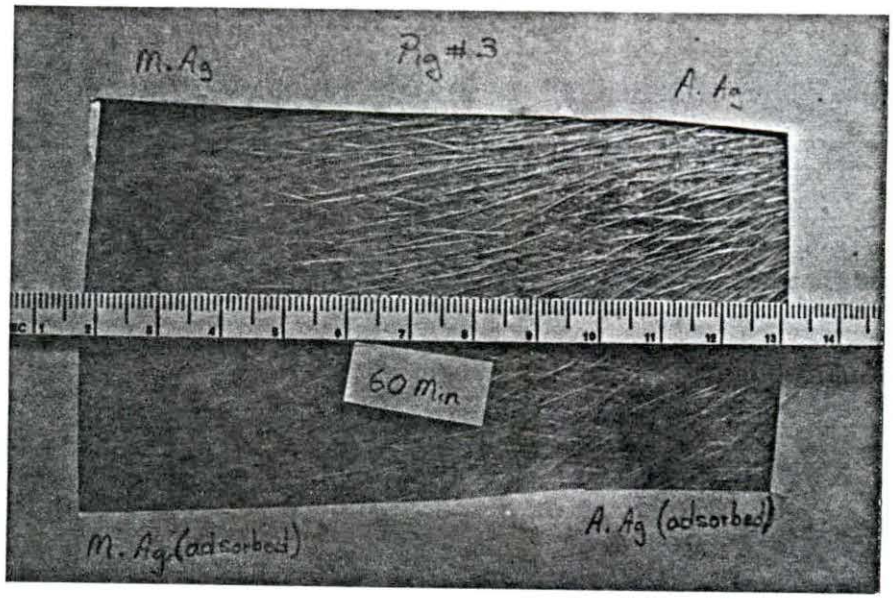
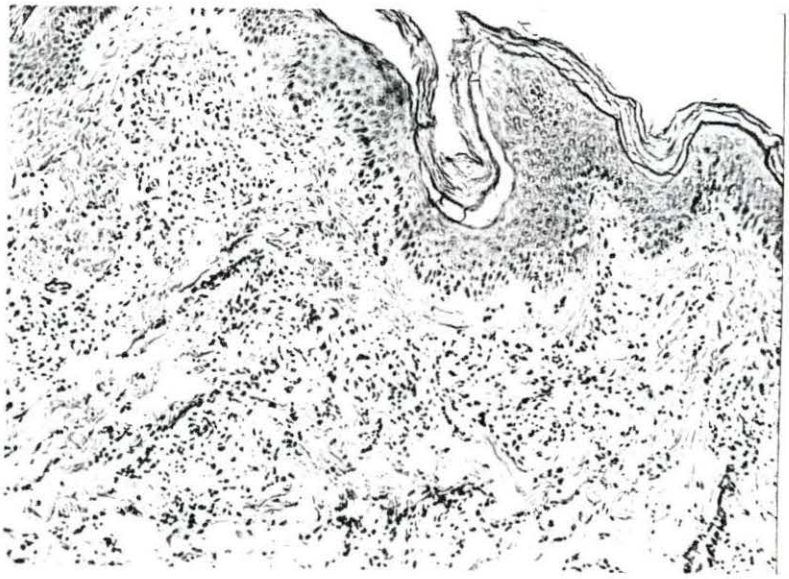

M. Ag
Pig #3
A. Ag
5 Min.
INCHES
M. Ag. (adsorbed)
A. Ag (adsorbed)

Figure 13. Antigen reactions in infected swine 3 at 60 minutes post injection

Figure 14. Microscopic section of M. Ag III injection site 24 hours post injection in infected swine 3 (about 95x)


M. Ag
Pig #3
A. Ag
60 min
M. Ag (adsorbed)
A. Ag (adsorbed)



induration was present at the 24-hour observation.

Histological examination of dialyzed M. Ag III sites

Histological examination of the indurated M. Ag III injection site in swine 3 and the analogous site in control swine 5 was performed. The indurated site in swine 3 was characterized by a perivascular reaction in the subcutaneous layer of the skin which consisted of deposition of fibrin and separation of collagenous bundles, presence of enlarged adventitial cells, and infiltration with neutrophils, eosinophils and a few mononuclear cells (Figure 14).

The analogous site in swine 5 did not show the perivascular reaction. However, a few eosinophils were present in the subcutaneous tissue and areas of mononuclear cell infiltration existed in the deeper dermis and fatty layers. Control sections from uninjected sites in the skin showed none of the changes mentioned above.

Parasite Load in Test Swine

Swine 1, 2 and 3 had wedge-shaped areas of emphysema on the posterior tips and latero-ventral borders of the diaphragmatic lobes. Seventy-two, 250 and 475 lungworms were counted from the lungs, respectively. Lungs from swine 4 and 5 were free of lesions and no lungworms were found.

Multiple focal lesions of chronic interstitial hepatitis were grossly visible on the livers of swine 1, 2 and 3. Swine 1 had 16 adult Ascaris suum in the small intestine. No ascarids were found in swine 2 and 3, and no other nematodes were found in the lungworm-infected swine. Control swine 4 and 5 had no liver lesions and no nematodes were found in the gastrointestinal tracts.

DISCUSSION

Products of the Extraction Procedures

A primary objective of this study was to establish a method by which Metastrongylus species, the lungworms of swine, could be extracted to provide stable antigens in solution. Many varied procedures have been employed by helminthologists to achieve such a goal; most have used buffered aqueous extracts. However, little is known to what extent such extraction procedures might denature or otherwise change protein structure from that found in their natural state. Such changes might impair meaningful results in antigenic studies.

Kent (1963) reflected on this possibility and conducted his extractions at cold temperatures to minimize denaturation. Based on this consideration, each extraction procedure used in this study was done at low temperature; however, the precaution did not provide stability in the solutions. Extraction procedures I and II, in which phosphate-buffered saline was used as the extracting medium, produced solutions which formed heavy precipitates after refrigerator and freezer storage. M. Ag I, M. Ag II and M. Ag III all had similar protein concentrations immediately after extraction, but after removal of the storage-induced precipitates in M. Ag I and M. Ag II, protein concentration was reduced. Evidence of the loss was observable in the electrophoresis results (Figure 1); identical volumes of all three extracts had been applied to the electrophoresis membrane, yet, M. Ag III gave more densely stained fractions than M. Ag I and M. Ag II, an indication of higher protein concentration in M. Ag III.

Chilson et al. (1965) have shown that trishydroxyaminomethane (tris)

acts as a cryoprotective substance to proteins in solution. Tris was found to act like certain other organic compounds, such as sucrose, ethylene glycol and dimethyl sulfoxide, in protecting protein from uncoiling and hybridizing during freezing and thawing. Based on these considerations, Jeska (1967) used trishydroxychloride-buffer for extraction of Toxocara canis antigens and considerably reduced denaturation of antigens in solution. Similar results were obtained in this study, where extraction procedure III, which utilized trishydroxychloride-buffered saline, provided a stable Metastrongylus antigen solution. However, direct evidence that the antigenic proteins in M. Ag III were not hybridized, or in some way changed from their natural state, was not shown in the study. Furthermore, such evidence would be difficult to obtain. It is conceivable that the proteins in M. Ag III were hybridized during extraction, and rabbit antibodies were produced against those hybridized proteins.

Precaution was taken in extraction procedures II and III to retain eggs and first-stage juveniles during washing of the lungworms. These developmental stages of the parasite may be antigenically important in the infected host. Eggs produced by lungworms must be passed up the respiratory bronchial tree, swallowed and pass through the alimentary tract to continue the life cycle. During the respiratory passage, eggs are in continual contact with host tissue. Microgranulomas surrounding eggs entrapped in alveoli have been described (Mackenzie 1958). These observations substantiate the hypothesis that lungworm eggs are important antigenically and should be included in a comprehensive antigen solution to be used in studying the immune response in swine lungworm disease. First-stage juveniles are likewise important, though evidence of host-tissue

contact is not available. Conceivably, entrapped eggs may open, releasing first-stage juveniles into the host-tissue environment, or leakage of antigen through the shell may occur and thereby introduce different antigens to the host. Furthermore, certain antigenic components in first-stage juveniles may be identical with those in third and fourth stage juveniles, which have extensive host-tissue contact during migration.

Lack of uniformity and reproducibility of results obtained by different laboratories using antigens originating from the same helminth species is not uncommon. Kent (1963) suggested that isolated antigen solutions be characterized by dry weight, protein concentration or carbohydrate concentration to minimize such occurrences. These parameters, measured on each extraction product in this study, as well as characterization by electrophoresis, should provide an adequate index to reproducibility, provided strict attention to the detail of the extraction procedure is employed.

Although variation in stability and variation in protein yield (Table 1) occurred between the three Metastrongylus antigen extracts, a similarity in protein fractions was in evidence in the electrophoretic patterns (Figure 1). This apparent similarity was substantiated by the occurrence of precipitin bands common to each antigen-extract with each respective antiserum (Figures 2, 3 and 4). However, careful study of all results obtained with the various precipitating systems revealed inconsistencies. To illustrate, M. Ag I vs. anti-M. Ag I serum and M. Ag III vs. anti-M. Ag III serum each had at least five precipitin bands (Figures 1 and 7), yet, the M. Ag II homologous system failed to produce more than three precipitin bands (Figure 3). Such inconsistency can only be explained by speculation. The denaturation which occurred in M. Ag I and M. Ag II may have been more

severe in M. Ag II, resulting in low concentration of certain proteins in that antigen-extract. The low protein concentration may have resulted in insufficient antigenic stimulation during immunization or may have been too low for correct antigen-antibody proportion necessary for precipitation in the test situation. Perhaps more important, different protein complexes having different antigenic specifities may have been produced in each antigen extract. These differences would have been reflected in respective homologous antisera.

Comparison of *Metastrongylus* and *Ascaris* Antigens

Metastrongylus species have been shown to cross react in serological tests with other unrelated parasites. Swine anti-*Metastrongylus* serum was found to react with *Dictyocaulus filaria* antigen in indirect hemagglutination tests (Becht 1960). Jaggers (1965) suspected cross-reaction between *Oesophagostomum dentatum* and *Metastrongylus* in intradermal tests in swine and in indirect hemagglutination tests. Soulsby (1957a) (1957b) studied skin test reactions to *Ascaris suum* antigen in swine, several of which had concurrent lungworm infection, but did not believe the presence of *Metastrongylus* influenced his results. However, Becht (1960) demonstrated a *Metastrongylus*-*Ascaris suum* relationship by observing cross-reaction with *Metastrongylus* antigen and anti-*Ascaris* serum in indirect hemagglutination tests.

Immunodiagnostic tests in helminthic diseases have often shown cross-reaction between various families and even various orders (Soulsby 1963). In most cases diagnostic test-antigens have not been single antigens. Only recently have attempts been made to determine which antigen in a mosaic is

responsible for the reaction that was elicited (Malley et al. 1968). That two or more unrelated helminths might have proteins of similar antigenic structure is very probable. Enzymes, membranous proteins or body-fluid proteins having the same biochemical or anatomical function within helminths are likely to be structurally similar and, hence, antigenically related. As a result of such reasoning, antigens from Dirofilaria immitis have been used for immunodiagnosis in human filarial infections (Soulsby 1963).

In this study at least one antigen common to both Ascaris suum and Metastrongylus species was demonstrated by immunoelectrophoresis (Figure 8). That this antigen represents a true protein relationship between the two parasites is conjectural. It is possible that the shared antigen was a host protein which contaminated both antigen extracts. However, in a trial preliminary to this study, anti-Metastrongylus rabbit serum was adsorbed with swine lung tissue and subsequently reacted with Metastrongylus antigen. Results were identical with those obtained using nonadsorbed antiserum, which indicated that no swine-protein contamination was present in the antigen extract. Furthermore, precaution was taken against swine-tissue contamination in the extraction procedures in this study. It was believed, therefore, that the relationship shown between Ascaris and Metastrongylus was not a result of host contaminant. The relationship, as shown, points out the need for further purification of lungworm antigens, which can ultimately be used with confidence in serological procedures.

Skin Sensitization Study

An immediate reaction, as defined by Kabat and Mayer (1961), was observed with intradermal injection of M. Ag III and A. Ag in swine that were infected with Metastrongylus. The two antigens could not be differentiated in the reacting swine on the basis of their respective reactivities. However, each reacting swine was harboring a lungworm infection and each had been exposed to, or was infected with, Ascaris suum.

The results with A. Ag are in agreement with those found by Soulsby (1957a). He found positive immediate skin reactions to Ascaris suum antigens in swine no longer harboring adult ascarids. However, all swine in which reactions were noted had been previously exposed to Ascaris, based on the presence of liver lesions.

Adsorption of M. Ag III and A. Ag with respective heterologous antiserum did not enable differentiation of the antigens by intradermal injection in the reacting swine. This was to be expected, since both swine tested with the adsorbed antigens had been exposed to Metastrongylus and to Ascaris. There was a detectable difference noted at 24 hours post injection, however. The adsorbed M. Ag III injection site was indurated, as were nonadsorbed M. Ag III and A. Ag sites, whereas the adsorbed A. Ag site was not indurated. This observation caused reflection on the possibility of a more prolonged immediate response to Metastrongylus antigen than to Ascaris antigen. Another possibility was that a delayed-type hypersensitivity was occurring with Metastrongylus antigen. Kabat and Mayer (1961) suggested that induration is a gross characteristic of delayed hypersensitivity.

Microscopic examination of histologic sections of the indurated test

site revealed a response that was primarily perivascular with edema and infiltration with neutrophils and eosinophils. This was interpreted as an aftermath of an immediate reaction that had occurred earlier. The presence of perivascular fluid may have been responsible for the grossly palpable induration. The presence of neutrophils was in contrast to an expected histiocyte infiltration, which is a differentiating characteristic of delayed-type reactions (Crowle 1962).

Andrews (1962) reviewed the literature on the occurrence of delayed hypersensitivity in parasite infections and concluded that the reaction was more an exception than the rule. With regard to Metastrongylus infections, Jaggers (1965) found only an immediate response after intradermal injection of antigen, and no delayed-type reaction was noted.

Immediate-type hypersensitivities can be transferred passively with serum antibodies, while delayed-type hypersensitivities normally are transferable with lymphoid cells or their derivatives. A subclassification of immediate hypersensitivity includes atopic sensitivity, which shares the basic immediate hypersensitivity characteristics. Thus an individual with atopic sensitivity will respond to intradermally placed antigen with an evanescent skin reaction, and the sensitivity has been passively transferred with serum. However, atopic sensitivity differs from other immediate-type reactions in several important respects. This hypersensitivity can not be artificially induced (by injection of antigen), but apparently occurs primarily when the antigens have been inhaled or ingested. Furthermore, the antibodies produced to such antigens are not the usual gamma globulins. The antibodies (usually called reagins) have unusual affinity for skin and mucous membranes, and all in vitro techniques to

demonstrate their presence require live tissues. (Crowle 1962, Raffel 1961).

It has been observed that monkeys infected with helminth parasites produce an antibody with the properties of reagins (Ogilvie 1964). However, no such antibodies were produced following vaccination with freshly homogenized worms or with soluble extracts. Recently atopic hypersensitivity was detected by intradermal test and passive transfer experiment in Ascaris lumbricoides-infected rhesus monkeys (Malley et al. 1968). Based on these considerations it is interesting to speculate on the occurrence of atopic hypersensitivity in swine lungworm disease. Certainly the results of the intradermal injection of Metastrongylus antigens and the subsequent immediate reaction would support such an occurrence. However, in vitro demonstration of reagins would be necessary to substantiate the hypothesis. It has been shown that humoral antibodies to Metastrongylus are produced in swine (Becht 1960) (Jaggers 1965). However, it is conceivable that some proteins of the Metastrongylus antigen-complex may induce reagin production as well.

CONCLUSIONS AND SUMMARY

A method by which Metastrongylus species, the lungworms of swine, could be extracted to provide antigens in solution was established in this study. Three different procedures were used. Two extraction procedures (I and II) were done using phosphate-buffered saline as the extracting medium and varied only in the way the lungworms were washed. A third extraction procedure was done using trishydroxychloride-buffered saline as the extracting medium.

Extraction procedures I and II provided final antigen solutions which were unstable during cold storage in that a heavy precipitate formed after thawing or prolonged refrigeration. This characteristic was not observed in the antigen solution obtained by the third extraction procedure.

The antigen solutions resulting from the three extraction procedures were very similar electrophoretically and antigenically. However, it was believed that the trishydroxychloride-extracted antigen solution was best suited for further study of Metastrongylus antigens because of its cold storage stability.

It was believed the washing procedure used in extraction procedures II and III resulted in a higher proportion of eggs and first-stage juveniles being saved for the extraction of antigens. The nature of separation of these stages from the adult lungworms during washing could be useful in a study on stage-specific antigens. The eggs and first-stage juveniles formed a buffy-coat layer over the adults, which could easily be separated.

An antigenic relationship was found between Metastrongylus and adult Ascaris suum. At least one shared antigen was found. This observation

suggests that further purification and fractionation of Metastrongylus antigens would be necessary to assure confidence in specificity if used in immunodiagnostic tests.

Immediate-type hypersensitivity was observed with Metastrongylus and Ascaris suum antigens in lungworm-infected swine. However, no differentiation between the antigens was possible based on their respective intradermal reactivities, because each lungworm-infected swine had also been exposed to Ascaris suum. The reaction was characterized by a wheal and flare that reached a maximum intensity at 15 minutes post injection and waned thereafter. Further study of the hypersensitivity reaction using experimental lungworm-infected swine should be done to see if Metastrongylus-Ascaris cross reaction occurs in infected swine. The nature of the hypersensitivity to lungworms should also be studied using in vitro methods which would further aid in learning about the immune response to lungworm infection in swine.

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