CHANGE OF INDIRECT HEMAGGLUTINATION REACTIONS IN SERUM AFTER THE TRANSFER OF ADULT *ANGIOSTRONGYLUS CANTONENSIS* TO THE ABDOMINAL CAVITY OF RATS

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INTRODUCTION

Angiostrongylus cantonensis is considered to be the causative agent of eosinophilic meningitis, which is distributed in South Pacific islands and Southeast Asia. Relating to the establishment of the sero-diagnosis of this disease, Kamiya and Tanaka (1969) reported the development of indirect hemagglutination (abbreviated as HA) antibodies in rats experimentally infected with *Angiostrongylus cantonensis*. An increase of HA titer was observed from the 6th week, almost at the same time as full maturation of the worms. Similar results were reported by Ishii *et al.*, (1968) in the study of the HA test in cotton rats infected with *Litomosoides carinii*. In their studies, they pointed out that the initial rise of HA titer is closely dependent on the maturation of the worms, and that the antigenic stimulation is released mainly from the adult worms. However, the role of male and female worms in relation to this increase of HA titer has not yet been analysed. In the experiments, it was concluded that changes of HA activities in the sera of albino rats showed remarkable differences according to the sex of the transferred worms. A possibility of the presence of sex-specific antigen is suggested.

MATERIALS AND METHODS

Transfer of adult worms into normal healthy rats : Living adult worms of *Angiostrongylus cantonensis* were collected from laboratory infected rats. The transfer of adult worms into the abdominal cavity of normal rats was carried out under aseptic conditions. Rats infected with *Angiostrongylus cantonensis* were fixed on a board under ether anaesthesia and were washed with alcohol. An incision was made along the sternum and the pleural cavity was opened by fixing both sides of the thoracic wall with pins. The adult worms, weighing approximately 6 mg for females and 1.5 mg for males, were collected from the pulmonary arteries of rats which had been infected 6 months previously with 100 third-stage larvae. Rats weighing 160 - 200 gm were divided into 3 groups as follows :

Group A .... 20 female worms were transferred into 5 rats, 5 female worms into 2 rats.
Group B .... 20 male worms into 4 rats, 80 male worms into 1 rat.
Group C .... 10 female and 10 male worms into 5 rats.

The rats were anaesthetized, fixed on a board, shaved and washed with alcohol. The worms were washed several times with physiological saline and were then transferred into the abdominal cavity through a subdiaphragmatic right or left sided paramedian incision. The wound was sutured with silk and 25,000 units of penicillin G per rat were injected. The rats were bled from the tip of tail at various intervals from the day of operation to 84 days. The sera were stored at –20°C after inactivation at 56°C for 30 minutes.

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Adult worms from the abdominal cavity were examined at the time the host died or was sacrificed.

Procedure of HA test

A standard serum obtained from a rabbit immunized with adult worms was used throughout the tests. The antigen was a phosphate buffered saline extract. The preparation of the antigen was described by Kamiya and Tanaka (1969). Sheep erythrocytes were preserved in Alsever's solution in a refrigerator. The method of sensitization of red cells with antigen was similar to that reported by Jacobs and Lunde (1957) for toxoplasmosis, and the same as that used in the *Angiostrongylus* study by Kamiya and Tanaka (1969). Microtiter plates were used instead of test tubes.

The cells were washed thrice with chilled phosphate buffered saline (0.15 M) at pH 7.2 (abbreviated as PBS). To make a 2.5% suspension, one ml of packed cells was diluted to 40 ml with PBS. For the determination of accurate concentration of sheep cells, one ml of 2.5% cell suspension was lysed with 14 ml of 0.1% Na_2CO_3 and the optical density of the lysate was measured by a spectrophotometer at 541 mμ. The original cell suspension was adjusted to give 0.38 absorbance. At this absorbance the concentration of sheep cells was 7×10^8/ml. Three ml of this cell suspension were then mixed with 3 ml of tannin diluted with PBS at 1 : 40,000 and the tanning treatment was allowed to continue in a water bath at 37°C for 15 minutes. During tanning treatment the mixture was stirred well every 5 minutes to provide a uniform suspension of the cells. After completion of the tanning reaction, the tanned cells were washed once with cold PBS and resuspended in 3 ml of physiological saline.

The resultant suspension of tanned cells was poured into a tube containing 3 ml of antigen (nitrogen contents, 64 μg/ml) that had been diluted with chilled 0.15 M phosphate buffer at pH 6.4 to its appropriate concentration as determined by the block titration. The mixture was kept 15 minutes at room temperature and shaken every 5 minutes. The cells were then centrifuged, washed twice with 1.0% normal rabbit serum (abbreviated as NRS) diluted with PBS which was used as diluent for all subsequent steps. The packed cells were suspended in 4.5 ml NRS, 1.5-fold volume of the initial volume of 3 ml, in the final procedure.

For the test, microtiter wells with radial (U) bottoms were filled with 3 drops (0.075 ml) of NRS using a calibrated dropper. Each serum was diluted in serial 4-fold dilution with a calibrated diluter. Into 3 drops of diluted serum, one drop (0.025 ml) of sensitized cell suspension was added. The microtiter wells were then covered with sealing tape and the plate was shaken to suspend the cells. Cells were allowed to settle for 3 hours at room temperature or over night in a cold room and the resulting patterns of cells at the bottom of the wells were read from below using a mirror mounted on a viewing rack. Patterns were classified into 5 categories: negative and 1 plus to 4 plus, following Jacobs and Lunde (1957). A positive reaction was considered to be 2 plus or more. A 2 plus reaction is defined as one in which the cells have not adhered to the steeper portions of the curvature and have formed a large ring-like pattern, with distinct margins.

RESULTS

Recovery of the transferred worms

Prior to the main experiment, 6 rats were implanted with female worms, male worms or both, and the worms were recovered after sacrifice or after normal death (Table 1).

All worms in rat Nos. 1, 3, 4, and 5 were alive 14 days after the transfer. Of 20 female

Table 1

Development of the HA serum activities in the rats after transfer of adult worms of *Angiostrongylus cantonensis* to their abdominal cavities.

Group	Rat No.	Rat Weight in gm.	Rat Sex	Number of worms transferred ♀	Number of worms transferred ♂	HA activity weeks after transfer 0	1	2	3	4
A	1	170	♀	20	0	—	+	dead*		
	2	160	♀	20	0	—	+	+	+	+** sacrificed
B	3	170	♀	0	20	—	—	—* sacrificed		
	4	200	♂	0	20	—	—	—* sacrificed		
C	5	160	♂	10	10	—	dead*			
	6	200	♂	10	10	—	+	+	+	+*** sacrificed

— = HA negative; + = HA positive; * All the worms were recovered alive; ** 20 female (19 living, 1 dead) worms were recovered; *** 10 female (all living) and 10 male (8 living, 2 dead) were recovered.

worms in rat No. 2, one was dead and encapsulated, and the rest were alive 28 days after transfer. Of 10 male and 10 female worms in rat No. 6, all 10 females were found living and 2 of 10 males were dead in a clump. All the worms collected from the 84th day after the transfer in the rats, Nos. 7, 8, 12, 13, 14, 15, 16 and 17 were found to be dead, fragmented and encapsulated.

Changes of HA activities in the rat sera

HA reactions were examined in 17 healthy rats and no reaction was detected. The sera were also tested in the above mentioned 6 rats at intervals (Table 1). Serum reactions of rats in Group A, Nos. 1 and 2, which were implanted with female worms only, turned positive one week after transfer. Sera of Group C rats implanted with male and female worms also turned positive one week after transfer. However, no HA activity was detected until three weeks in sera of rats in Group B with male worms only.

The change of HA titers in rats shown in the previous preliminary study was further examined in detail. HA titers in the rat sera in Group A (Nos. 7, 8, 9, 10 and 11 in Fig. 1) showed positive as early as 4 days after transfer and persisted progressively through the period of observation. The change of HA reactions in the time course after the transfer in these rats was similar irrespective to the number of worms transferred as shown in Fig. 1.

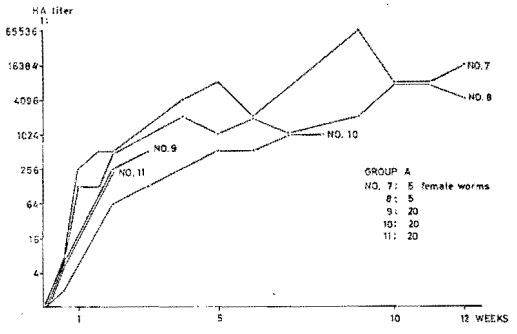

Fig. 1—Sera HA titers in the rats of Group A (Nos. 7, 8, 9, 10 and 11) after peritoneal transfer of 5, 5, 20, 20 and 20 female worms of *Angiostrongylus cantonensis*, respectively.

However, HA activities were not detectable in rats of Group B (Nos. 12, 13 and 14) implanted with 20 males in the first 5 weeks. The reaction of rat Nos. 13 and 14 turned positive 6 weeks after transfer with male worms. The serum of rat No. 12 did not show any HA activity through 11 weeks, but turned positive after 12 weeks, even though the rat

was implanted with 80 males, as shown in Fig. 2.

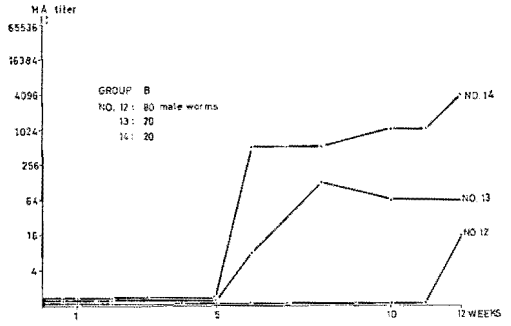

Fig. 2—Sera HA titers after the transfer of *Angiostrongylus cantonensis* male worms to abdominal cavity of rats, Group B.

The appearance and the change of HA reactions in rats of Group C (Nos. 15, 16 and 17) which were implanted with both sexes of worms were nearly the same as those in Group A as shown in Fig. 3.

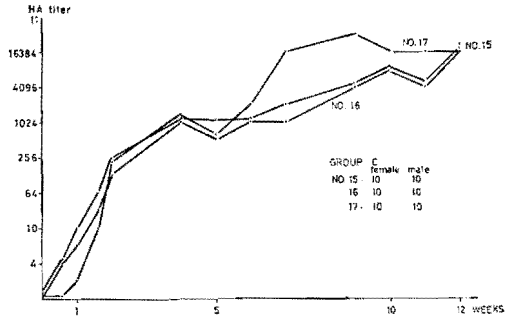

Fig. 3—Sera HA titers after the transfer of *Angiostrongylus cantonensis* female and male worms to abdominal cavity of rats, Group C (Nos. 15, 16 and 17).

DISCUSSION

Litomosoides carinii was implanted to the abdominal cavity of normal cotton rats by Fujita and Kobayashi (1969). The same technique was applied to the present investigation on *Angiostrongylus*. The implanted worms survived for at least 2 weeks. Remarkable differences were observed between the sera of rats transferred with male and female worms. Rats transferred with female worms showed HA activity as early as 4 days after transfer. On the other hand, rats sera transferred with male worms only were completely lacking in HA activity up to 5 weeks, and some of them turned positive from the 6th week. Jacobs *et al.*, (1965) carried out experiments on the *in vitro* cultivation of *Angiostrongylus*, male and female worms in tissue culture medium. The culture fluids were used for the antigen of the indirect HA test. Incubates containing male worms were completely lacking in demonstrable antigenicity by this test. Incubates from female worms were antigenic during the time when the females were depositing eggs. Considered with these findings, the appearance of antibody previously reported by Kamiya and Tanaka (1969) in experimentally infected rats seems to be correlated to maturation of the adult worms, especially to the female worms. Since the implanted worms seem to live at least 2 weeks, antibody detected with the extract of the male and female worms as early as 4 days after transfer was not due to the stimulation of the somatic antigen but the stimulation of a metabolic antigen.

The value of a somatic and a metabolic antigen of *Trichinella spiralis* has been studied by Sadun and Norman (1957). In their studies, the metabolic antigen showed greater sensitivity in the flocculation test than a somatic antigen, but the reactions with the two antigens were similar. Also, it is indicated that metabolic antigen produced higher titers of flocculation antibody during the early stage of an infection when worms were still living and metabolizing, and a somatic antigen produced higher titers when worms were considered to be dead and degenerating in the tissues. This may indicate that the HA titers in the rats implanted with male worms has been caused by the death and degeneration of the worms in the peritoneal cavities of the rats.

Many of the existing studies, except for the work on females of *Ascaris suum* by Kent (1960), dealt with the mixed extract of male and female worms as an antigen for serological study of the nematode infection, and the sex specific antigens have not been well studied. The present results, as well as the results of Jacobs *et al.*, (1965) suggests the presence of a female specific antigen which is lacking in male worms.

Further study is required to evaluate the antigens respective to the sex of the worm in the serological study of the nematode infection.

SUMMARY

The adult worms of *Angiostrongylus cantonensis*, weighing approximately 6 mg for females and 1.5 mg for males, were collected from rats that had been infected with 100 third-stage larvae 6 months previously, and transferred to the abdominal cavity of normal rats by surgical operation. Seventeen healthy rats were divided into 3 groups and adult worms were transferred as follows :

Group A : Living adult female worms in 7 rats.
Group B : Living adult male worms in 5 rats.
Group C : Living female and male worms in 5 rats.

HA activity was detected in the sera of Group A and C as early as 4 days after transfer and persisted through the period of observation. HA activities by using whole adult worm saline extract were not detected in the sera of Group B during the period earlier than 5 weeks. Our present results support the conclusion of our previous study (Kamiya and Tanaka, 1969) that appearance of circulating antibody, 6 weeks after infection, is apparently related to maturation of the adult female worms and oviposition.

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