Studies on Immunodiagnosis of Angiostrongyliasis. 2. Preliminary Experiment for Preparing Specific Antigen from *Angiostrongylus cantonensis*

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The recovery of *Angiostrongylus cantonensis* in the cerebro-spinal fluid of a patient suffering from meningitis was first reported by Nomura and Lin (1945) in Taiwan. However, little attention had been paid to this parasitic disease until Rosen *et al.* (1962) reported the finding of the worm in the brain of a man who died from eosinophilic meningitis in Hawaii. Subsequently, this parasite is now generally recognized as an etiologic agent of eosinophilic meningoencephalitis in the Pacific Islands and Southeast Asia.

The diagnosis of angiostrongyliasis is generally presumptive and based upon characteristic clinical symptoms such as eosinophilicpleocytosis, since it is rarely the case that the parasite can be detected in the cerebro-spinal fluid.

Several investigators have made attempts to develop useful immunodiagnostic adjuncts by using worm extract as antigens. However, satisfacory results were not obtained, because of many cross reactions with other helminthic infections (Alicata and Brown, 1962a; Anderson *et al.*, 1962; Kagan and Zaiman, 1964).

This study was conducted to elucidate the complexity of the antigens from *Angiostrongylus cantonensis* adult worms, and to determine which antigenic components may be useful in immunodiagnostic tests.

Materials and Methods

Infection of rats: *A. cantonensis* larvae were obtained from naturally infected land snails, *Achatina fulica*, which were collected from southern Taiwan. Long Evans rats, weighing 150 to 200 g each, were used in all experiments. The rats were infected with 100 larvae by stomach tube and the animals sacrificed 50 days after infection. Adult worms and the sera were obtained from these rats.

Preparation of antigen: The adult worms were washed several times with 0.05 M phosphate buffered saline, pH 7.4 (PBS) and emulsified thoroughly in PBS in a mortar. After centrifugation at 3,000 rpm for 30 minutes, a brown colored supernatant was obtained. The supernatant was then dialyzed against PBS for 12 hours at 4°C, and was stored in a deep freezer (−60°C) until used.

Preparation of antisera: Sera were collected from infected rats, as described above.

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Antisera against adult worm extract were prepared in 2 rabbits, weighing 2.5 kg each. To one ml of adult worm extract, an equal volume of Freund's complete adjuvant was mixed and injected subcutaneously 4 times to foot pads and backs of rabbits at intervals of 10 days. Rabbits were bled periodically from the marginal ear veins, and the sera wer examined for antibodies by double-diffusion and immunoelectrophoresis. Finally, rabbits were bled from carotid artery one week after the last injection, and the sera were stored at −60°C. Similarly, antisera against normal rat serum and red blood cells were prepared.

Five rabbits were also infected with 200 third-stage larvae of *A. cantonensis*, and sera were collected every 5 days for 100 days after infection.

Preparation of immunoglobulin : To the antiserum, an equal volume of PBS was added and the same volume of saturated ammonium sulfate solution was slowly added at constant stirring in an ice bath. The mixture was allowed to stand for 30 minutes at 4°C, then centrifuged at 8,000 rpm for 30 minutes at 4°C. The sediment was washed twice with a large volume of 40 per cent cold saturated ammonium sulfate solution, dissolved again in PBS and dialyzed against several changes of this buffered solution. The preparation was stored at −60°C until used.

Fractionation of antigen with saturated ammonium sulfate solution : The saturated ammonium sulfate solution was added dropwise to the whole worm extract to give 60 per cent saturation. The mixture was kept in an ice bath for 30 minutes. After centrifugation at 8,000 rpm for 30 minutes, the supernatant and precipitate were separately dialyzed against several changes of PBS to remove ammonium sulfate.

Chromatographic fractionation of antigen on DEAE-cellulose column : DEAE-cellulose was washed with deionized water, activated by washing with sodium hydroxide – hydrochloric acid – sodium hydroxide in due order, equilibrated with 0.01 M phosphate buffer, pH 7.2 (PB) and packed into 2.5×23 cm column. The fractions were eluted stepwise by molarity gradients of sodium chloride with 0.01, 0.1 and 0.5 M in 0.01 M PB solution, respectively. The fractions collected were concentrated by lyophilization and tested by gel-diffusion techniques.

Gel-diffusion techniques : Double-diffusion was performed according to the method of Ouchterlony (1933) using 1.2 per cent agarose in 0.05 M sodium barbital – hydrochloric acid buffer, pH 8.2 containing one per cent merthiolate on 5×5 cm glide. The depth of the gel was approximately 1.5 mm. Precipitin bands were allowed to develop for 48 hours at room temperature. The slide was then washed for 72 hours in several changes of normal saline, dried under filter paper and stained with amido black 10 B.

Immunoelectrophoresis was performed by the micro-method of Scheidegger (1955) using the same gel as above on 2.5×8 cm glass slide. A potential of 100 volts was maintained across the slide for 3 hours, and precipitin bands were allowed to develop for 48 hours at room temperature. The drying and staining procedure was similar to the above.

Results

Antigenic componets of whole worm extract : When the whole worm extract (WWE) was tested against serum of rabbit immunized with the same material (Anti-WWE) by gel-diffusion, 6 precipitin bands were appeared (Fig. 1). On the other hand, 13 precipitin bands were produced by immunoelectro-

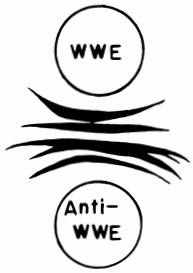

Fig. 1 Double-diffusion between whole worm extract (WWE) and anti-whole worm extract (Anti-WEE).

phoresis, and 3 of them were more distinct than the other bands. These bands were named A, B and C, as shown in Fig. 2.

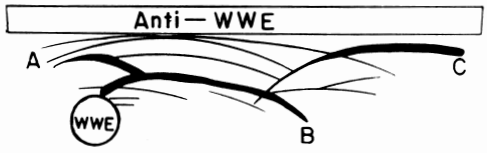

Fig. 2 Immunoelectrophoretic pattern between whole worm extract and anti-whole worm extract.

Host components contained in whole worm extract: It is naturally thought that some antigenic components originated from the host, especially from the host blood may be contained in the WWE. For this reason, existence of the components from rat serum and red blood cells in the WWE was tested. When the WWE was tested against serum of rabbit immunized with normal rat serum (Anti-NRS) by double-diffusion, 2 clear bands were produced, and 5 bands were also seen by immunoelectrophoresis (Figs. 3 and 4). Three bands were also produced between the

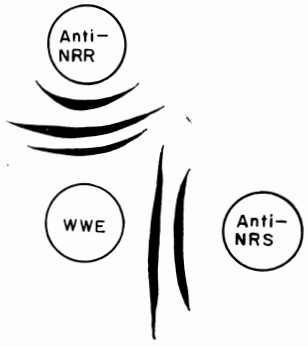

Fig. 3 Double-diffusion between whole worm extract and anti-normal rat serum (Ant-NRS) and anti-normal rat red cells (Anti-NRR).

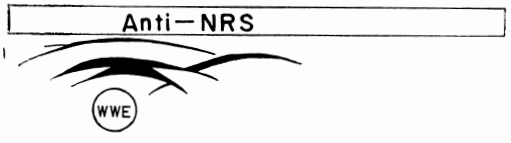

Fig. 4 Immunoelectrophoresis between whole worm extract and anti-normal rat serum

WWE and serum of rabbit immunized with normal rat red blood cells (Anti-NRR) by either double-diffusion or immunoelectrophoresis (Figs. 3 and 5). On the contrary, when normal rat serum (NRS) and red blood cells (RBC) were tested against the Anti-WWE by double-diffusion and immunoelectrophoresis, only one band was produced in the former combination and two bands in the latter (Figs. 6 and 7). This suggests that a considerable amount of host components may be contained in the WWE.

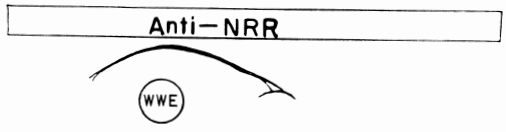

Fig. 5 Immunoelectrophoresis between whole worm extract and anti-normal rat red cells.

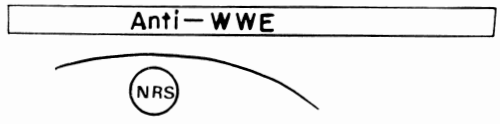

Fig. 6 Immunoelectrophoretic pattern between normal rat serum (NRS) and anti-whole worm extract.

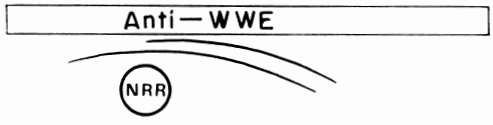

Fig. 7 Immunoelectrophoretic pattern between normal rat red cells (NRR) and anti-whole worm extract.

Antibody production in definitive host: In this study, 3 precipitin bands were seen between the WWE and the infected rat serum by double-diffusion, and 8 bands were observed by immunoelectrophoresis (Fig. 8). Among them, 2 appeared to be identical with Bands A and B, but the other was difficult to differentiate or classify.

Antibody production in insuitable host: Since this parasite does not develop to the fully differentiated adult stage in man, the antibodies produced in such host may not

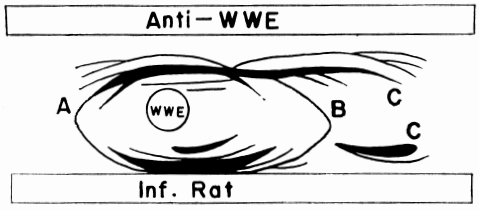

Fig. 8 Comparison of immunoelectrophoretic bands deveploed by anti-whole worm extract and infected rat serum (Inf. Rat) against whole worm extract.

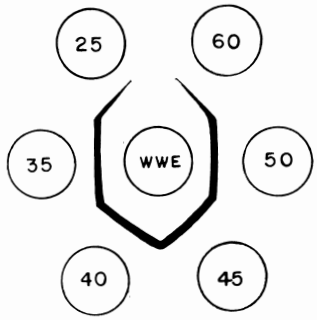

Fig. 9 Double-diffusion between whole worm extact and sera of rabbit infected with 200 larvae. Well numbers represent days after infection.

be the same as those in the definitive host. In antiserum of a rabbit infected with 200 larvae, one precipitin band appeared at 25 days and disappeared at 65 days after infection (Fig. 9). When the rabbit was reinfected with 200 larvae 75 days after the first infection, the precipitin band reappeared 5 days later. In immunoelectrophoresis, only one precipitin band was also seen between the WWE and the infected rabbit serum. By concentrating immunoglobulin of the infected rabbit serum, however, the number of bands increased up to 5 or more and its immunoelectrophoretic pattern was similar with that of the infected rat serum (Fig. 10). This may indicate that there are no qualitative difference in the antibody formation between the suitable and insuitable hosts but only quantitative.

Fractionation of whole worm extract using saturated ammonium sulfate solution : In

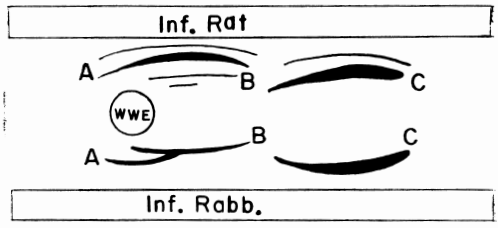

Fig. 10 Comparison of immunoelectrophoretic patterns of sera from infected rat and infected rabbit (Inf. Rabb).

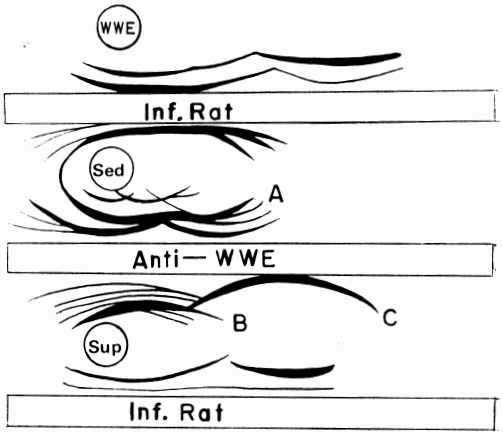

Fig. 11 Immunoelectrophoresis of whole worm extract, sediment (Sed) and supernatant (Sup) fractionated by 20–60 % saturation of ammonium sulfate against infected rat serum and anti-whole worm extract.

oredr to isolate 3 bands as described above, a fractionation techuique with saturated ammonium sulfate solution was utilized. As demonstrated in Fig. 11, Band A was seen in the sediment produced by 20–60 per cent saturation and Bands B and C were contained in the supernatant. Contaminations from each, however, were also observed in both fractions.

Chromatographic fractionation on DEAE-cellulose column : To separate Bands B and C, the supernatant was further fractionated by DEAE-cellulose column chromatography as shown in Fig. 12. When the fractions were collected, concentrated and tested against the infected rat serum and the Anti-WWE by immunoelectrophoresis, Band B

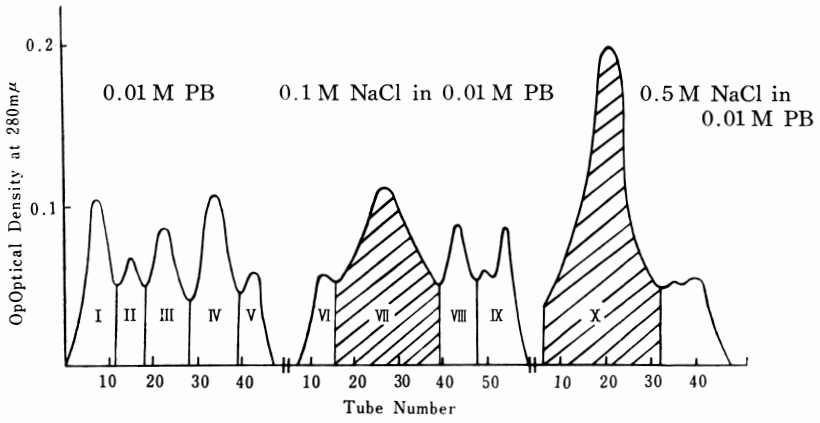

Fig. 12 Elution pattern of DEAE-cellulose column chromatography of the supernatant yielded by 60 % saturation of ammonium sulfate. Column dimdnsions: 2.5×23 cm; Eluants: 0.01 M phosphate buffer, pH 7.2, and the same buffer containing sodium chloride in 0.1 M and 0.5 M; Flow rate: 18 ml/hr.; Sample: volume 10 ml, total protein 30 mg.

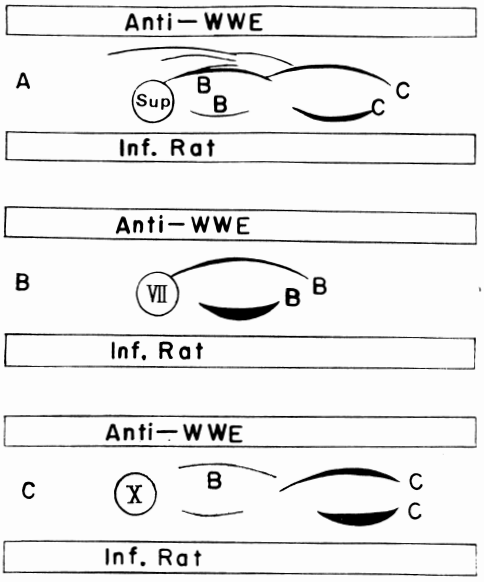

Fig. 13 Immunoelectrophoresis of fractions obtained by DEAE-cellulose column chromatography of 60 % supernatant against anti-whole worm extract and infected rat serum.

was seen in Fraction VII and Band C in Fraction X (Fig. 13).

Discussion

Immunodiagnostic tests using antigen prepared from *A. cantonensis* adults have been reported by several investigators; Alicata and Brown (1962 a) reported that skin test using crude extract from the adult worms elicited a positive reaction in cases of eosinophilic meningitis in Tahiti. Kagan and Zaiman (1964) conducted skin test with adult worm extract on adult hospitalized group of 347 tuberculosis patients in New York City and found 16 (18.2 %) out of 88 Puerto Rican patients with a positive reaction while none of 259 non-Puerto Ricans reacted to the antigen. They supposed that the positive reactions among Puerto Ricans were probably due to non-specific cross reactions with other helminthic infections, since *A. cantonensis* has not been found in Puerto Rico. Subsequently, Alicata and Brown (1962 b) suggested that the efficiency of the skin test with crude antigen may be limited and the negativity of test is sufficiently to rule out angiostrongyliasis. Anderson *et al.* (1962) made an attempt to detect antibodies in sera and cerebro-spinal fluids of Tahitian patients suffering from eosinophilic meningitis by means of a complement fixation test using somatic and metabolic antigens of *A. cantonensis*. They were, however, unable to obtain conclusive

result, since positive reactions were seen in the limited number of cases and also in some cases of control group. Kamiya (1972) tested Thai patients suffering from eosinophilic meningoencephalitis by an indirect hemagglutination test using crude *A. cantonensis* antigen and reported that positive reactions were seen in 14 (3.3 %) out of 19 patients. Suzuki *et al.* (1973) found that the majority of the positive cases for the indirect hemagglutination test with *A. cantonensis* antigen was negative for a Japanese cncephalitis hemagglutination inhibition test in Taiwan. Moreover, the most of these cases were the inhabitants of endemic angiostrongyliasis focus, southern parts of the Island. These findings may suggest that the immunologic tests employing *A. cantonensis* adult extract have a potential value for epidemiological study of the disease.

Williams and Soulsby (1970) demonstrated the existence of common components between parasites and their definitive hosts in an experiment using *Ascaris suum* and porcine serum. In the present experiment, some components showing the common antigenicity were demonstrated in both the adult worm extract and normal rat blood. However, it was thought that these components should be of host blood components or their decomposed substances contained in the worm, especially in the digestive tracts of the worm, rather than of true parasitic origin.

Bouthemy *et al.* (1972) analyzed the antigenic components of worm extract of *A. cantonensis* by double-diffusion and immunoelectrophoresis, and indicated that this parasite shared several common components with other helminths, particularly with nematodes. A similar information was also given by Tsuji (1972). In the present study, 13 precipitin bands were demonstrated in the whole worm extract of *A. cantonensis* adults and 3 of them were also antigenic against serum of the infected rat. Consequently, it was thought that the major antigenic components might mostly be involved in these bands.

An attempt to separate these precipitin bands was then made by means of the fractionation techniques. However, any conclusive result was not obtained by these techniques. Subsquently, other methods are now being tried in our laboratory and the results of this study will be reported in another paper.

Smmary

In an attempt to obtain specific antigen for immunodiagnosis of angiostrongyliasis, a preliminary study on antigenic analysis of whole worm extract of *Angiostrongylus cantonensis* adults was made. At least 13 precipitin bands were demonstrated in the whole worm extract by immunoelectrophoresis. Three of these bands appeared to be highly antigenic against serum of the infected rat.

Subsequently, these 3 bands were tried to isolate by fractionations with ammonium sulfate and DEAE-cellulose. However, satisfactory results were not obtained by these techniques.

Acknowledgements

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広東住血線虫症の免疫診断に関する研究
2. 広東住血線虫よりの特異的抗原作製のための予備実験

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広東住血線虫症の多発地においては，通常脳脊髄液中の好酸球増多などのような臨床症状より本症が疑がわれるが，実際脊髄液中に本線虫を検出して確定診断が下されるという例はきわめて稀である．有効な免疫診断法の確立のためには特異的抗原の作製が最も重要な課題であろうとの考えから本研究に着手したが，本報では抗原精製のための予備実験として行なつた広東住血線虫成虫からの全抽出液の抗原分析と抗原性成分の予備精製実験成績について述べた．

免疫電気泳動法を用いて全抽出液の抗原性成分を調べると，全抽出液で免疫した家兎血清との間に13本の沈降線がみられ，そのうち明瞭にあらわる3本の沈降線は全抽出液と感染ラット血清との間にあらわれる沈降線と同一のものと考えられた．

次いで，これら3本の沈降線を形成する成分を単離するため，飽和硫安溶液での塩析法ならびに DEAE-cellulose カラムクロストグラフィーを行なつた．しかし，これらの手段によつては充分満足すべき成績が得られなかつたので，他の方法による精製法を検討中である．