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Penetration of Third-Stage Larvae of *Angiostrongylus cantonensis In Vitro* II. Invasion to and Passage through the Rat Stomach, Duodenal, Rectal Walls and Skin

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Key words: *Angiostrongylus cantonensis,* third-stage larva, penetration, invasion, passage, *in vitro*

Introduction

Studies for clarifying the tissue invasiveness of parasitic nematode larvae are important for better understanding of their infection routes. Previously, a quantitative experiment was conducted on the penetration of the third-stage larvae of *Angiostrongylus cantonensis,* which are known as the major causative agent of tropical eosinophilic meningoencephalitis in man, through the rat duodenal wall *in vitro* (Araki *et al.,* 1986). As a result, it was ascertained that after invasion to the rat duodenal wall, depending on the experimental conditions, the larvae either passed through the tissue, or stayed in the tissue. The authors attempted to clarify the conditions which made the larvae possible to move from the tissue. In addition, the authors also investigated the possibility of the larvae invading the rat stomach, duodenal, rectal walls, and intact or abraded abdominal skin.

Materials and Methods

Preparation of the third-stage larvae of *A. cantonensis* and the test apparatus was performed as described in the previous report (Araki *et al.,* 1986). Preparation of tissues used in this experiment was as follows. The female Sprague Dawley rats (200–300 g) were anesthetized and killed with ether. Immediately after death, the stomach, duodenum and rectum were removed and were cut into about 1-cm long or 1-cm^2 pieces. To obtain skin, abdominal hairs of freshly killed rats were removed by using an electric shaver, and the subcutaneous muscles were removed as far as possible (intact skin). Furthermore, the skin surface was rubbed with sand paper (Mitsuya, G-120) and wounded several times with a razor (abraded skin).

Harvested third-stage larvae from *Biomphalaria glabrata* were thoroughly (more than 3 times) washed with distilled water and 50 larvae in 1 ml of distilled water as an inner liquid were placed in each syringe. The stomach, duodenal, rectal walls and the skin were fixed with thread over the cut tip of the syringe turning luminal or outer surface in. Then, the test apparatus was brought into contact with the surface of the outer liquid in a glass tube (16 mm in diameter, 16 mm in height).

The outer liquids applied were NCTC 109 with 50% horse serum (NCTC-50HS), NCTC 109, horse serum (HS) and each solution listed in Table 1. The osmotic pressure of each solution listed in Table 1 was adjusted by Sodium Chloride Equivalent Method with NaCl so as to give the same osmotic pressure as that of physiological salt solution (S-A) except for

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Table 1 Abbreviation for solutions used as the outer liquid and their constituents

Abbreviation for solution	Constituents
S-A	H_2O, 1000 ml; NaCl, 9.0 g
S-B	H_2O, 1000 ml; NaCl, 8.7 g; KCl, 0.4 g
S-C	H_2O, 1000 ml; NaCl, 8.56 g; KCl, 0.4 g; $CaCl_2$, 0.2 g
S-D	H_2O, 1000 ml; NaCl, 8.54 g; KCl, 0.4 g; $CaCl_2$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g
S-E	H_2O, 1000 ml; NaCl, 8.49 g; KCl, 0.4 g; $CaCl_2$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $NaH_2PO_4 \cdot 2H_2O$, 0.14 g
S-F	H_2O, 1000 ml; NaCl, 7.06 g; KCl, 0.4 g; $CaCl_2$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $NaH_2PO_4 \cdot 2H_2O$, 0.14 g; $NaHCO_3$, 2.2 g
S-G*	H_2O, 1000 ml; NaCl, 6.88 g; KCl, 0.4 g; $CaCl_2$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $NaH_2PO_4 \cdot 2H_2O$, 0.14 g; $NaHCO_3$, 2.2 g; glucose, 1.0 g
S-H	H_2O, 1000 ml; $NaHCO_3$, 2.2 g
S-I	H_2O, 1000 ml; $NaHCO_3$, 2.2 g; NaCl, 7.57 g
S-J	H_2O, 1000 ml; $NaHCO_3$, 2.2 g; NaCl, 7.27 g; KCl, 0.4 g
S-K	H_2O, 1000 ml; $NaHCO_3$, 2.2 g; NaCl, 7.13 g; KCl, 0.4 g; $CaCl_2$, 0.2 g
S-L	H_2O, 1000 ml; $NaHCO_3$, 2.2 g; NaCl, 7.11 g; KCl, 0.4 g; $CaCl_2$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g

* Modified Earle's BSS.

S-H (Matsumura *et al.*, 1972). The ionic strength of each solution except for S-H was 0.15–0.16. The pH of S-A, S-B, S-C, S-D and S-E was 4.8–5.0, while that of S-F, S-G, S-H, S-I, S-J, S-K and S-L was 7.6–7.8.

The test apparatus was placed in an incubator with a gas phase of 5% CO_2 and 95% air at 37°C for an appropriate incubation period. At the end of the incubation period, the number of larvae remaining in the inner liquid and of those which passed into the outer liquid were counted under a dissecting microscope. Invasion rate (IR) and passage rate (PR) were calculated from the following formulas:

$$IR(\%) = 100 \times (TL-RL)/TL$$

$$PR(\%) = 100 \times ML/(TL-RL)$$

In these formulas, TL implied the initial number of larvae applied; RL, number of remaining larvae in the inner liquid; and ML, number of larvae passed into the outer liquid. IR represents the probability of the larvae which invaded the tissues, while PR represents the probability of the larvae which migrated to the outer liquid among the larvae which invaded the tissue.

Results

The time courses of IR and PR of the larvae were investigated using the rat duodenal wall in the following conditions: The inner liquid was distilled water and the outer liquid was NCTC-50HS (EXP. 1), and both the inner and outer liquids were distilled water (EXP. 2). As shown in Table 2, PR in EXP. 1 increased with the increase of incubation period, but PR in EXP. 2 was 0% at any incubation periods.

In the next experiment, after 2-hr incuba-

Table 2 Time course of invasion rates (IR) and passage rates (PR) of *A. cantonensis* third-stage larvae using distilled water as the inner liquid and NCTC-50HS or distilled water as the outer liquid

Experiment	Outer liquid	IR(%) & PR(%)	Incubation period (hr)				
			0.5	1.0	2.0	3.0	5.0
EXP. 1	NCTC-50HS*	IR	89.0±7.1	98.0±0.0	96.0±2.8	94.0± 0.0	91.0± 4.2
		PR	16.5±9.8	38.8±5.8	73.0±5.1	77.7±10.5	84.9±11.7
EXP. 2	Distilled water	IR	78.0±0.0	N.D.	71.0±1.4	N.D.	56.0± 0.0
		PR	0	N.D.	0	N.D.	0

Values are means ± standard deviations of two replicates using rat duodenal wall.
* NCTC 109 with 50% horse serum.
N.D.: Not done.

tion under the condition of EXP. 2, the outer liquid was replaced with NCTC-50HS for another 3 hr. As a result, IR was 85.0 ± 15.6 and PR was 70.6 ± 0.4 which were similar to those in EXP. 1 (Table 2). This study revealed that NCTC-50 HS was involved in the passage of the larvae into the outer liquid.

For the purpose of investigating what constituent(s) of NCTC-50HS is related to the passage of the larvae, IR and PR of the larvae were calculated using following outer liquids; NCTC 109, HS (these are constituents of NCTC-50HS) and S-G which is a partial modification of Earle's BSS, the BSS of NCTC 109. As shown in Table 3, there were no marked differences in IR and PR among the outer liquid containing NCTC-50HS, NCTC 109, HS and S-G. Accordingly, it was suggested that S-G, having a simple composition, is suitable for studying conditions relating to the passage phenomenon.

The effect of the constituents of S-G on PR was further studied. A series of outer liquids was prepared by adding each constituent of S-G one by one (Table 1, S-A–S-F). As shown in Table 4, when S-A, S-B, S-C, S-D or S-E was used as the outer liquid, IR ranged between 59–88% at 5 hr incubation. On the other hand, PR showed low value ranging between 11–22%. When S-F with $NaHCO_3$ was employed as the outer liquid, IR and PR showed much higher values as 91% and 90%, respectively.

The pH of S-A, S-B, S-C, S-D and S-E ranged from 4.8 to 5.0, while that of S-F was 7.6–7.8. Consequently, the weakly alkaline pH of the outer liquid was considered favorable for the passage of the larvae.

The other series of outer liquids was prepared by adding each constituent of S-F one by

Table 3 Invasion rates (IR) and passage rates (PR) of *A. cantonensis* third-stage larvae after 5-hr incubation by using distilled water as the inner liquid and various outer liquids

Outer liquid	NCTC-50HS*	NCTC 109	HS†	S-G‡
IR (%)	95.5 ± 2.1	89.0 ± 4.2	82.0 ± 17.0	92.0 ± 5.7
PR (%)	74.9 ± 2.4	77.1 ± 17.0	77.2 ± 8.2	79.1 ± 9.0

Values are means ± standard deviations. See footnote in Table 2.
* NCTC 109 with 50% horse serum.
† Horse serum.
‡ Modified Earle's BSS (see Table 1).

Table 4 Invasion rates (IR) and passage rates (PR) of *A. cantonensis* third-stage larvae after 5-hr incubation by using distilled water as the inner liquid and various outer liquids

Outer liquid	S-A	S-B	S-C	S-D	S-E	S-F
IR (%)	68.0±19.8	88.0±8.5	60.0±33.9	59.0±32.5	81.0± 4.2	91.0±4.2
PR(%)	10.5±11.4	11.8±7.6	21.9±23.0	17.2± 7.1	15.6±18.4	90.1±2.1

Values are means ± standard deviations. See footnote in Table 2.

Table 5 Invasion rates (IR) and passage rates (PR) of *A. cantonensis* third-stage larvae after 5-hr incubation by using distilled water as the inner liquid and various outer liquids

Outer liquid	S-H	S-I	S-J	S-K	S-L	S-F
IR (%)	72.0± 2.8	68.0± 5.7	86.0±0.0	80.0± 0.0	67.0± 1.4	72.0±17.0
PR(%)	31.5±20.4	40.7±13.2	52.3±8.2	70.0±10.6	86.8±14.5	90.5±10.1

Values are means ± standard deviations. See footnote in Table 2.

Table 6 Invasion rates (IR) and passage rates (PR) of *A. cantonensis* third-stage larvae after 5-hr incubation using various rat tissues

Rat tissue	Stomach wall	Rectal wall	Intact skin	Abraded skin
IR (%)	95.0 ± 1.4	89.0 ± 4.2	60.0 ± 5.7	74.0 ± 17.0
PR (%)	75.8 ± 4.1	71.7 ± 9.3	0	50.4 ± 43.8

Values are means ± standard deviations of two replicates using distilled water as the inner liquid and S-F (see Table 1) as the outer liquid.

one to S-H (H_2O, 1000 ml; $NaHCO_3$, 2.2 g) while the osmotic pressure, ionic strength and pH were adjusted to the same as those of S-F (Table 1, S-H–S-L). As shown in Table 5, in S-H, IR was 72% but PR showed a low value as 32%. With the increase in the sorts of added constituents, PR increased as shown in the values of 87% for S-L and 91% for S-F. Summarizing the results, it was clearly shown that the frequent passage of the larvae into the outer liquid occurred when (1) the outer liquid is weakly alkaline, (2) the outer liquid has the same osmotic pressure and ionic strength as physiological salt solution, and (3) the outer liquid contains KCl, $CaCl_2$, $MgSO_4$ and NaH_2PO_4. Since PR in case of S-F as the outer liquid was higher than that in case of S-G, it is clear that glucose is not necessary for the passage of the larvae into the outer liquid.

The possible invasion of the larvae to the stomach, rectal walls and skin was investigated by using S-F as the outer liquid. As shown in Table 6, IR and PR after 5-hr incubation for the stomach and rectal walls were as high as those of the duodenal wall. In addition, IR was 74% for abraded skin and 60% for intact skin, while PR was 50% for abraded skin, but 0% for intact skin.

Discussion

The study showed that the larvae did not pass into the outer liquid, although they invaded to the rat duodenal wall when the inner and outer liquids were distilled water (Table 2). But, in the case that the outer

liquid was NCTC-50HS, NCTC 109, HS and modified BSS of NCTC 109 (S-G), the larvae passed into the outer liquid at a high probability (Table 3). Consequently, in order to clarify the favorable conditions for the passage of the larvae into the outer liquid after invading the tissue, studies were carried out by employing distilled water as the inner liquid and S-G as the outer liquid because S-G had a simple composition. As a result, the pH of the outer liquid was the most effective factor on PR under the constant osmotic pressure and ionic strength (Table 4). Besides, the addition of $CaCl_2$ and $MgSO_4$ are especially effective on the enhancement of PR (Table 5). Therefore, it is surmised that the reason the larvae passed into the outer liquid is that the larvae in the tissue respond to the osmotic pressure, ionic strength, pH, KCl, $CaCl_2$, $MgSO_4$ and NaH_2PO_4 for active passage.

Jindrák (1968) indicated in rats that one hour after the oral infection, most third-stage larvae of *A. cantonensis* were in the walls of the stomach and intestine. Our *in vitro* study also indicated that the third-stage larvae quickly invaded to the walls of the stomach and intestine, and passed into the outer liquid (Tables 5, 6). It is supposed that as the larvae passed into the outer liquid in this experiment, a part of the larvae ingested may move from the walls of hte stomach and intestine into the abdominal cavity and again invade to the blood vessels *in vivo*. Ubelaker *et al.* (1981) also indicated no significant differnces in the recovery of adult worms of *Angiostrongylus costaricensis* from *Sigmodon hispidus* between oral and intraperitoneal routes.

Alicata and Brown (1962) experimentally confirmed that the larvae of *A. cantonensis* could penetrate rat skin only when the surface of the skin had been abraded. Ubelaker *et al.* (1981) also established cutaneous penetration of third-stage larvae of *A. costaricensis* in *S. hispidus* only when the skin had been abraded. From these 2 reports, it is clear that infection could be established only in the abraded skin. In the present study, the passage of the larvae into the outer liquid was seen only when the skin was abraded. On the basis of these facts, it may be stated that the percutaneous infection of larvae occurs without fail only when the skin was abraded.

Summary

Studies were carried out to find out the factors which stimulate the passage of the third-stage larvae of *Angiostrongylus cantonensis* from invaded rat tissues to the outer liquid. It was found that the high invasion rate and passage rate were obtained when S-F (H_2O, 1000 ml; NaCl, 7.06 g; KCl, 0.4 g; $CaCl_2$, 0.2 g; $MgSO_4 \cdot 7\ H_2O$, 0.1 g; $NaH_2PO_4 \cdot 2\ H_2O$, 0.14 g; $NaHCO_3$, 2.2 g) was employed as the outer liquid, and that weakly-alkaline pH (7.6–7.8), physiological osmotic pressure, ionic strength (0.15–0.16), $CaCl_2$ and $MgSO_4$ in the outer liquid were necessary for the passage of the larvae into the outer liquid. The state of larval invasion to each rat tissue was studied by employing distilled water as the inner liquid and S-F as the outer liquid. The larvae invaded to and passed through the stomach, duodenal and rectal walls at a high probability. Besides, the larvae invaded to and passed through the abraded abdominal skin, but did not pass through the intact skin.

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In vitro における広東住血線虫3期幼虫の穿通について
II. ラットの胃，十二指腸，直腸壁及び皮膚への侵入とこれら組織からの移行

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ラット十二指腸壁に侵入した広東住血線虫3期幼虫の組織外への移行について *in vitro* で検討した．実験方法は以下のとおりである（荒木ら，1986年）．即ち，1 mℓのディスポーザブル注射筒の先端を切断し，新鮮なラットの十二指腸壁で覆う．*Biomphalaria glabrata* から採集した3期幼虫50匹を含む1 mℓの水を，内液としてこの注射筒内に入れ，先端に固定した組織面がガラス容器中の外液に接触するように垂直に固定する．37℃，湿度飽和，5％炭酸ガス培養器に入れ，5時間後，内・外液の幼虫数を実体顕微鏡下で調べた．外液に S-F（H_2O, 1000mℓ; NaCl, 7.06g; KCl, 0.4g; $CaCl_2$, 0.2g; $MgSO_4 \cdot 7H_2O$, 0.1g; $NaH_2PO_4 \cdot 2H_2O$, 0.14g; $NaHCO_3$, 2.2g）を用いた時，最も高い幼虫の組織への侵入率及び組織からの外液への移行率がみられた．幼虫の外液への移行に，外液が弱アルカリ性（pH7.6～7.8）であること，生理的浸透圧，イオン強度が0.15～0.16であること，及びS-F の各成分，特に $CaCl_2$, $MgSO_4$ が必要であることが明らかになった．外液を S-F にした場合のラットの各組織に対する侵入率は，胃壁（95％）；十二指腸壁（82％）；直腸壁（89％）；傷を付けた腹部皮膚（74％）及び無傷の皮膚（60％）であった．各組織に侵入した幼虫の外液への移行率は，胃壁（76％）；十二指腸壁（91％）；直腸壁（72％）；傷を付けた皮膚（50％）及び無傷の皮膚（0％）であった．