STUDIES ON THE ANTIGENICITY OF METABOLITES OF THE CANINE FILARIAE IN URINE

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(Received for publication January 10, 1966)

An antigenic substance originated from the helminthic parasite appearing in the urine of infected host has been reported by Welt (1941), from his studies on Trichinella spiralis. Welt observed, during his experiment on monkeys artificially infected with Trichinella spiralis, that antigen was excreted into the urine of the monkeys several weeks after antibody had been produced in their sera. Subsequently, Okabe and Tanaka (1961) and Okabe and Ono (1961) discovered that an antigenic substance was transferred to the urine of a human patient infected with Schistosoma japonicum, and asserted that this fact had an important significance in the clinical diagnosis of schistosomiasis japonica. Furthermore, Okabe and his associates detected antigenic substances in the urine of human patients infected with liver and lung flukes, and reported the results of application of these substances to the clinical diagnosis of the respective diseases.

It is difficult to collect adult worms of Wuchereria bancrofti for material in the preparation of antigen. Currently, an antigenic substance is extracted from adult worms of Dirofilaria immitis, instead of Wuchereria bancrofti, and used for the immunological diagnosis of the human filariasis.

Should any antigenic substance originating from Wuchereria bancrofti appear in the urine of a human patient and be isolated from the urine, it will be a useful material for the immunological diagnosis of Wuchereria infection. Thus, a study on the urine of dogs infected with Dirofilaria immitis, was undertaken. It was confirmed that an antigenic substance, presumably derived from this filaria, had been excreted into the urine of the dogs. Furthermore, this substance was isolated, purified, and examined for biological and immunological properties. The results of the study are reported in the present paper.

MATERIALS AND METHODS

1. Treatment of urinary samples.
   Urinary samples were collected from dogs infected with Dirofilaria immitis and

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dialysed in cellophane bag in running water for 48 hours. The resultant fluid was centrifuged (3,500 r.p.m. for 15 minutes) to obtain the supernatant. The supernatant was concentrated and dried at 40°C under reduced pressure. The resulting product was dissolved in distilled water, or, if necessary, in physiological saline solution, and used as a crude antigen.

2. Examination of antigenicity.

The antigenicity of the resulting product was determined by the results of intradermal reaction and precipitin reaction caused by the ring method overlaying urinary antigen on antiserum of rabbit immunized with the substance derived from the worm of the canine filaria.

The antiserum used was prepared in the following manner. A substance was extracted from the worm with physiological saline solution (1:500), in accordance with the method of Koyama (1959). The supernatant was harvested from the extraction. A rabbit was inoculated subcutaneously with 0.5ml/kg of the supernatant daily for seven consecutive days. The rabbit was sensitized in this manner and produced a serum, the antigen titer of which was not lower than 1:3,000.

3. Isolation of an antigenic substance from the urine by paper chromatography.

A mixture of butanol, acetic acid, and distilled water (4:1:2) and filter paper No. 52 of Toyo Brand (40 cm × 40 cm) were used to develop a paper chromatogram of the antigenic substance in the urine by the ascending method. A piece of paper about 3 cm in length was cut off from the end of the filter paper and subjected to examination of the color reaction with ninhydrin and the qualitative reaction of sugar with ammoniacal silver nitrate. Each section of the resultant colored zone was extracted with distilled water in a cool chamber for 24 hours and then centrifuged. The resulting supernatant was concentrated under reduced pressure to be desiccated and solidified. The materials obtained in this manner from every section of the colored zone was examined for antigenicity by means of the forementioned method. Furthermore, the sheet of filter paper on which the paper chromatogram had been developed was divided into four equal portions, without any consideration on the expansion of the colored zone. Each of the four portions was subjected to extraction and examination for antigenicity.

4. Isolation and purification by paper electrophoresis.

Paper electrophoresis was carried out in accordance with the method of Okada (1959). Using barbital (veronal was adopted) buffer solution at pH 8.6 and the ion intensity of 0.05. The breadth of the sheet of filter paper was 1 cm per 0.3 mA. Electrophoresis was conducted for 7 hours. The filter paper was then cut at two places 1 cm distant from the original point on the anodic and the cathodic side, respectively. Each piece was placed in a test tube and extracted with distilled water in a cool chamber for 24 hours and the extraction was centrifuged. The resulting supernatant was desiccated and solidified under reduced pressure. The product was dissolved in physiological saline solution and examined for potency as an antigen.

5. Purification by the Sephadex G 25 column.

The Sephadex G 25 column used was 3 cm by 20 cm in size. Distilled water was employed for development. The samples were collected at the rate of 50 ml an hour in 10-ml amounts by means of a fraction collector, desiccated and solidified by process of concentration under reduced pressure. The resultant product was dissolved in physiological saline solution and examined for antigenicity by the precipitin
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reaction with antiserum. This reaction was performed by the accumulation method.

RESULTS

1. Antigen appearing in the urine of rabbits injected intravenously with *Dirofilaria immitis* antigen.

Rabbits, weighing 2.1 to 3.0 kg, were injected slowly with 5 to 10 ml of the extract of the body substance of *Dirofilaria immitis* in physiological saline solution (1:50) intravenously. Urinary samples were collected immediately, 7, 24, and 48 hours, and 3, 4, and 5 days, following injection. The samples were centrifuged and the resulting supernatants subjected to dialysis through a cellophane membrane in running water for 24 hours. Then, they were concentrated under reduced pressure to be made desiccated and solidified. The resultant products were dissolved in physiological saline solution and tested for precipitin reaction by the accumulation method. The results obtained are indicated in Table I.

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Body weight (kg)</th>
<th>Does injected (ml)</th>
<th>No. of days</th>
<th>Before injection</th>
<th>After injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7 hrs.</td>
<td>24 hrs.</td>
</tr>
<tr>
<td>I</td>
<td>2.3</td>
<td>5</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>2.1</td>
<td>5</td>
<td>2</td>
<td>-</td>
<td>×</td>
</tr>
<tr>
<td>III</td>
<td>3.0</td>
<td>10</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>2.5</td>
<td>10</td>
<td>1</td>
<td>-</td>
<td>-</td>
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</table>

Remarks: Positive .......... +
Doubtful .......... ±
Negative .......... −
Not tested .......... ×

As shown in Table I, when a rabbit was injected intravenously with 5 ml of the extract of the worm body daily for two consecutive days, the results of the precipitin reaction confirmed that an antigenic substance is excreted in the urine of the rabbit about 24 to 48 hours after injection. Four days after injection, all the rabbits gave a negative reaction.

Rabbits injected intravenously with a single dose of 10 ml, a positive precipitin reaction was observed in the urine collected 7 hours after injection. However, the precipitin was negative in this rabbit earlier than in that one which had received for two consecutive days.

Thus, it has been confirmed that the substance of the worm body of the canine filaria injected into a host animal is excreted into the urine of the animal within a
2. Isolation of antigenic substance from the urine of a dog infected with *Dirofilaria immitis*.

Urinary samples were collected from a dog which harbored microfilariae in its peripheral blood. It was subjected to dialysis through a cellophane membrane in running water for 24 hours and then centrifuged. The resulting supernatant was concentrated to about one-tenth of the original quantity under reduced pressure. Using this product as material, paper chromatography was carried out by the method mentioned above.

![Figure 1. Treatment of metabolites by paper chromatography.](image)

**Figure 1.** Treatment of metabolites by paper chromatography.  
Development: Filter paper No. 51 of Toyo Brand, 40×40 mm in size. Butanol: acetic acid: water (4:1:2)  
Color reaction: Ninhydrin.

A: Metabolites, B: Extraction of worm body, o.p.: Original point.

Following development, the sheet of filter paper was divided into four equal portions, I to IV, as shown in Fig. 1. Extraction from each of the portion was taken with distilled water in a cool chamber for 24 hours and centrifuged. The resulting supernatant was desiccated and solidified by process of concentration under reduced pressure. The product was dissolved in physiological saline solution and examined in the manner mentioned above for precipitin reaction by the accumulation method and for intradermal reaction upon sensitized rabbits. The results obtained are given in Table II and Fig. 2.

When examined for the precipitin reaction, fraction I, as classified by chro-
### TABLE II

*Precipitin Reaction with Extract of Each Fraction as Antigen*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Time in minutes</th>
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<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Fraction I</td>
<td>–</td>
</tr>
<tr>
<td>Fraction II</td>
<td>–</td>
</tr>
<tr>
<td>Fraction III</td>
<td>–</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>–</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
</tr>
</tbody>
</table>

*Figure 2.* Results of intradermal reactions with four fractions isolated by paper chromatography upon sensitized rabbits.

- ●: Fraction I
- △: Fraction II
- ○: Fraction III
- ×: Fraction IV

Matography, showed a particularly strong positive reaction. Both fractions II and IV gave negative results. Fraction III showed a positive reaction after a lapse of 60 minutes.

Thus, it was assumed that the antigenic substance of the canine filaria appearing in the urine might be located relatively close to the original point of the chromatogram. The following experiment was then carried out confirming the position of this substance.

3. **Comparison of the antigenic fractions between the urine of dogs infected with filariae and the solution used for cultivation of canine filariae.**

In the experiment mentioned in the preceding section, the urine of dogs infected with *Dirofilaria immitis* was subjected to fractionation by chromatography. As a
result, it was elucidated that the antigenic substance in the urine was situated relatively close to the original point of the chromatogram. In the present experiment, the urine of dogs infected with canine filariae and the physiological saline solution used for cultivation of canine filariae were developed by chromatography and examined for the presence of a colored zone by the color reaction with ninhydrin. The results obtained are indicated in Fig. 3.

\[\text{Figure 3. Paper chromatograms of the solution used for cultivation of canine filariae (A) and the urine of dogs infected with filariae (B).} \]

\[\text{Remarks: Color development: Ninhydrin.} \]

\[\text{o.p.: Original point.} \]

In the paper chromatograms of both urine and solution used for cultivation, a ninhydrin-colored zone appeared with Rf 0.28 as the ascending point. Coloration, with Rf 0.2 as its center, was detected at 0.16 in the urine and 0.18 in the solution used for cultivation. Furthermore, antigenicity was strongly active at these points. Thus, it can be assumed that the antigenic substance appearing in the urine of dogs, infected with canine filariae, may have been derived from the metabolites of these filariae.

4. Separation of the antigenic fractions of the urine by paper electrophoresis.

The results of fractionation of the urine by paper chromatography has demonstrated that the antigenic substance exhibit a strong reaction in its fraction I, which is close to the original point than any other fraction. Further investigation was performed by means of paper electrophoresis to obtain any data on the purification of this substance. The method used by Okada (1959) was applied to it.

The paper chromatogram bearing the fractions was divided into portions of 1 cm in breadth, with the original point as their center, and subjected to precipitin
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5. Isolation and purification of the active fractions by means of Sephadex G 25 columns.

The fractions of the antigenic substance which had been transferred to the urine were isolated and purified by paper chromatography and paper electrophoresis. In the present experiment, they were subjected further to column chromatography using Sephadex G 25 columns, so that the active fractions might be purified.

The water derived from the columns was developed by means of the fraction collector. Each of the resulting products was examined for qualitative reaction on sugar using the Anthron reagent. The results obtained are indicated in Fig 5. A positive sugar reaction was shown by fraction I (test tubes Nos. 5 to 10) and fraction II (test tubes Nos. 25 to 37).

Furthermore, each of the products obtained by development was concentrated under reduced pressure, dissolved in physiological saline solution, and subjected to the precipitin reaction by the accumulation method. The results obtained are indicated in Table 3.

A pronounced positive precipitin reaction appeared in fraction I, which had shown a positive sugar reaction at test tubes Nos. 5 to 10. The precipitin reaction was negative in fraction II, which had given rise to a positive sugar reaction at test tubes Nos. 25 to 37.

The two fractions were then subjected for intradermal reaction on sensitized rabbits. Both fractions gave a positive intradermal reaction, as shown in Fig 6.

The two portions of the antigenic substance in the urine, which take part in the precipitin reaction and the intradermal reaction, respectively, transferred simultaneously. The antigenic substance, however, could be divided into two portions, one active in the precipitin reaction and the other active in the intradermal reaction, by column reaction for judgment as reflected in Fig 4. As a result, a positive precipitin reaction was observed in only one portion next to the original point (1 cm distant from it) on the anodic side. It was, therefore, presumed that the antigenic substance transferred to the urine had been hardly moved from the viewpoint of paper electrophoresis.
Purification of active fractions of antigenic substance in the urine by means of Sephadex G 25 columns. Anthron reagent was applied.

I, II: Fractions I and II, respectively.

Table III

Results of Precipitin Reaction in Fractions I and II Obtained by Means of Sephadex Columns

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Fraction I</td>
<td>++</td>
</tr>
<tr>
<td>Fraction II</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
</tr>
</tbody>
</table>

Results of intradermal reactions with fractions I and II on sensitized rabbits.

\[\text{\textbullet} : \text{Fraction I} \quad \text{\textbullet} : \text{Fraction II} \quad \times : \text{Control}\]
chromatography using Sephadex G 25 columns. Thus, it was presumed that these two portions may be heterogenous to each other.

**DISCUSSION**

When a host is infected with helminths, a substance originated from the helminths appears in the urine of the host. This substance possesses a potency as an antigen. These findings were reported by Welt (1941), who had conducted his studies on *Trichinella spiralis*. Subsequently, Okabe and Tanaka (1961) and Okabe and Ono (1961) performed investigation of *Schistosoma japonicum*, and liver and lung flukes, and determined that an antigenic substance derived from the worm body of each species had been transferred to the urine of the host harboring worms of the respective species. Moreover, Okabe and his associates studied the antigenic substance which had been demonstrated in the urine of the host, by applying the precipitin reaction with antiserum, so some relationship might be established between the appearance of this substance in the urine and the occurrence of infection with helminths. As a result, they determined that a high value should be placed to this reaction in the diagnosis of helminthic infection.

The present studies were designed to determine whether the antigenic substance originated from filariae could be transferred to the urine of their host and to elucidate the immunological properties of this substance. Rabbits were injected with filarial antigen and examined for the process of excretion of the antigen into the urine with the lapse of time.

As a result, it was affirmed by means of the precipitin reaction that the filarial antigen injected parenterally into rabbits had appeared in the urine of these animals. Then such antigenic substance was isolated from the urine of dogs infected with *Dirofilaria immitis* and purified. Comparative studies were made on the antigenic substance having appeared in the urine of the host and the same substance prepared from the fluid used for cultivation of canine filariae, by using paper chromatography. As a result, these two substances were found to show the same Rf value (0.16 to 0.28).

Based on these results, it is assumed that the antigenic substance transferred to the urine may have been derived from nothing more than the metabolites of helminthic parasites harbored in the host. Further isolation and purification of this antigenic substance were carried out by means of paper electrophoresis. A portion with a positive reaction was recovered from this substance and purified by column chromatography using Sephadex G 25 columns. It contained a fraction which was positive for sugar and precipitin reaction.

These findings seem to indicate that the antigenic substance originated from the metabolites of the filaria and transferred to the urine of the host may have an active factor in one of its fractions which is rather strongly positive for sugar reaction.

It should be noted that this substance, which possesses a potency as an antigen, also gives rise to a positive intradermal reaction in rabbits which have undergone sensitization with the substance derived from the body of the canine filaria. This fact gives evidence for the presence of a factor which is common to one substance that has participated in the reaction with antiserum *in vitro* and the other substance that has appeared in the body as a result of the reaction having taken place *in vivo*,


or tissue antibody.

SUMMARY

The author isolated a substance from the urine of a dog infected with *Dirofilaria immitis*. The substance had been derived from the body of the parasite was examined for biological and immunological properties. The results obtained are summarized as follows.

An antigenic substance, which showed activity in the precipitin reaction and in the intradermal reaction, had been transferred to the urine of the host infected with filariae. It was proven by paper chromatography that this substance was located in the vicinity of Rf 0.2. Another substance was isolated from the fluid used for artificial cultivation of canine filariae. It was demonstrated that the substance isolated from the urine had essentially the same properties as that isolated from the fluid of filarial culture.

ACKNOWLEDGMENT

The author is greatly indebted to Professor Shigeya Shimizu, major adviser to him for writing a dissertation, for his valuable advice and review of this manuscript and Dr. Minoru Akusawa, instructor in Medical Zoology, for his cooperation and assistance in performing the experiments.

The outline of this paper was read before the 22nd general meeting of the Eastern Japan Division of the Japanese Society of Parasitology in 1962.

REFERENCES