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Citation	Japanese Journal of Veterinary Research, 49(4), 273-285
Issue Date	2002-02-28
DOI	<a href="https://doi.org/10.14943/jjvr.49.4.273">https://doi.org/10.14943/jjvr.49.4.273</a>
Doc URL	<a href="https://hdl.handle.net/2115/2925">https://hdl.handle.net/2115/2925</a>
Type	departmental bulletin paper
File Information	KJ00002400403.pdf



## *Taenia taeniaeformis* larval product induces gastric mucosal hyperplasia in SCID mice

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(Accepted for publication : December 21, 2001)

### Abstract

The effects of intraperitoneal implantation of *Taenia taeniaeformis* larvae and inoculation of *in vitro* larval products on gastric mucosa of SCID mice were investigated in this study. Mice surgically implanted with *T. taeniaeformis* larvae developed slight and moderate gastric hyperplasia. When *in vitro* cultured *T. taeniaeformis* larval excretory-secretory (TtLES) products containing 1 mg of protein were injected daily into mice, they caused gastropathy after 5-7 days. Mice injected daily with 0.5mg of TtLES products also showed slight gastric hyperplasia after day 14 and 28. The gastropathy was characterized by reduction of both parietal and zymogenic cell number and increased number of alcian blue-periodic acid Schiff (AB-PAS)-positive cells and by two-fold extension of proliferative zone of gastric units. Larval implantation demonstrated a more potent effect in inducing gastropathy than did *in vitro* larval culture products. Significant decrease in number of parietal cells with concomitant increase of proliferative zone and AB-PAS-positive cell number indicated their important roles in inducing the hyperplastic lesion. Similarities with other gastropathies indicated that there is a common fundamental regulatory mechanism involved, and that the host response may not be specific to parasites. Present study validated the induction of gastric mucosal hyperplasia by larval ES products of *T. taeniaeformis*. This proved the hypothesis of previous studies suggesting the role of larvae-derived products in inducing gastric mucosal hyperplasia in *T. taeniaeformis*-infected rats.

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Key words : *Taenia taeniaeformis*, SCID mice, gastric hyperplasia, excretory-secretory products

### Introduction

It has been reported that *Taenia taeniaeformis* larvae induce gastric mucosal hyperplasia in rats in the presence of physical separation between the liver, where parasites are located, and the stomach<sup>1,2,3,12,23</sup>. The inducing factor that mediated between larval hepatic infection and gastric mucosal hyperplasia is unclear. Larvae of *T. taeniaeformis* were reported to release larval excretory-secretory (ES) products *in vivo* and *in vitro*<sup>22</sup>. A number of reports provided strong evidence asserting the hypothesis that this larval ES product is the primary cause.

Substantial data supporting the above claim were the development of gastric mucosal hyperplasia in non-infected rats surgically joined by parabiosis to *T. taeniaeformis* infected rats<sup>4</sup>, and in recipient rats intraperitoneally implanted with larvae<sup>13</sup>. Observations in the growth of cultured stomach cells stimulated by larval *in vitro* culture products<sup>22</sup> and localization of larval products in epithelial cell cytoplasm of hyperplastic mucosa<sup>24</sup> further reinforced this hypothesis.

Recently, inoculation of *T. taeniaeformis* eggs or *in vitro*-hatched oncospheres into severe combined immune-deficient (SCID) mice resulted in gastric mucosal hyperplasia with similar lesions as in rats<sup>15</sup>. This qualified the SCID mice as suitable animal models for the study of this phenomenon. Hyperplastic gastropathy in SCID mice was described histologically as alcian blue-periodic acid-Schiff reaction (AB-PAS)-positive mucous cell hyperplasia. Loss of parietal cells and decrease of zymogenic cells was noted in severe conditions.

We conducted a series of experiments in

order to validate the hypothesis on the involvement of *T. taeniaeformis* larval excretory-secretory (TtLES) products in inducing gastric mucosal hyperplasia. First we determined the effect of surgical implantation of larvae intraperitoneally on gastric mucosa of SCID mice. Finding gastropathy in the first experiment, sequel studies were conducted to determine the role of *in vitro* cultured TtLES products by daily intraperitoneal inoculation into SCID mice.

### Materials and Methods

#### *T. taeniaeformis* larval implantation

Male Wistar rats, 5 weeks old, served as donor animals. Rats were orally inoculated with approximately 3,000 *T. taeniaeformis* eggs using a stomach tube. Donor rats were sacrificed at 9 weeks post inoculation (WPI) and the larvae were collected aseptically from hepatic cysts. Larvae were placed in Petri dishes and washed with physiologic saline containing penicillin (100 IU/ml) and streptomycin (100 µg/ml). To check viability, the larvae were incubated at 37°C for 5 min and only actively moving larvae were used for implantation. Fifteen male C.B-17-*scid/scid* (SCID) mice (5 weeks old) were used as recipient animals. Ten SCID mice were intraperitoneally implanted with 40 viable *T. taeniaeformis* larvae by abdominal surgical operation. A midline abdominal incision was made and the larvae were inserted using a modified funnel-shaped instrument and sutured immediately. Five control SCID mice were subjected to a sham operation. Necropsy was done following the death of mice or sacrificed when they became lethargic or comatose from 3 to 7 days post implantation (DPI). During sacrifice the implanted larvae were re-collected from the

abdominal cavities, counted and weighed. Control mice were also sacrificed at 7 DPI.

#### *T. taeniaeformis* larval *in vitro* culture and TtLES product injection

To determine the role of *in vitro* cultured larval products, another group of male Wistar rats (n=10) was orally infected with approximately 3,000 viable *T. taeniaeformis* eggs. These donor rats were sacrificed at 9-12 WPI and larvae were collected aseptically from hepatic cysts for *in vitro* culture. Larvae were washed five times by sedimentation in sterile Hank's Balanced Salt Solution (HBSS; Sigma, USA) and two times in sterile Medium 199 (Gibco BRL, USA). Both media contained penicillin (100 IU/ml) and streptomycin (100 µg/ml) (Penicillin-Streptomycin solution; Sigma, USA). Larvae were transferred to sterile flasks containing sterile Medium 199 at a ratio of 0.25 ml per larva. A total of 3,000 larvae were cultured in a CO<sub>2</sub> incubator at 37°C for 48hr. Supernatant fluid was then collected as a *T. taeniaeformis* larval excretory-secretory (TtLES) product and stored at -80°C. TtLES products were thawed and warmed to 37°C prior to intraperitoneal inoculation.

Male SCID mice (5 weeks old) were used for the TtLES products injection study. The first group of mice (n=5) was injected intraperitoneally, either with 2.5 or 5 ml of TtLES products per day, equivalent to 0.5 or 1 mg of protein, respectively. Mice that died or comatosed were necropsied immediately and the rest of the surviving mice were sacrificed at 7 days of injection (DOI). The second group included SCID mice (n=9) injected with 5ml of TtLES products (0.5 mg of protein) per day and control SCID mice (n=6) injected with an equal volume of Medium 199 per day. Three TtLES product injected-mice and 2 control mice were sacrificed on schedule at 7, 14 and 28 DOI.

Protein concentration of TtLES products was estimated according to microassay procedure of the Bio-Rad Protein Assay (Bio-Rad, UK). All animals in this experiment were obtained from a commercial source (Clea, Japan).

#### *Histopathology*

Upon sacrificing the SCID mice, the stomach was removed, weighed and opened at the greater curvature extending from the squamocolumnar junction to the gastroduodenal junction. Tissues were fixed using 10% neutral buffered formalin, dehydrated and embedded in paraffin. Sections were then cut approximately 5 µm thick and stained with hematoxylin and eosin (HE), and with AB-PAS. Counting of the total number of cells, AB-PAS-positive, parietal and zymogenic cells per gastric unit in the corpus region was made in 3 - 5 complete longitudinally-cut gastric units of 5 subserial sections at least approximately 25 µm apart.

Degrees of hyperplasia were classified into 3 categories according to histopathological observations. Gastric corpus mucosa showing less than a 50% increase in the number of cells per gastric unit with observable lesions concentrated at the pit region of the gastric glands were considered as having slight hyperplasia. Moderate hyperplasia was characterized by a greater than 50% increase in the number of cells per gastric unit with observable pathologic changes at the upper half of the gastric unit. Severe gastric hyperplasia was characterized by a 2 to 3 fold increase in the number of cells with observable pathologic changes in more than half or in the whole length of the gastric unit. Moreover, it was characterized by the presence of cystic cavities dilating the crypts in gastric mucosa. Proliferating cellular nuclear antigen (PCNA) immunostaining was conducted to determine

Table 1. Gastric hyperplasia in control (C) and recipient SCID mice (R)

Recipient SCID mice	Re-collected larvae [total weight (g)/n]	Days post-implantation	Stomach weight (g)	Mean total no. of cells / gastric unit	Gastric hyperplasia
C-1	0/0	7	0.42	103.8	-
C-2	0/0	7	0.4	102.2	-
C-3	0/0	7	0.32	100	-
C-4	0/0	7	0.23	86.8	-
C-5	0/0	7	0.3	91	-
R-1	ND	4*	ND	ND	ND
R-2	2.66/38	7	1.27	196.8	++
R-3	2.35/39	7	0.57	190.6	++
R-4	1.93/39	7	0.48	148	+
R-5	1.88/39	7	0.39	141	+
R-6	ND	3*	ND	ND	ND
R-7	2.03/38	5*	0.61	131.4	+
R-8	2.09/38	5*	0.42	137.4	+
R-9	2.17/40	6	0.42	145.2	+
R-10	2.33/39	6	0.75	184.6	++

\* Died

ND=not determined ; + = slight ; ++ = moderate

changes in the proliferative zone of gastric mucosa. The staining procedure was followed as recommended by the manufacturer of the PCNA staining kit (Zymed, USA), except for minor modifications mentioned below. Paraffin sections were mounted on a slide coated with poly-L-lysine (Sigma, USA). Endogenous peroxidase activity was blocked with peroxidase quenching solution (one part of 30% H<sub>2</sub>O<sub>2</sub> to 9 parts of methanol) for 20 min. Heat Induced Epitope Retrieval (HEIR) was applied to enhance specific staining by submerging the tissue sections in 0.01 M citrate buffer solution. The solution with sections was heated until boiling, kept boiling for 10 min and then allowed to cool down. The blocking step was incubated for 20 min. Biotinylated mouse anti-PCNA was diluted with 1% BSA in 10 mM phosphate buffered saline (PBS) at 1:10 and incubated with tissues for 30 min. PCNA stained slides were used to determine an in-

crease of proliferative zone by measuring the length between the highest and lowest PCNA-positive cells in a complete gastric unit. Proliferative zone was expressed as percentage of PCNA-occupied length over the mean length of gastric units. Measurement was made using an Olympus video micrometer (Model VM-30).

#### Statistics

Significant differences between groups were analyzed using the Student's *t*-test for unpaired data. The correlation coefficient (*r*) was determined using Pearson's correlation and *P* value by Kendall test. *P* values of < 0.05 were considered statistically significant.

#### Results

##### Larval implantation

Table 1 showed the weights of re-collected larvae and stomach, mean total number of

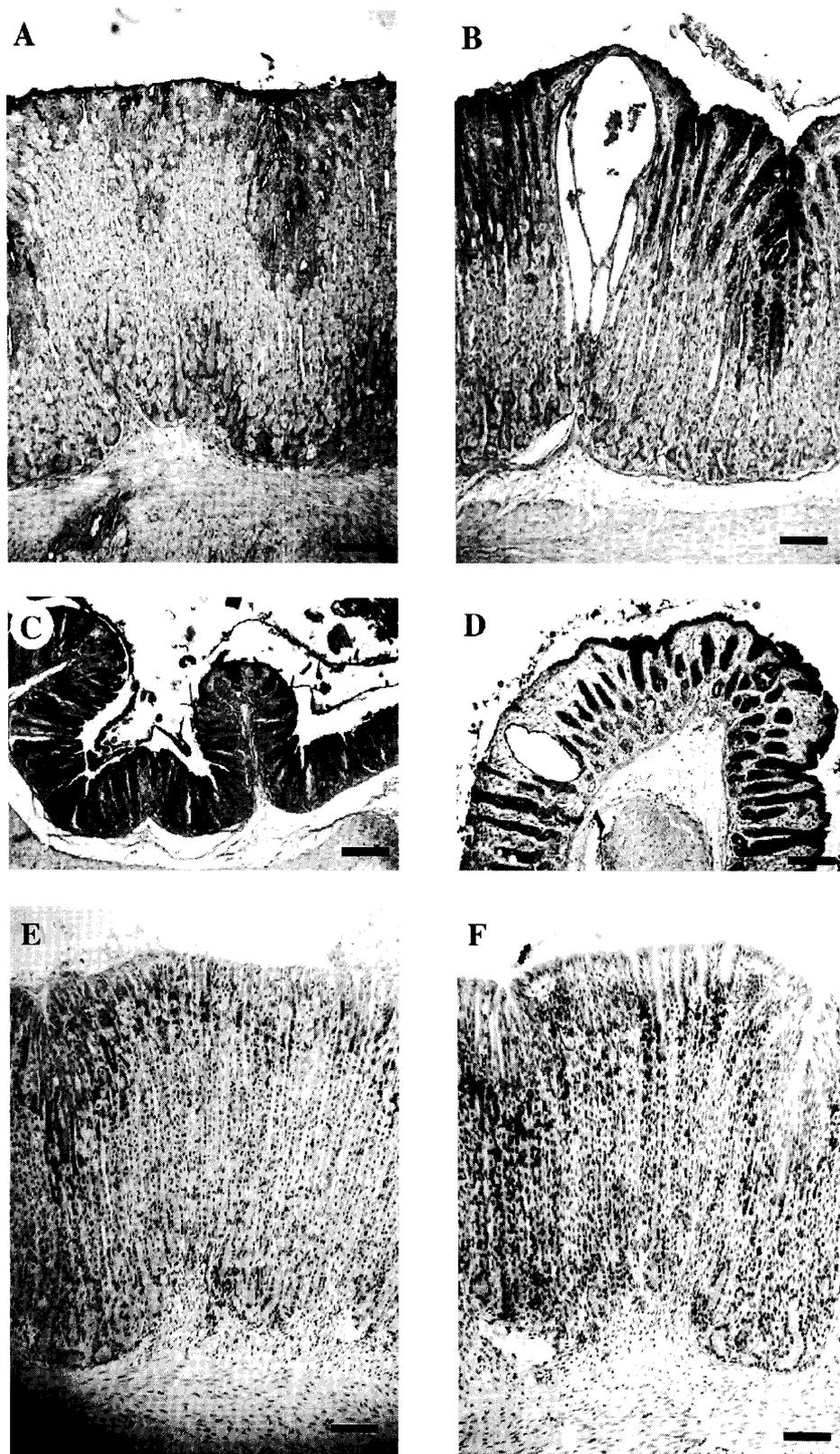


Fig. 1. A. Corpus gastric mucosa of control mice. B. Moderate gastric mucosal hyperplasia in surgically implanted recipient mice with 40 *T. taeniaeformis* larvae. Reduction of parietal and zymogenic cells is observed. C. Antral gastric mucosa of control mice. D. Antrum mucosal hyperplasia in recipient mice. E–F. Proliferating cell nuclear antigen (PCNA) immunostaining in mucosa of control mice injected with 5 ml of Medium 199 (E), and TtLES products (F) after 28 days of injection. Bars : 100 $\mu$ m.

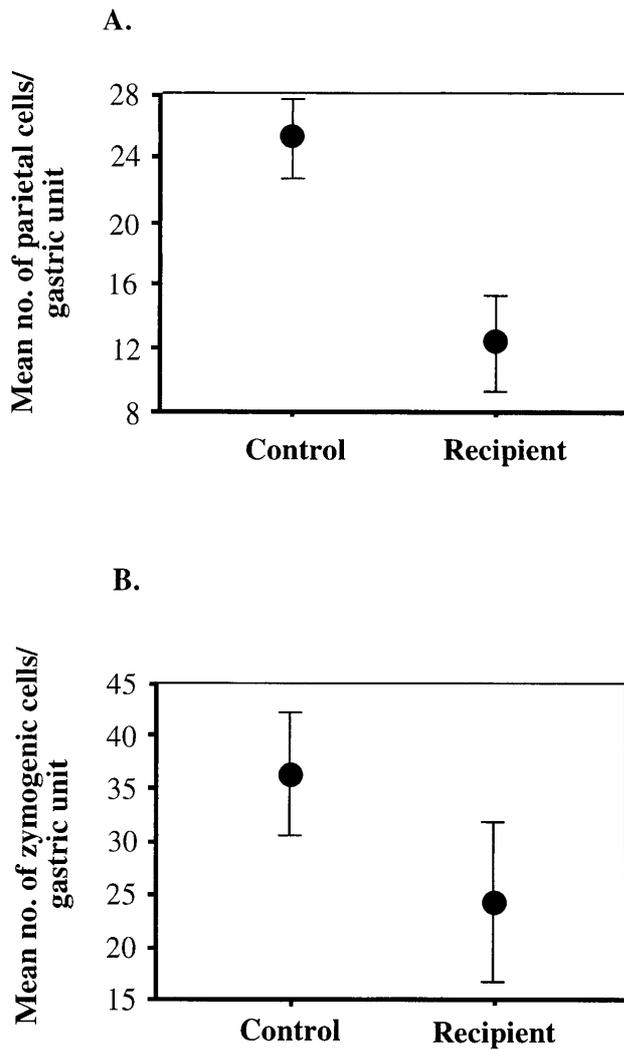


Fig. 2. A. Mean number of parietal cells per gastric unit in control and recipient mice surgically implanted with 40 *T. taeniaeformis* larvae at 5-7 DPI. The difference is statistically significant ( $P < 0.0001$ ). B. Mean number of zymogenic cells per gastric unit in control and recipient mice surgically implanted with 40 *T. taeniaeformis* larvae at 5-7 DPI with moderate hyperplasia. The difference is statistically significant ( $P = 0.0003$ ). Bar = SD

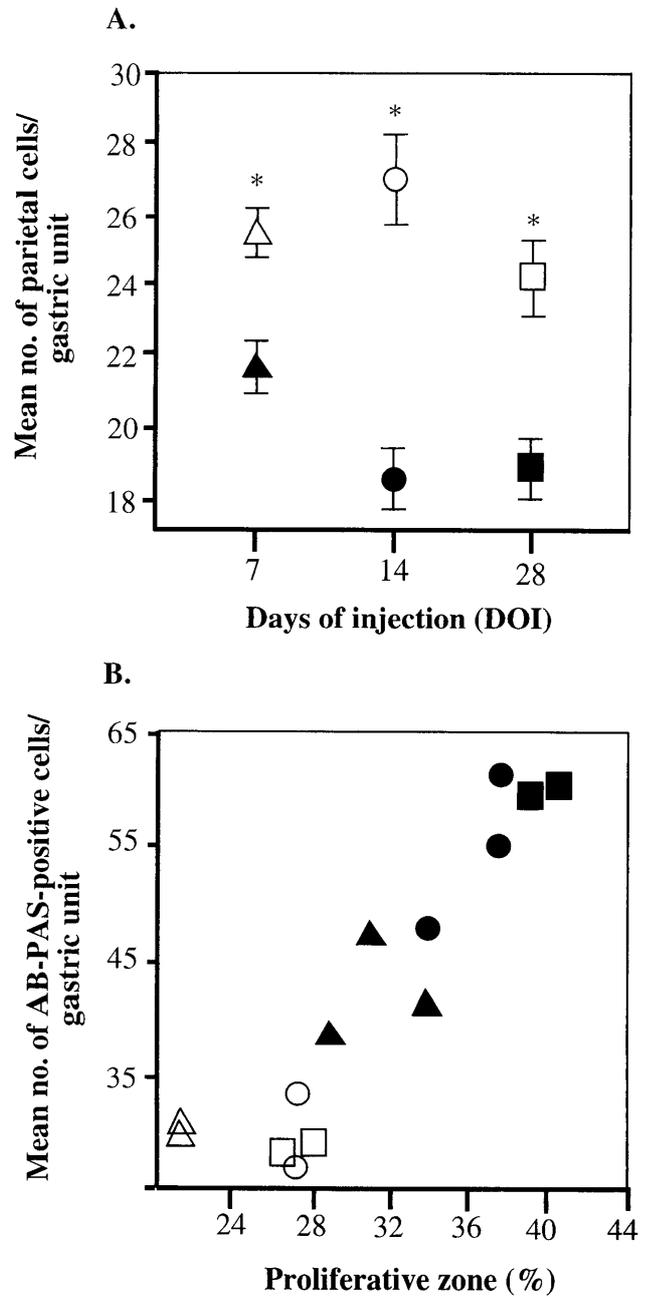


Fig. 3. A. Mean number of parietal cells per gastric unit in mice injected daily with Medium 199 and TtLES products. \* $P < 0.05$ ; Bar = SD. B. Graph showing correlation of mean number of AB-PAS-positive cells/gastric unit and proliferative zone length (%), 7 DOI (triangle), 14 DOI (circle), 28 DOI (rectangle), Medium 199 (open), TtLES products (closed).  $P < 0.05$ ;  $r = 0.91$ .

cells per gastric unit and gastric hyperplasia in control and recipient SCID mice. During necropsy, larvae were re-collected and appeared active when incubated at 37°C in physiologic saline. Weights of re-collected larvae did not significantly increase as compared with those prior to implantation. Body and stomach weights between recipient and control SCID mice did not differ significantly, although the recipient mice appeared to be emaciated.

Corpus and antrum gastric mucosa from control SCID mice was apparently normal (Fig. 1 A, 1C). Spherical to pyramidal shaped parietal cells with eccentric nuclei and pale eosinophilic spaces in cytoplasm representing secretory canaliculi were abundant throughout the gastric units. Zymogenic cells stained with hematoxylin were located in the base region, and AB-PAS-positive mucous cells were located in the surface pit region. Few PAS-positive mucous cells were found in the neck region, and immature cells with strongly blue nuclei were abundant in the isthmus region immediately below the pit region.

Table 1 revealed 8 of 10 *T. taeniaeformis* larvae-recipient SCID mice with slight and moderate (Fig. 1 B) gastric mucous cell hyperplasia at 5-7 DPI. The length of gastric units was comparable between groups, although an

increase of the AB-PAS-positive cell region was noticeably apparent. Consequently, an increase of the gastric unit region occupied by AB-PAS-positive cells was noted, causing a decreased length of the neck and base regions. Numerous cystic cavities were found at the lamina propria of the isthmus and pit regions of the corpus of recipient mice. Lumens of gastric units in recipient mice were wider than in control mice and edematous thickening of the lamina propria at the pit region was observed. Antral mucosa also showed hyperplastic lesions and increased length of mucous glands in recipient mice (Fig. 1 D).

The counting of major cells types in gastric units revealed a significant reduction of the mean number of parietal cells in recipient mice, as compared with control mice ( $P < 0.0001$ , Fig. 2 A). The mean number of zymogenic cells per gastric unit in recipient mice with moderate hyperplasia was significantly decreased compared to the mucosa in control mice ( $P = 0.0003$ , Fig. 2 B).

#### *TtLES products injection*

Two of the 3 mice injected with 5 ml TtLES products (1.0 mg protein / day) in Group 1 (Table 2) showed a slight hyperplasia of the gastric mucosa. The lesions were similar to the gastric mucosa of recipient mice

Table 2 . Gastric hyperplasia in TtLES products injected SCID mice (Group 1 )

Injected SCID mice	Vol. of TtLES product/day (protein content, mg/ml)	Duration of injection	Stomach weight (g)	Mean total no. of cells/gastric unit	Gastric hyperplasia	Mean length of proliferative zone
A1	2.5ml (0.5)	5*	0.20	72.3	—	24.4
A2	2.5ml (0.5)	7	0.35	108.4	—	27.7
B1	5ml (1.0)	3*	0.25	72.7	—	23.2
B2	5ml (1.0)	5 <sup>c</sup>	0.43	135.7	+	39.4
B3	5ml (1.0)	7	0.60	148.4	+	42.3

\* Died

<sup>c</sup> Comatosed

Table 3 . Gastric hyperplasia in Group 2 SCID mice injected with 5ml TtLES products (0.5mg protein) or Medium 199 (control) per day

Duration of injection (days)	Treatment grouping	Experimental SCID mice	Stomach weight (g)	Mean total no. of cells/gastric unit	Gastric hyperplasia	Mean length of proliferative zone	
7	control	1	0.19	88.0	—	25.2	
		2	0.20	93.4	—	25.3	
	TtLES	1	0.21	94.0	—	27.9	
		2	0.35	103.4	—	30.7	
		3	0.25	92.7	—	28.7	
	14	control	1	0.27	102.2	—	27.1
2			0.25	100.0	—	28.7	
TtLES		1	0.27	103.8	—	33.8	
		2	0.30	104.8	—	37.5	
		3	0.35	138.8	+	40.3	
28		control	1	0.30	102.3	—	28.3
	2		0.32	86.8	—	25.5	
	TtLES	1	0.35	140.2	+	39.6	
		2	0.42	143.0	+	39.2	
			3*	ND	ND	ND	ND

\* Died

ND=not done

implanted with the larvae. The widening of spaces between gastric units and cystic cavities formation at the pit region were found, whereas those were absent in the mucosa of control mice. Parietal cell number per gastric unit was reduced in the cases with slight mucosal hyperplasia, while the zymogenic cell was not affected. PCNA immunostaining revealed an increase of proliferative zone in mice injected with 5ml TtLES products (Table 2). Labeled cells were observed abundantly at the isthmus, neck, and upper base region. In the gastric mucosa of mice injected with 2.5 ml TtLES products, PCNA labeled cells were located mostly at the isthmus region.

Table 3 showed Group 2 mice injected with TtLES products equivalent to 0.5 mg protein / day with a slight effect on the gastric mucosal integrity at 7 DOI. Injected mice showed a slight increase in the number of AB-

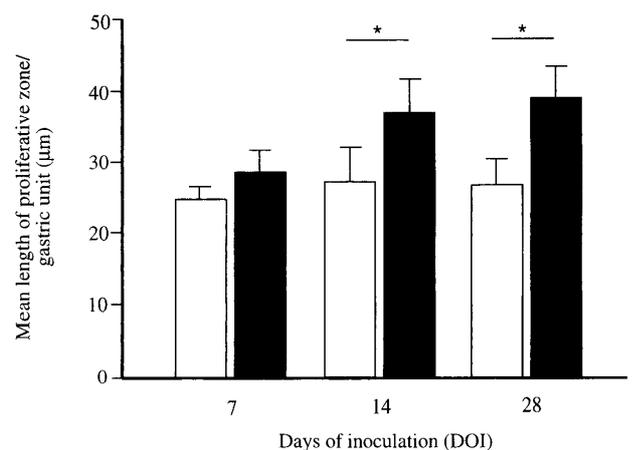


Fig. 4 . Mean lengths of proliferative zones in Medium 199 (open bar) and TtLES products (close bar) inoculated SCID mice at different days of intraperitoneal injection. \* $P < 0.05$  ; Bar = SD.

PAS-positive cells. The number of parietal cells per gastric unit was significantly different between control and TtLES product injected mice ( $P=0.0014$ ; Fig. 3 A).

At 14 DOI, slight hyperplastic changes was evident in 1 of 3 mice injected with TtLES products. Significant reduction in number of parietal cells were noted ( $P<0.0001$ ; Fig. 3 A). PCNA labeled cells in mucosa of control mice were found mostly in the isthmus region (Fig. 1 E). Labeled cells in hyperplastic mucosa were located abundantly in the middle third of gastric units occupying the isthmus and neck regions (Fig. 1 F). Figure 4 showed the increase in proliferative zone length in gastric units of TtLES inoculated mice compared with control mice. Observations in serial sections stained with AB-PAS revealed that the isthmus region of slightly hyperplastic mucosa was located deeper than in control mucosa, and that the length of the pit covered by AB-PAS-positive cells was significantly increased.

On day 28 of TtLES product injection, two of three mice demonstrated slight mucosal hyperplasia. Mean numbers of parietal cells per gastric unit were significantly reduced in TtLES product-injected mice ( $P=0.0008$ ; Fig. 3A). Increase in the total number of AB-PAS-positive cells correlated with an increase of proliferative zone (Fig. 3B, 4).

### Discussion

Generally, parasites produce pathologic disturbances at their sites of predilection. In gastrointestinal parasitism, however, parasite free-regions might show histological changes as compensatory mechanisms to affected sites<sup>10</sup>. Gastropathy during *T. taeniaeformis* larval infection in the liver of rats demonstrated an atypical relationship in parasitism. An unusual host-parasite relationship was also reported in Spirometrid tapeworms

where plerocercoids synthesized and released growth hormone-like factors, causing accelerated body growth of their host<sup>21</sup>.

A number of studies have strongly supported the hypothesis that the larval ES product was the chief inducing factor for gastric mucosal hyperplasia in *T. taeniaeformis*-infected rats. Nevertheless, in all these reports, no concrete evidence yet could be presented that the larval ES product alone could indeed evoke such pathologic lesions. Blaise and Williams<sup>2)</sup> attempted to prove this by serial inoculation of saline-soluble extracts of larvae and *in vitro* cultured larval ES products into the peritoneal cavity of rats for 3 weeks, but failed to observe any histopathological changes. Based on a recently established model of *T. taeniaeformis*-induced gastric hyperplasia using immunodeficient mice<sup>15)</sup>, we undertook several experiments that could possibly pinpoint larvae-derived products as the sole inducing factor.

Recipient SCID mice implanted with larvae showed slight and moderate gastric hyperplasia, whereas those injected with TtLES products showed slight mucosal hyperplasia and various stages in the early phase of hyperplastic gastropathy. The milder effect of *in vitro* TtLES product inoculation than larval implantation might have indicated different characteristics between the products released *in vivo* and *in vitro* culture. Products excreted or secreted *in vivo* might contain more molecules that were responsible for inducing gastropathy, thus becoming more potent than metabolic products produced by larvae *in vitro*<sup>22)</sup>. Moreover, comparable lesions observed in hyperplastic lesions of mice injected with TtLES products (0.5 mg/day) at 14 and 28 DOI might indicate the dose as insufficient to induce a severe pathologic effect and the host response may probably have compensated for the milder effect.

Hammerberg and Williams<sup>7)</sup> reported that *T. taeniaeformis* larval *in vitro* products contained polysulfated glycosaminoglycan. These molecules have been detected on surfaces of various infectious organisms. Glycosaminoglycans were suggested as playing a role in the healing process of acetic acid ulcer in rat stomach<sup>29)</sup>. Parasitic glycosaminoglycans were suggested to stimulate the growth of gastric epithelial cells<sup>22)</sup>.

Previous studies suggested ES product to have direct inducing action on the stem cell pool of gastric units<sup>22)</sup>. An increase of proliferative zone as revealed by PCNA immunostaining was accompanied by an increase of AB-PAS-positive cells in this study. PCNA is a protein found in nuclei of cycling cells that play a role in initiation of cell proliferation by mediating DNA polymerase<sup>30)</sup>. By indirect immunoperoxidase staining, Rikihisa et al.<sup>24)</sup> showed ES product localization in supranuclear cytoplasm of epithelial cells lining gastric units. *In vitro* stimulation by *in vitro* culture products on rat and dog stomach cells suggested a direct action of the products on epithelial cell growth<sup>22)</sup>.

Indirect action of TtLES products in inducing hyperplastic lesions could be possible due to significant reduction in number of parietal cells that concomitantly occurred with increases in proliferative zone length and AB-PAS-positive cells. Parietal cell loss was reported to be associated with the development of characteristic changes to mucous cell hyperplasia<sup>26,28)</sup>. The cause of parietal cell loss in this study is unknown. Accordingly, parietal cell inhibition might be caused by blocking the proton pump or by interfering with its complex physiological regulation<sup>25)</sup>. Larvae derived substances from *Ostertagia circumcincta* were suggested to have caused parietal cell loss after transplantation of adult and larval parasites<sup>26,27)</sup>. Indirect suppression of

parietal cells by inhibiting the secretory activity of enterochromaffine-like (ECL) cells was induced by *Haemonchus contortus* ES products<sup>8)</sup>. Analogous to these, acute parietal cell loss was induced in rats dosed with DMP 777, a cell-permeant inhibitor of neutrophil elastase<sup>6)</sup>. During acute treatment with DMP 777, loss of parietal cells was accompanied by a rapid increase in the number of PAS-positive mucous cells and by a marked increase in bromodeoxyuridine (BrdU)-labeled S-phase cells. Goldenring et al.<sup>6)</sup> further claimed that after 2 days of treatment, increased BrdU staining was observed in cells at the isthmus region and in mucosal cells located deeply below the gastric units. Parietal cell loss had been implicated as playing a vital role in several gastric mucous cell hyperplastic conditions. Diminution of parietal cells induced gastric mucous cell hyperplasia by the compensatory mechanism of stem cells, to produce parietal precursor cells<sup>17)</sup>. Simpson<sup>28)</sup> suggested that parietal cell loss was the key event that led to mucous cell hyperplasia in abomasal nematodosis.

Simultaneous action of direct and indirect mechanisms in inducing stem cell proliferation is a conceivable possibility that could also be suggested. Presuming that the TtLES product did contain cytotoxic molecules, a probable concomitant effect on parietal cell death and injury to gastric mucosa may occur. Mucosal proliferation was observed following aspirin-induced injury in rat stomachs<sup>20)</sup>. Acute injury from hypertonic saline-induced denudation of gastric mucosa elicited a rapid up-regulation of pathways leading to production of pit mucous cells<sup>19)</sup>. Responses utilizing these cells seemed logical since they have a life span of only approximately 4 days<sup>11)</sup>. This assumption agreed with the occurrence of mucosal hyperplasia in antrum regardless the involvement of parietal cells that are devoid in

this region<sup>16)</sup>.

Similarities between forms of gastric mucosal hyperplasia have been observed in several gastric diseases of mice<sup>5)</sup> to infections in human beings<sup>14)</sup>, indicating that common fundamental regulatory mechanisms occurred in all of these syndromes. Very different etiologies, such as genetically engineered ablation of parietal cells<sup>18)</sup> to DMP 777 drug dosing<sup>6)</sup>, produced similar cellular changes, suggesting that this host response was not specific to parasites. Further clarifications on components of TtLES products could help us understand the pathogenesis of this atypical pathologic phenomenon, as well as the mechanism by which gastric mucosal cell proliferation and growth is initiated.

Based on our results, gastric hyperplasia could be induced in SCID mice by the intraperitoneal surgical implantation of *T. taeniaeformis* larvae, and by the injection of TtLES *in vitro* culture products. Although larval ES products released *in vivo* were more potent in inducing gastropathy, *in vitro* culture products alone were proven to stimulate an increase in stem cell proliferation and in the number of AB-PAS-positive cells that developed into gastric mucosal hyperplasia.

#### Acknowledgment

We would like to acknowledge the valuable help of Dr. Naoko Kato during the course of our experiment. This study was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (No. 09041137).

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