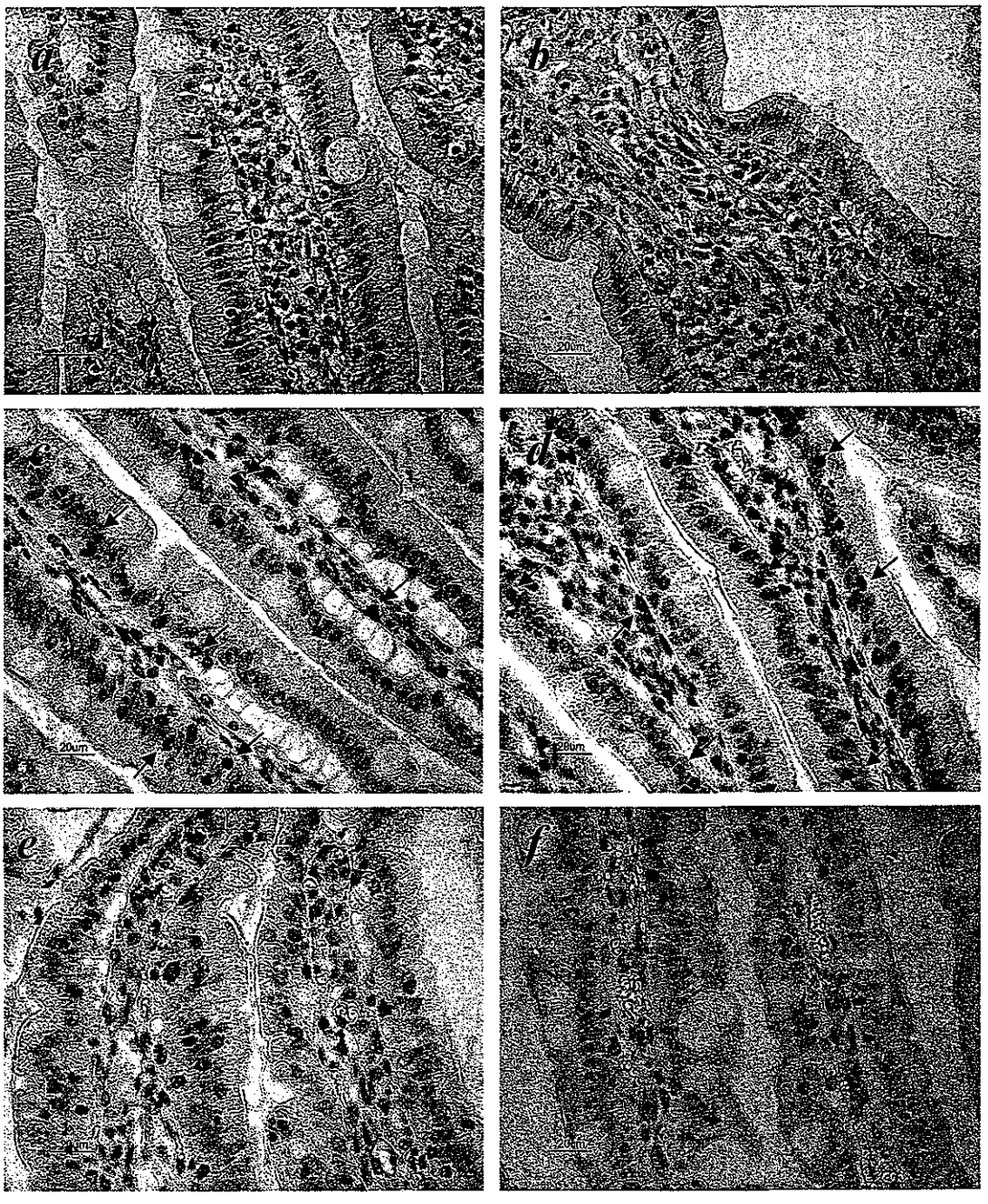

FIGURE 3. Immunohistochemical localization of granzyme B in the jejunum of rnu/+ (**a–c, e**) and rnu/rnu rats (**d, f**) performed on formalin-fixed paraffin-embedded tissue sections. (**a–b**) Negative control sections of rnu/+ rats, uninfected and 10 days after infection, respectively. Sections were incubated with normal IgG instead of granzyme B–specific IgG. (**c–d**) Granzyme B localization in uninfected rnu/+ and rnu/rnu rats, respectively. (**e–f**) Granzyme B localization in rnu/+ and rnu/rnu rats 10 days after *Nippostrongylus brasiliensis* infection, respectively. Granzyme B immunoreactivity (indicated by arrows in **c** and **d** but not indicated in **e** and **f**) shows granular staining localized in the perinuclear cytoplasmic region of small-sized mononuclear cells. Nuclei are lightly counterstained with hematoxylin.

Because the granzyme B expression level was increased in euthymic rats in association with the progression of villus atrophy, the granzyme B^+ cell distribution in the jejunal mucosa was examined by immunohistochemistry. Granzyme B immunoreactivity showed granular staining mainly in the perinuclear cytoplasmic region and was confined mostly to mononuclear cells (Fig. 3). The majority of granzyme B^+ cells were observed in the villus epithelium of euthymic as well as athymic rats, although some granzyme B^+ cells were also found in the propria mucosa. Unexpectedly, the granzyme B^+ cell numbers in the epithelium and propria mucosa of uninfected athymic rats were significantly more than those in euthymic rats (Table II). However, intraepithelial and propria mucosal granzyme B^+ cells showed a remarkable increase in euthymic rats 10 days after infection, the numbers being significantly more than those in corresponding athymic rats. Twenty days after infection, gran-

TABLE II. Numbers of granzyme B+ cells in the jejunum of euthymic rnu/+ and athymic rnu/rnu rats infected with *Nippostrongylus brasiliensis*.*

	Granzyme B+ cells (no./VCU)			
	rnu/+		rnu/rnu	
Day	IE	PM	IE	PM
0†	9.3 ± 0.8‡	4.1 ± 0.6‡	18.9 ± 1.3	6.8 ± 0.7
10	29.3 ± 1.0‡§	10.2 ± 0.1‡§	11.2 ± 2.0§	6.5 ± 0.3
20	8.5 ± 1.8‡	5.9 ± 0.7	20.2 ± 1.3	7.6 ± 1.3

* Granzyme B immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections. Positive cells in the epithelium (IE) and the propria mucosa (PM) were counted in 10 villus-crypt units (VCU) and then the cell number per VCU was calculated. All data are means ± SE of 4 rats.
† Uninfected control.
‡ Significantly different from rnu/rnu rats ($P < 0.05$).
§ Significantly different from day 0 ($P < 0.05$).

zyme B+ cell numbers returned to preinfection levels. In athymic rats, granzyme B+ cell numbers were decreased 10 days after infection.

Granzyme B expression is reportedly found mainly in CTL and NK cells (Jenne and Tschopp, 1988). To examine the distribution of T cells in the jejunal mucosa, immunohistochemical studies were performed using antibodies to pan-T-cell marker CD3. In athymic animals, substantial numbers of CD3+ cells were observed in the villus epithelium, whereas few cells were found in the lamina propria mucosa (Table III), consistent with previous reports that IEL are not depleted in athymic rats (Vaage et al., 1990; McKay et al., 1995). In euthymic rats, nematode infection induced significant increases of propria mucosal CD3+ cell numbers, whereas intraepithelial CD3+ cell numbers decreased after infection (Table III). CD8 is expressed on large proportions of mature CTL and some populations of NK cells. Despite the fact that athymic rats had large numbers of granzyme B+ cells, only small numbers of CD8+ cells were found in the epithelium as well as the propria mucosa (Table IV). In euthymic rats, intraepithelial CD8+ cell numbers decreased significantly after infection, whereas propria mucosal CD8+ cell numbers did not change significantly. We tried double immunostaining of CD8 and granzyme B on formalin-fixed paraffin-embedded tissue sections, but it was unsuccessful.

DISCUSSION

It is known that T cell–associated damage to the small intestine, such as that which occurs in graft-versus-host disease, produces villus atrophy (Felstein and Mowat, 1990; Lionetti et al., 1993). Ferguson and Jarrett (1975) reported that thymus-deprived (B) rats did not develop villus atrophy after *N. brasiliensis* infection, suggesting that T cell–dependent mechanisms have an important role in the progression of villus atrophy in nematode infection as well. These results showed that villus atrophy did not develop in nematode-infected athymic rats at least until 20 days after infection. However, as reported previously (D'Inca et al., 1992), crypt hyperplasia developed in athymic rats at a level comparable with that in euthymic rats, suggesting that certain levels of epithelial damage occurred even in athymic rats. In fact, nematode-infected athymic rats showed morphological alterations such as mucosal edema and sloughing off of some enterocytes from the villus tips, although these alterations were less intense than those in euthymic rats. Collectively, these results suggest that epithelial injuries and cell loss occurred in both euthymic and athymic rats but that the enteropathy in athymic rats was not sufficiently severe to cause villus atrophy and could have been compensated for by the crypt stem cell replication. D'Inca et al. (1992) reported that partial villus atrophy developed 21 days after infection in athymic rats together with decreases in disaccharide enzyme activities and increases in epithelial permeability, although 10 days after infection there was no villus atrophy. In their study, the number of L3 larvae inoculated in athymic rats was 3 times more than that in this study. It seems that T cell–dependent mechanisms play a major role in the development of villus atrophy, although under conditions of high worm burdens, T cell–independent mechanisms might also play a certain role.

TABLE III. Numbers of CD3+ cells in the jejunum of euthymic rnu/+ and athymic rnu/rnu rats infected with *Nippostrongylus brasiliensis*.*

	CD3+ cells (no./VCU)			
	rnu/+		rnu/rnu	
Day	IE	PM	IE	PM
0†	9.1 ± 1.0‡	5.3 ± 0.3‡	4.3 ± 0.7	0.02 ± 0.02
10	7.0 ± 2.4	24.5 ± 1.8‡§	4.2 ± 0.1	0.03 ± 0.02
20	4.0 ± 0.6§	8.1 ± 0.2‡§	4.0 ± 0.1	0.05 ± 0.01

* CD3 immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections. Positive cells in the epithelium (IE) and the propria mucosa (PM) were counted in 10 villus-crypt units (VCU) and then the cell number per VCU was calculated. All data are means ± SE of 4 rats.
† Uninfected control.
‡ Significantly different from rnu/rnu rats ($P < 0.05$).
§ Significantly different from day 0 ($P < 0.05$).

TABLE IV. Numbers of $CD8^+$ cells in the jejunum of euthymic rnu/+ and athymic rnu/rnu rats infected with *Nippostrongylus brasiliensis*.*

	$CD8^+$ cells (no./VCU)			
	rnu/+		rnu/rnu	
Day	IE	PM	IE	PM
0†	5.2 ± 0.6‡	8.5 ± 0.6‡	0.3 ± 0.2	1.1 ± 0.2
10	3.0 ± 0.7‡§	6.0 ± 1.2‡	0.5 ± 0.4	1.7 ± 0.2
20	1.9 ± 0.5‡§	8.0 ± 0.3‡	0.2 ± 0.1	1.3 ± 0.2

* CD8 immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections. Positive cells in the epithelium (IE) and the propria mucosa (PM) were counted in 10 villus-crypt units (VCU) and then the cell number per VCU was calculated. All data are means ± SE of 4 rats.
† Uninfected control.
‡ Significantly different from rnu/rnu rats ($P < 0.05$).
§ Significantly different from day 0 ($P < 0.05$).

The mechanisms whereby T cells regulate enteropathy or the development of villus atrophy are not thoroughly understood. Granzyme B is a cytotoxic cell granule protease that plays a critical role in mediating cytotoxicity and is expressed mainly in $CD8^+$ T cells and NK cells, as well as in some populations of $CD4^+$ T cells. Immunohistochemical studies showed that granzyme B^+ cells were found not only in euthymic but also in athymic rat intestinal mucosa, consistent with previous reports that IEL and NK cells are not depleted in athymic rats (Sfaksi et al., 1985; Vaage et al., 1990; Hougen, 1991; McKay et al., 1995). After infection, granzyme B transcript levels and granzyme B^+ cell numbers increased significantly in euthymic rats in association with the development of villus atrophy. In contrast, athymic rat granzyme B^+ cells in the epithelial compartment decreased in number after infection, whereas those in the propria mucosa did not change significantly. These results clearly indicate that granzyme B^+ cytotoxic cells did not proliferate in athymic rats, probably for lack of growth factors, such as interleukin (IL)-2, derived from activated T cells. The precise mechanisms causing the significant reduction in granzyme B–immunoreactive cells 10 days after infection in athymic rats were not clarified in this study. Athymic rats developed crypt hyperplasia, which peaked 10 days after infection, suggesting that epithelial turnover was accelerated during that period. Given the acceleration of epithelial turnover, a large proportion of intraepithelial granzyme B^+ cells may also have been lost while repopulation of the cells was delayed until 20 days after infection. Alternatively, it is also possible that the reduction of granzyme B^+ cells was partly due to reduced staining caused by secretion of the enzyme in response to the worm.

FasL is also expressed on some populations of $CD4^+$ T cells, $CD8^+$ T cells, and NK cells and mediates target cell killing through FasL–Fas (CD95) interaction. Unlike granzyme B expression, FasL expression was not increased significantly after infection in either athymic or euthymic rats, suggesting that FasL–Fas interaction might not play important roles in mediating villus epithelial injury or apoptosis after nematode infection (or both). The discrepancy in the expression of granzyme B and FasL after infection suggests that granzyme B and FasL are not necessarily regulated in a coordinated fashion.

TNF-α is produced by a wide variety of cells, including T cells, NK cells, macrophages, and mast cells. In macrophages, TNF-α synthesis can be induced by various pathogens, including viruses, bacteria, and parasites, as well as by cytokines (IL-1, IL-2, interferon-γ, granulocyte-macrophage–colony stimulating factor fluid, and TNF-α itself) (Herbein and O'Brien, 2000). In the euthymic rats in this study, the TNF-α transcript levels increased significantly in association with villus atrophy. Surprisingly, infected athymic rats showed higher TNF-α transcript levels than euthymic rats, and the high levels were sustained even 20 days after infection. The high and sustained TNF-α upregulation in infected athymic rats and the transient TNF-α upregulation in euthymic rats seemed to be positively correlated with the worm burden in the small intestine in each animal, suggesting the possibility that TNF-α upregulation was induced by nematode-related factors. TNF-α is a key cytokine in the inflammatory processes of various diseases such as inflammatory bowel disease and rheumatoid arthritis. It has been reported that neutralization of TNF-α by administration of a monoclonal antibody induced a detectable decrease in the inflammatory parameters of colitis (Van Deventer, 1999), and TNF-α antagonist ameliorated joint inflammation, although the antagonist had little effect on tissue destruction (Joosten et al., 1999). Thus, TNF-α upregulation after nematode infection might be associated with the development of some aspects of inflammatory responses in the intestinal mucosa that could occur without T cell regulation. It has been reported that villus atrophy did not occur in *Trichinella spiralis*–infected $TNFR^{-/-}$ or $iNOS^{-/-}$ mice, indicating that TNF-α and nitric oxide are involved in intestinal pathology in nematode infection (Lawrence et al., 1998, 2000). In this study, no association was observed between TNF-α upregulation and villus atrophy, suggesting that TNF-α–induced inflammation was not sufficiently intense to cause tissue damage. The mechanisms involved in the intestinal pathology might differ among parasite species as well as among host species. Collectively, the present RT-PCR and immunohistochemical studies showed that development of villus atrophy was associated most significantly with the proliferation of granzyme B^+ cells in the epithelium and propria mucosa and was not associated with the transcript levels of FasL or TNF-α.

In euthymic rats, $CD3^+$ T cells increased significantly in the intestinal propria mucosa, whereas intraepithelial $CD3^+$ T cell numbers were not increased but rather decreased 20 days after infection. CD8 is expressed on large proportions of mature CTL and some populations of NK cells. The $CD8^+$ cell numbers also decreased in the epithelium after infection, whereas those in the propria mucosa showed no significant change. The reduction of IEL numbers after *N. brasiliensis* infection is partly comparable with the effects on IEL kinetics in *T. spiralis*–infected mice, in which IEL numbers increased 4–7 days after infection but displayed a significant reduction to below normal levels from 14–29 days after infection (Bozic et al., 1998). Athymic rats, as reported previously, had substantial numbers of $CD3^+$ T cells in the epithelium but virtually none in the propria mucosa (Vaage et al., 1990; Hougen, 1991; McKay et al., 1995). Because our attempt to perform double immunostaining of CD8 and granzyme B on formalin-fixed paraffin-embedded tissue sections was unsuccessful in this study, it was not clarified whether some granzyme B^+ cells express CD8. However, our finding of the presence of large numbers of granzyme B^+ cells in athymic rats, which had few $CD8^+$ cells, as well as the dissociated kinetics of granzyme B^+ cells and $CD8^+$ cells in the propria mu-

cosa of euthymic rats after infection, suggests that the majority of granzyme B$^+$ cells might not express CD8.

It has been suggested that mucosal mast cell activation has an important role in *N. brasiliensis*–induced enteropathy in both immunocompetent and athymic rats (D'Inca et al., 1990, 1992; McKay et al., 1995). We reported previously that in mast cell–deficient *Ws/Ws* rats, villus atrophy developed as early as in wild-type +/+ rats after *N. brasiliensis* infection (Hyoh et al., 1999). Thus, despite the fact that mast cell activation aggravates the nematode-induced enteropathy, other effector mechanisms are more likely to be involved in the induction of villus atrophy.

Granzyme B–containing cells have the potential to induce apoptosis in closely contacted target cells after exposure to granzyme B together with perforin (Jenne and Tschopp, 1988). Thus, large numbers of activated granzyme B$^+$ cells might play a significant part in inducing enhanced cellular damage or apoptosis in epithelial cells, which could result in villus atrophy. The failure of villus atrophy to develop in athymic rats despite the presence of relatively large numbers of granzyme B$^+$ cells before infection might be due to the lack of proliferation or invasion of the cells from the propria mucosa into the epithelium during infection. It has been suggested that IL-4 and IL-13 are critical for *N. brasiliensis* worm expulsion, and mucin secretion or muscle contraction (or both) has been proposed as being directly involved in expelling worms from the intestine (Nawa et al., 1994; Urban et al., 1998; Zhao et al., 2003). It would be of interest to determine whether granzyme B$^+$ cells could also act as effector cells involved in worm expulsion. Although granzyme B reportedly has an antibacterial property (Shafer et al., 1991), it is not clear whether it has an antihelminthic effect as well or whether granzyme B$^+$ cell proliferation is dependent on IL-4 or IL-13 (or both).

Taken together, these results show for the first time that cells expressing the cytotoxic protease granzyme B infiltrate the villus epithelium and propria mucosa of the small intestine in association with the occurrence of villus atrophy after nematode infection. The types of granzyme B–expressing cells that infiltrate after nematode infection still remain to be identified, and their role in the progression of enteropathy should be further clarified.

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LITERATURE CITED

BANKFALVI, A., H. NAVABI, B. BIER, W. BOCKER, B. JASANI, AND K. W. SCHMID. 1994. Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. Journal of Pathology **174:** 223–228.

BOZIC, F., D. FORCIC, R. MAZURAN, A. MARINCULIC, Z. KOZARIC, AND D. STOJCEVIC. 1998. Gamma delta TCR+ intestinal intraepithelial lymphocytes (i-IEL) in reaction against intestinal nematode Trichinella spiralis. Comparative Immunology, Microbiology and Infectious Diseases **21:** 201–214.

D'INCA, R., P. ERNST, R. H. HUNT, AND M. H. PERDUE. 1992. Role of T lymphocytes in intestinal mucosal injury. Inflammatory changes in athymic nude rats. Digestive Diseases and Sciences **37:** 33–39.

———, J. K. RAMAGE, ———, AND ———. 1990. Antigen-induced mucosal damage and restitution in the small intestine of the immunized rat. International Archives of Allergy and Applied Immunology **91:** 270–277.

DUNNILL, M. S., AND R. A. WHITEHEAD. 1972. A method for the quantitation of small intestinal biopsy specimens. Journal of Clinical Pathology **25:** 243–246.

FELSTEIN, M. V., AND A. M. MOWAT. 1990. Experimental studies of immunologically mediated enteropathy. VI. Inhibition of acute intestinal graft-versus-host reaction in mice by 2′-deoxyguanosine. Scandinavian Journal of Immunology **32:** 461–469.

FERGUSON, A., AND E. JARRETT. 1975. Hypersensitivity reactions in small intestine. I. Thymus dependence of experimental 'partial villous atrophy'. Gut **16:** 114–117.

GREEN, D. R. 1998. Apoptotic pathways: The roads to ruin. Cell **94:** 695–698.

HERBEIN, G., AND W. A. O'BRIEN. 2000. Tumor necrosis factor (TNF)-alpha and TNF receptors in viral pathogenesis. Proceedings of the Society for Experimental Biology and Medicine **223:** 241–257.

HOUGEN, H. P. 1991. The athymic nude rat. Immunobiological characteristics with special reference to establishment of non-antigen-specific T-cell reactivity and induction of antigen-specific immunity. Acta Pathologica Microbiologica et Immunologica Scandinavica Supplementum **21:** 1–39.

HYOH, Y., S. ISHIZAKA, T. HORII, A. FUJIWARA, T. TEGOSHI, M. YAMADA, AND N. ARIZONO. 2002. Activation of caspases in intestinal villus epithelial cells of normal and nematode infected rats. Gut **50:** 71–77.

———, M. NISHIDA, T. TEGOSHI, M. YAMADA, R. UCHIKAWA, S. MATSUDA, AND ———. 1999. Enhancement of apoptosis with loss of cellular adherence in the villus epithelium of the small intestine after infection with the nematode Nippostrongylus brasiliensis in rats. Parasitology **119:** 199–207.

JENNE, D. E., AND J. TSCHOPP. 1988. Granzymes, a family of serine proteases released from granules of cytolytic T lymphocytes upon T cell receptor stimulation. Immunological Reviews **103:** 53–71.

JOOSTEN, L. A., M. M. HELSEN, T. SAXNE, F. A. VAN DE LOO, D. HEINEGARD, AND W. B. VAN-DEN-BERG. 1999. IL-1αβ blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-alpha blockade only ameliorates joint inflammation. Journal of Immunology **163:** 5049–5055.

KURODA, A., R. UCHIKAWA, S. MATSUDA, M. YAMADA, T. TEGOSHI, AND N. ARIZONO. 2002. Up-regulation of fas (CD95) and induction of apoptosis in intestinal epithelial cells by nematode-derived molecules. Infection and Immunity **70:** 4002–4008.

LAWRENCE, C. E., J. C. PATERSON, L. M. HIGGINS, T. T. MACDONALD, M. W. KENNEDY, AND P. GARSIDE. 1998. IL-4-regulated enteropathy in an intestinal nematode infection. European Journal of Immunology **28:** 2672–2684.

———, X. Q. WEI, F. Y. LIEW, P. GARSIDE, AND ———. 2000. Nitric oxide mediates intestinal pathology but not immune expulsion during Trichinella spiralis infection in mice. Journal of Immunology **164:** 4229–4234.

LIONETTI, P., E. BREESE, C. P. BRAEGGER, S. H. MURCH, J. TAYLOR, AND T. T. MACDONALD. 1993. T-cell activation can induce either mucosal destruction or adaptation in cultured human fetal small intestine. Gastroenterology **105:** 373–381.

LUNN, P. G., C. A. NORTHROP, R. H. BEHRENS, J. MARTIN, AND M. WAINWRIGHT. 1986. Protein losing enteropathy associated with Nippostrongylus brasiliensis infestation and its impact on albumin homoeostasis in rats fed two levels of dietary protein. Clinical Science **70:** 469–475.

MACDONALD, T. T. 1992. Epithelial proliferation in response to gastrointestinal inflammation. Annals of the New York Academy of Sciences **664:** 202–209.

MCKAY, D. M., M. BENJAMIN, E. M. BACA, R. D'INCA, K. CROITORU, AND M. H. PERDUE. 1995. Role of T lymphocytes in secretory response to an enteric nematode parasite. Studies in athymic rats. Digestive Diseases and Sciences **40:** 331–337.

NAWA, Y., N. ISHIKAWA, K. TSUCHIYA, Y. HORII, T. ABE, A. I. KHAN, BING-SHI, H. ITOH, H. IDE, AND F. UCHIYAMA. 1994. Selective effector mechanisms for the expulsion of intestinal helminths. Parasite Immunology **16:** 333–338.

NOLLA, H., J. R. BRISTOL, AND L. F. MAYBERRY. 1985. Nippostrongylus brasiliensis: Malabsorption in experimentally infected rats. Experimental Parasitology **59:** 180–184.

PERDUE, M. H., J. K. RAMAGE, D. BURGET, J. MARSHALL, AND S. MAS-

SON. 1989. Intestinal mucosal injury is associated with mast cell activation and leukotriene generation during Nippostrongylus-induced inflammation in the rat. Digestive Diseases and Sciences **34:** 724–731.

SCUDAMORE, C. L., A. M. PENNINGTON, E. THORNTON, L. MCMILLAN, G. F. NEWLANDS, AND H. R. MILLER. 1995. Basal secretion and anaphylactic release of rat mast cell protease-II (RMCP-II) from ex vivo perfused rat jejunum: Translocation of RMCP-II into the gut lumen and its relation to mucosal histology. Gut **37:** 235–241.

SFAKSI, A., M. BONNEVILLE, AND J. P. SOULILLOU. 1985. Cellular immune response in rnu/rnu rats. I. Lectin responsiveness and IL-2 production kinetics of natural cytotoxicity and spleen-cell surface marker expression. Immunology **55:** 655–662.

SHAFER, W. M., J. POHL, V. C. ONUNKA, N. BANGALORE, AND J. TRAVIS. 1991. Human lysosomal cathepsin G and granzyme B share a functionally conserved broad spectrum antibacterial peptide. Journal of Biological Chemistry **266:** 112–116.

SYMONS, L. E. A. 1965. Kinetics of the epithelial cells and morphology of villi and crypts in the jejunum of the rat infected by the nematode *Nippostrongylus brasiliensis*. Gastroenterology **49:** 158–168.

URBAN, J. F. JR., N. Noben-Trauth, D. D. Donaldson, K. B. Madden, S. C. Morris, M. Collins, and F. D. Finkelman. 1998. IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. Immunity **8:** 255–264.

VAAGE, J. T., E. DISSEN, A. AGER, I. ROBERTS, S. FOSSUM, AND B. ROLSTAD. 1990. T cell receptor-bearing cells among rat intestinal intraepithelial lymphocytes are mainly alpha/beta+ and are thymus dependent. European Journal of Immunology **20:** 1193–1196.

VAN DEVENTER, S. J. 1999. Review article: Targeting TNFα as a key cytokine in the inflammatory processes of Crohn's disease—The mechanisms of action of infliximab. Alimentary Pharmacology and Therapeutics **13**(Suppl.): 3–8.

WEIBEL, E. G. 1963. Principles and methods for the morphometric study of the lung and other organs. Laboratory Investigation **12:** 131–155.

WOODBURY, R. G., H. R. MILLER, J. F. HUNTLEY, G. F. NEWLANDS, A. C. PALLISER, AND D. WAKELIN. 1984. Mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematode infections in rat. Nature **312:** 450–452.

ZHAO, A., J. MCDERMOTT, J. F. URBAN JR., W. Gause, K. B. Madden, K. A. Yeung, S. C. Morris, F. D. Finkelman, and T. Shea-Donohue. 2003. Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves. Journal of Immunology **171:** 948–954.

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Case Report

Case of acute ileus caused by a spirurina larva

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A growing body of clinical cases suggests that a kind of nematode larva, type X larva of the suborder Spirurina that inhabits firefly squids (*Watasenia scintillans*, or 'Hotaru-ika' in Japanese), can cause acute ileus in humans. However, the larva itself has rarely been found in the wall of the obstructed intestine. We describe here a case of acute ileus, in which a type X spirurina larva was found histologically. A 60-year-old Japanese man suffered from acute abdomen, and an emergency laparotomy revealed a marked stenosis of the ileum. Histological study of the surgically resected ileum showed severe eosinophilic enteritis and a nematode larva. The morphological features of this larva were identical to those of the type X spirurina larva. Interestingly, the larva that was found existed within a small blood vessel, suggesting that the larva migrans of type X spirurina can take place via vasculature. The patient in the present case did not recall ingesting raw squids prior to the onset of his disease. Hence, this indicates that even if the ingestion of raw firefly squids is uncertain, spirurina infection should be included in the differential diagnosis of acute ileus or eosinophilic enteritis.

Key words: eosinophilic enteritis, ileus, larva migrans, raw fish, spirurina larva

Obstruction of the small intestine, or ileus, is caused by various kinds of disorders. Parasitic infections can cause acute ileus; anisakiasis is one such infection and is well known in countries where raw fish is one of the national culinary staples.[1,2]

Recently in Japan there has been an increasing number of clinical cases of acute ileus, which were believed to be caused by a parasite other than *anisakis*.[3–9] These cases arose from eating raw 'firefly' squids (*Watasenia scintillans*, or 'Hotaru-ika' in Japanese),[4–9] and the patients' peripheral blood showed eosinophilia,[6,7,9] compatible with a parasite infection. *W. scintillans* is known to be a host of type X larva of the suborder Spirurina,[10,11] a kind of nematode larva that was originally characterized by Hasegawa.[12,13] Many of the patients were shown to have serum antibodies against this larval nematode.[6–9] Thus, type X spirurina larva is suspected of infecting humans and causing acute ileus. However, cases in which the body of the spirurina larva was found in the obstructed intestine of patients are extremely rare.[3–5]

In contrast, there are a number of cases of cutaneous creeping eruption that have occurred after the eating of raw *W. scintillans* and histologically they have shown a type X spirurina larva in the skin eruption.[14,15] It is likely that type X spirurina larvae migrate from the intestine through the skin, but little is known about the route(s) of the spirurina larva migrans.

In the current paper, we describe a case of acute ileus, which showed a severe stenosis with eosinophilic enteritis of the ileum. A type X larva of the suborder Spirurina was shown histologically in the ileal wall, giving eloquent evidence that type X spirurina larva is one of the causative agents of acute ileus and eosinophilic enteritis in humans. A brief discussion about the possible vascular route of the spirurina larva migrans is also provided.

CLINICAL SUMMARY

A 60-year-old Japanese man complained of abdominal pain several hours after he had eaten several kinds of raw fish. He did not recall eating any squids. The pain became more severe the next day and he was admitted to Shakaihoken Kobe Central Hospital. On admission, he presented with symptoms of acute abdomen and the laboratory data showed that the serum C-reactive protein (2.3 mg/dL) and peripheral

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white blood cell count (12 900/mm^3) were significantly elevated over the normal ranges. Peripheral blood eosinophils were not counted. Emergency laparotomy revealed a severe stenosis of the ileum, and the ileum just oral to the stenosis was markedly dilated with ischemic changes. Partial resection of the ileum was performed. The patient's postoperative course was unremarkable.

The serum antibody against anisakis was examined 5 days after the operation, but was negative.

PATHOLOGICAL FINDINGS

The wall of the obstructed ileum was markedly thickened and the mucosa showed a small low prominence, approximately 4 mm in diameter, whose surface was eroded (Fig. 1).

Microscopically, there was a severe and extensive phlegmonous inflammation in the ileal wall (Fig. 2). Many of the inflammatory cells were eosinophils (Fig. 2). In the subserosa just below the small mucosal prominence, the body of a nematode larva was discovered (Fig. 3). It was found within a dilated venule (Fig. 4). The body of the larva was approximately 100 μm in diameter. The cuticle had no spines, but it had surface transverse annulations approximately 3 μm in width (Fig. 5). The muscle layer was of the polymyarian (24–27 muscle bundles in a quadrant) coelomyarian type. The lateral chords projected bilaterally and occupied the body cavity. Cross-section of the glandular esophagus measured approximately 38 × 45 μm. These morphological features are summarized in Table 1 and were identical to those of type X larva of the suborder Spirurina.

Figure 1 Macroscopic view of the ileal mucosa at the site of stenosis. The mucosa is edematous, and there is a small low prominence of the mucosa whose surface is eroded.

DISCUSSION

Acute ileus with eosinophilic enteritis can be caused after the ingestion of raw fish that are the normal hosts of parasites, and anisakiasis is the most popular of the parasite-induced acute ileus.[1,2] Freezing of raw fish prior to market delivery is effective in destroying the anisakis larvae and has succeeded in preventing anisakiasis in the Netherlands.[16]

Over the previous 20 years in Japan, it has been noted that acute ileus was brought on after the ingestion of a kind of raw squid.[4–9] This squid, *W. scintillans,* is not the host of anisakis, but recent parasitological investigations have found that the visceral organs of *W. scintillans* are inhabited by a kind of larval nematode, type X larva of the suborder Spirurina.[10,11] Serological examination of patients has often revealed the presence of antibodies against the type X spirurina larva, indicating infection with this parasite.[6–9] Thus,

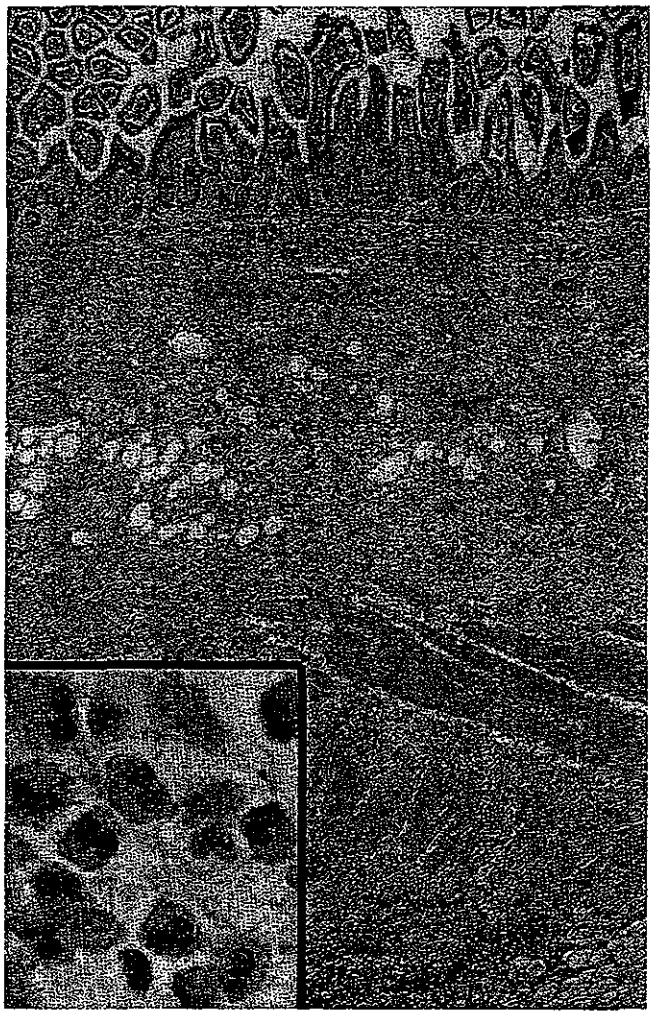

Figure 2 The ileal wall at the site of stenosis (HE). The ileal submucosa is markedly thickened, and there is a severe phlegmonous inflammation. Many of the inflammatory cells are eosinophils (inset).

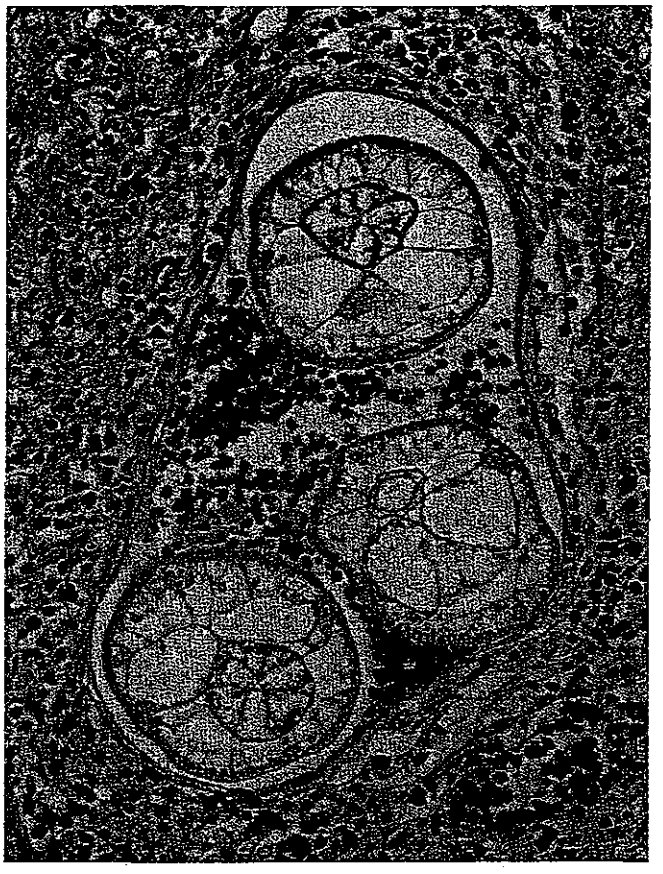

Figure 3 Three cross-sections of the body of a larval nematode found in the ileal subserosa (HE).

Figure 4 The larva is present within the lumina of a dilated venule, which is outlined by a thin layer of reticulin (elastica van Gieson).

acute ileus following ingestion of *W. scintillans* is believed to be an infectious disease caused by the type X spirurina larva. Although there was a large decrease in the number of clinical cases in 1995 when the squids were frozen to destroy any

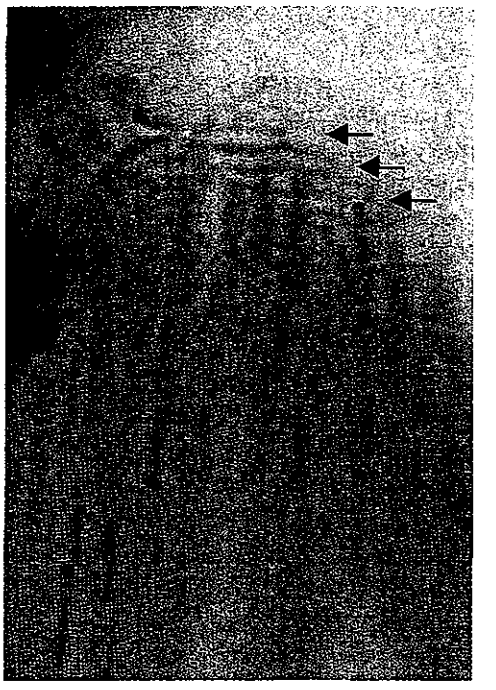

Figure 5 Transverse annulations (arrows) of the surface of the larval cuticle, which are characteristic to spirurina larvae.

Table 1 Morphological features of the larva

Body width	78–92 × 97–101 μm
Cuticle	Thin (1 μm)
Annulation	2–3 μm
Somatic musculature	Polymyarian coelomyarian type
Muscle cells in a quadrant	24–27
Dorsal and ventral chords	Small, clear
Lateral chords	Small, clear
Glandular esophagus	34–42 × 43–47 μm
No. branches	9
Intestine	18–24 × 25–29 μm
No. cells	6

larvae before they came into the market, a number of cases were still reported every year after 1996, probably because of inappropriate freezing procedures.[17] However, cases in which the body of the spirurina larva was found in the obstructed intestine of the patients are extremely rare; there are only four such cases reported in the literature.[3–5]

In the present case of acute ileus, we successfully showed the body of a larval nematode in the wall of the obstructed ileum, which showed severe eosinophilic enteritis. The structure of this larva was different from that of anisakis larvae;[18] for example, its body was much smaller than an anisakis larval body (approximately 200–600 μm in diameter), its muscle cells were much fewer than those of anisakis (60–90 muscle cells per quadrant), and its lateral chords expanded to occupy the body cavity (the lateral chords of anisakis larvae locally project into but do not occupy the body cavity).

We found that the surface of the larva had no spines, unlike Gnathostoma larvae, and its cuticle had annulations, unlike filariae.[12,13] The nematode larva was found to be morphologically identical to type X larva of the suborder Spirurina. The present case proves that this larva is one of the causative agents of acute ileus in humans.

The patient in the present case remembered eating raw fish but no squids before the onset of his disease. Previous parasitological studies have made it evident that type X spirurina larvae can inhabit several kinds of fish and squids other than *W. scintillans*.[3,10,11] Therefore, it is important to be aware of the possibility of spirurina infection in cases of acute ileus following ingestion of raw fish, even if the history of ingesting raw *W. scintillans* is uncertain.

Recent dermatological case studies have documented that type X spirurina larvae causes cutaneous larva migrans in humans; the body of this larva was shown histologically in the skin of a creeping eruption that occurred after a patient ate raw *W. scintillans*.[14,15] It is reasonable that type X spirurina larvae migrate from the intestine through the skin, but little is known about the route(s) of the spirurina larva migrans. A vascular route of larva migrans for some other nematodes is proposed from experimental studies; in mice, visceral larva migrans of *Baylisascaris transfuga* can take place by way of blood vessels.[19] The human case presented in the current paper showed, for the first time, the presence of type X spirurina larva within a blood vessel of the intestinal wall, suggesting that a vascular route is (one of) the way(s) of the spirurina larva migrans.

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REFERENCES

1 Liu LX, Weller PF. Intestinal nematodes. In: Braunwald E, Wilson JD, Martin JB, Fauci AS, Kasper DL, eds. *Harrison's Principles of Internal Medicine*, 13th edn. New York: McGraw–Hill, 1994; 919.

2 Ishikura H, Kikuchi K, eds. *Intestinal Anisakiasis in Japan. Infected Fish, Sero-Immunological Diagnosis, and Prevention.* Tokyo: Springer-Verlag, 1990.

3 Otsuru M, Shiraki T, Kenmotsu M. Some cases of larval or adult nematode migrations in the human tissue. *Kiseichu-Gaku Zasshi* 1974; **23**: 106–15 (in Japanese).

4 Kagei N, Kumazawa H, Miyoshi K, Kosugi I, Ishih A. A case of ileus caused by a spiruroid nematode. *Int J Parasitol* 1992; **22**: 839–41.

5 Matsuda K, Nakamura N, Maeda Y *et al.* A case with a larva of a spiruroid nematode in the intestinal wall. *Nippon Shokakibyo Gakkai Zasshi* 1994; **91**: 1344–7 (in Japanese).

6 Morita M, Nakamura H, Urade M, Hirosawa H. Clinical study of ileus like symptom due to eating of raw firefly squid, *Watasenia scintillans*. *Nippon Shokakibyo Gakkai Zasshi* 1995; **92**: 26–31 (in Japanese).

7 Aoyama S, Hinoue Y, Takahashi H *et al.* Clinical study of ten cases with acute abdomen after eating raw firefly squid (*Watasenia scintillans*, Hotaruika), which are probably due to type X larvae of the suborder spirurina. *Nippon Shokakibyo Gakkai Zasshi* 1996; **93**: 312–21 (in Japanese).

8 Sugane K. A case report of ileus caused by larval spiruriniasis. *Clin Parasitol* 1997; **8**: 117–19 (in Japanese).

9 Naganuma K, Aoyama K, Murakami J, Terada Y. A case of paralytic ileus due to eating of raw firefly squid, *Watasenia scintillans*. *Rinsho Shokaki Naika* 1997; **12**: 1795–8 (in Japanese).

10 Ando K, Sato Y, Miura K, Chinzei Y, Ogawa S. Further observation on the larva of the suborder Spirurina suspected as the causative agent of creeping eruption. *Jpn J Parasitol* 1992; **41**: 384–9.

11 Okazawa T, Akao N, Ohyama T, Kondo K. Prevalence and habitat of the type X larvae of the suborder Spirurina in squids. *Jpn J Parasitol* 1993; **42**: 356–60.

12 Hasegawa H. Larval nematodes of the superfamily Spiruroidea – A description, identification and examination of their pathogenicity. *Acta Med Biol (Niigata)* 1978; **26**: 79–116.

13 Hasegawa H, Sekikawa H, Kenmotsu M *et al.* A case of cutaneous larva migrans due to spirurin nematode found in Niigata prefecture, Japan, with special reference to the significance of cuticular morphology on identification. *Kiseichu-Gaku Zasshi* 1993; **42**: 12–7 (in Japanese).

14 Shinozaki M, Akao N, Okazawa T, Fukui Y, Kondo K. Detection of type X larva of the suborder Spirurina from a patient with a creeping eruption. *Jpn J Parasitol* 1993; **42**: 51–3.

15 Hattori S, Niimi Y, Kawana S. Creeping eruption caused by a larva of the suborder Spirurina type X. *Eur J Dermatol* 2003; **13**: 87–9.

16 VanTheil PH, Bakker PM. Worm granulomas of the stomach in the Netherlands and Japan. *Ned Tijdschr Geneeskd* 1981; **125**: 1365–70.

17 Kawanaka M, Sugiyama H. Infectious Disease Weekly Report. Home page of Infectious Disease Surveillance Center (http://idsc.nih.go.jp), 2004 (in Japanese).

18 Ishikura H, Namiki M, eds. *Gastric Anisakiasis in Japan. Epidemiology, Diagnosis, Treatment.* Tokyo: Springer–Verlag, 1989.

19 Papini R, Renzoni G, Malloggi M, Casarosa L. Visceral larva migrans in mice experimentally infected with *Baylisascaris transfuga* (Ascarididae: Nematoda). *Parassitologia* 1994; **36**: 321–9.

特集

幼虫移行症の薬物治療

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SUMMARY

幼虫移行症は動物を本来の終宿主とする寄生虫がヒトに感染し，幼虫が体内をさまよう（移行する）ことによって生じる寄生虫症である．決してまれなものではなく，現在日本で遭遇する寄生虫症の中でも，重要な疾患群の１つと位置づけられている．幼虫移行症は診断が必ずしも容易でなく有効な抗寄生虫薬が知られていないものも多いが，原因不明の好酸球増多症，特有の症状，特有の食歴などを認めたときはこれを疑い，早期に診断し適切な治療を行うことが必要である．

はじめに

幼虫移行症とは，ヒトを固有宿主としない寄生虫，例えばイヌ回虫や顎口虫がヒトに感染した時，幼虫がヒト体内で成虫にまで発育することなく体内諸臓器や皮膚を移行することによって生じる寄生虫症である．中には皮膚爬行疹のように独特の症状を示すものもある．幼虫移行症の多くは線虫が原因となっている．末梢血好酸球増多症が見られる例が多いが，病型によっては好酸球増多症は見られない．主要な幼虫移行症とその病型を**表1**に示した．

表1　主要な幼虫移行症の病型

	病型	寄生虫
内臓，眼，神経系などの幼虫移行症	内臓幼虫移行症	各種動物の回虫*
	眼幼虫移行症	各種動物の回虫*，広東住虫線虫**，顎口虫**
	神経系幼虫移行症	各種動物の回虫*，広東住虫線虫，顎口虫**，マンソン孤虫**
	その他	イヌ糸状虫（肺や軟部組織の腫瘤）
皮膚の幼虫移行症	遊走性限局性皮膚腫脹	顎口虫，マンソン孤虫
	皮膚爬行疹	顎口虫，ブラジル鉤虫，旋尾線虫
消化管の幼虫移行症	急性炎症型	アニサキス，旋尾線虫
	肉芽腫形成型	アニサキス

＊イヌ回虫，ネコ回虫，ブタ回虫，アライグマ回虫など
＊＊まれに生じるもの

Ⅰ 幼虫移行症の治療の原則

幼虫移行症には薬物治療の対象になるものと，必ずしも必要でないものがある．抗寄生虫薬による治療の対象となるのは，幼虫が一定期間以上人体内で生存しつづけ，種々の病態を惹起し，かつ幼虫の外科的摘出が困難な場合である．一方，イヌ糸状虫による肺腫瘤やアニサキスによる肉芽性腫瘤のように，発見されたときには多くの場合虫体がすでに死亡・変性している場合は，原則的に薬物治療は必要でない．

寄生虫症の治療に用いられる化学療法薬の多くは成虫を対象とした薬物であり，とくに腸管寄生虫を対象とした抗寄生虫薬の多くは，幼虫移行症に対しては全く無効である．幼虫移行症の薬物治療には，**ベンズイミダゾール誘導体**のチアベンダゾール（ミンテゾール®）やアルベンダゾール（エスカゾール®）が多く用いられている．これら薬剤はベータチュブリンに結合し，ミクロチュブルスの形成を阻害することが知られている．一方，土壌放線菌が産生する大環状ラクトン類 avermectin の誘導体，**イベルメクチン**（ストロメクトール®）も一部の幼虫移行症に有効なことが明らかになっており，今後期待できる薬剤である．

しかし，すべての幼虫移行症に対して薬物治療が確立しているわけではなく，とくに眼や中枢神経系に侵入した幼虫移行症の治療は困難なことが少なくない，薬剤選択にあたっては専門家に相談することが望ましい．なお，寄生虫学会ホームページ（http://jsp.tm.nagasaki-u.ac.jp/~parasite/welcome-2.html）でも相談を受け付けている．また「寄生虫症薬物治療の手引き」を同サイトからダウンロードできる．

Ⅱ 各種幼虫移行症に対する薬物治療

① 動物由来の回虫症

土壌中に排出された**イヌ回虫，ネコ回虫，ブタ回虫，アライグマ回虫**などの虫卵が，偶発的にヒトに経口的に摂取された場合に感染が生じる．これら回虫の幼虫の人体内における生存期間はきわめて長く，薬物治療が必要である．動物由来の回虫症は基本的に次の3病型に分類できる．(1) 肝，肺病変を中心とする**内臓幼虫移行症**，(2) 眼球内に侵入する**眼幼虫移行症**，(3) 神経系に侵入する**神経系幼虫移行症**．これらの病型が混在する場合もある．

a. 動物由来の回虫による内臓幼虫移行症

動物由来の回虫症の中で最も一般的な病型である．発熱，咳嗽，全身倦怠などで発症し，肝腫大や末梢血好酸球増多症が見られ画像にて肝または肺に多発性小結節性病変が見出されることが多い．生検で幼虫が発見されることはまれで，通常血清診断が行われる．

治療にはベンズイミダゾール誘導体のエスカゾール®が最もよく使用されている．

処方例：エスカゾール®200mg 錠，10～15mg/kg/日，分3，2～4週間．5日間の投与を一定期間毎に2～3回繰り返すことも欧米では行われている．治療開始1週間以内に一過性に好酸球数が増加することがある．白血球減少，肝機能低下などの副作用に対する注意が必要である．

b. 動物由来の回虫による眼幼虫移行症

イヌ回虫（トキソカラ）によるものが最も多く，**眼トキソカラ症**と呼ばれている．小児や思春期で

の発症が多く，大半が片眼性である．眼内炎型，後極部肉芽種型，周辺部腫瘤型に病型分類されている．好酸球増多症や肺・肝病変などの内臓幼虫移行症の症状を伴うことは少なく，眼に症状が限局して見られることが多い．血清診断のほか，眼房水中の抗体価の測定が診断に役立つ．

眼トキソカラ症に対する薬物治療の有効性はかならずしも確立していない．薬物治療の場合，ステロイド剤の単独使用または抗寄生虫薬とステロイド剤の併用が試みられている．抗寄生虫薬の使用にあたっては，死亡虫体によるアレルギー性炎症の発生を考慮する必要がある．内臓幼虫移行症と同じくエスカゾール®が使用されているほか，クエン酸ジエチルカルバマジン（スパトニン®）の使用例もある．

c．動物由来の回虫による神経系幼虫移行症

動物由来の回虫の感染により，髄膜脳炎，脊髄炎，脳症などを生じる症例が存在する．イヌ回虫で本病型が生じることはまれだが，ブタ回虫の感染では脊髄炎を生じた例が報告されている．一方，アライグマ回虫では重症の神経系幼虫移行症が高頻度に発現することが北米から報告されている[1]．

アライグマ回虫による神経系幼虫移行症の予後はきわめて不良で，死をまぬがれても重度の後遺症を残す例が多い．エスカゾール®とステロイド剤の併用による薬物治療が試みられているが，その有効性は低いと考えられている．日本国内における人体感染例はまだ報告されていないが，今後注意を払う必要のある幼虫移行症の1つである．

② 広東住虫線虫症

広東住虫線虫はネズミを終宿主，陸産・淡水産の貝類やナメクジを中間宿主，淡水産のエビなどを待機宿主とする寄生虫である．ヒトが貝やエビを摂食することによって広東住血線虫に感染すると，幼虫はクモ膜下腔に侵入し**好酸球性髄膜脳炎**を惹起する．幼虫はクモ膜下腔で幼若成虫にまでは発育するが，それ以上は発育せずいずれ死滅する．頭痛のほか，各種の髄膜炎症状を発現し，好酸球増多症が末梢血や脳脊髄液に見られる．診断は血清診断による．

重症度は感染数に依存する．一般的にはステロイド剤の投与や，脳圧降下剤による治療などで対処されるが，多数感染の場合は死の転帰をとることも少なくない．抗寄生虫薬の効果は不明だが，中国の症例ではエスカゾール®（200mg/日，5日間，その後400mg/日，5日間）とステロイド剤の併用[2]，台湾の症例ではメベンダゾールとステロイド剤の併用により症状が緩和されたと報告されている．

③ 顎口虫症

顎口虫は世界で約10種類存在するが，日本では有棘顎口虫，剛棘顎口虫，ドロレス顎口虫および日本顎口虫の4種による幼虫移行症が報告されている．自然界における終宿主は各種哺乳類，第1中間宿主はケンミジンコ，第2中間宿主および待機宿主はドジョウ，カエル，マムシ，渓流魚，雷魚などである．第2中間宿主または待機宿主の摂食によりヒトに感染する．多くの例で**皮膚幼虫移行症**を生じる．好酸球増多症は認められる例と認められない例がある．有棘顎口虫は主に皮下を遊走するために**遊走性限局性皮膚腫脹**を生じるが，その他3種の顎口虫は皮内を移動するため線状の**皮膚爬行疹**を生じる．このほか，内臓移行や**中枢神経系への侵入**が生じることもあり，とくに後者の場合予後は不良である．

虫体摘出が可能なら実施する．皮膚腫脹部や爬行疹の先端とそのやや前方の皮膚を切除し，連続組織切片やその他の方法で虫体を検索する．薬物治療も行われている．

処方例：エスカゾール®200mg錠，10～15mg/kg/日，分3，3週間．再発した場合は治療を繰り返す．このほか，ストロメクトール3mg錠，200μg/kg，単回服用が有効との報告もある．

④ その他の皮膚幼虫移行症

ブラジル鉤虫，イヌ鉤虫，旋尾線虫などの感染によっても顎口虫と同様の線状の皮膚爬行疹が生じる．基本的には診断をかねて皮膚切除し虫体摘出を試みるべきだが，薬物治療も行われている．ブラジル鉤虫，イヌ鉤虫の場合，エスカゾール®4日間連用やストロメクトール®単回投与が有効との報告がある．

条虫類の**マンソン孤虫**は皮下を遊走することにより遊走性限局性皮膚腫脹を生じる．体長10～20cmで，乳白色紐状のため肉眼で識別でき，外科的摘出が容易なことが多い．内臓移行したマンソン孤虫症に対してプラジカンテル（ビルトリシド®）の投与が有効であったとの報告もあるが，無効との報告もあり薬物療法は確立していない．

⑤ 消化管の幼虫移行症

海産魚類から感染する**アニサキス**幼虫は胃壁または腸壁に侵入する．胃アニサキス症に対しては内視鏡的虫体摘出が行われる．腸アニサキス症は，腹膜炎症状を伴い小腸部分切除によって発見される例が多いが，対症療法のみで回復した例もある．アニサキス症に対して抗寄生虫薬が使用された例はほとんど見られないが，動物実験ではイベルメクチンとアルベンダゾールの有効性が報告されている．

ホタルイカから感染する**旋尾線虫症**の多くはイレウスや腹膜炎症状を示す．旋尾線虫幼虫は人体内における生存期間が比較的長く，後に皮膚爬行症を生じることがあり，抗寄生虫薬による治療の対象とならないわけではない．しかし，一部の症例を除いては保存的療法で改善を見ており，薬物療法は試みられていない．

(参考文献)

1) Patrick G, et al：Neural larva migrans caused by the raccoon roundworm Baylisascaris procyonis. Ped. Infect. Dis. J, 21：971-975, 2002.
2) Wang Xiaotong, et al：A clinical study of eosinophilic meningoencephalitis caused by angiostrongyliasis. Chi. Med. J, 115：1312-1315, 2002.

A MULTIPLEX PCR FOR DISCRIMINATION BETWEEN *PARAGONIMUS WESTERMANI* AND *P. MIYAZAKII* AT THE METACERCARIAL STAGE

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Abstract. A multiplex polymerase chain reaction (PCR) system was developed for accurate species discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. The interspecies-conserved and species-specific primers designed from the sequences of the second internal transcribed spacer (ITS2) region of nuclear ribosomal DNA (rDNA) were all incorporated into single tubes and PCR amplification was carried out. The method allowed us to identify *P. westermani* and *P. miyazakii*, and discriminate them from *P. ohirai* at the metacercarial stage in a single tube reaction.

INTRODUCTION

The lung flukes, *Paragonimus westermani* and *P. miyazakii*, are of known medical importance as pathogens causing human paragonimiasis in Japan. The metacercariae of these species are found in the same freshwater crab species and are morphologically quite similar (Miyazaki, 1991). Therefore, the development of sensitive and objective diagnostic methods is required for accurate species discrimination and identification of the individual metacercariae of these species. These methods could be used for epidemiological investigations of the prevalence of the metacercariae in the crab host, and thus have important implications for controlling lung fluke disease.

We recently reported the establishment of molecular methods based on the use of polymerase chain reaction (PCR) for accurate discrimination of individual metacercariae of *P. westermani* and *P. miyazakii* (Sugiyama *et al*, 2002). The methods included direct cycle sequencing of the PCR products, PCR-restriction fragment length polymorphism (RFLP) and direct PCR-amplification using species-specific primers. All of these methods utilize nucleotide differences in the second internal transcribed spacer (ITS2) region of nuclear ribosomal DNA (rDNA) for discrimination. Of these methods, direct PCR-amplification provides a more rapid differential identification of species, because only a single-round PCR is required. However, this method limits identification to a single species and requires concurrently-run controls. In the present paper, we report the development of a multiplex PCR utilizing interspecies-conserved and species-specific primers designed from the sequences of the ITS2 region, which allows species discrimination of the individual metacercaria in a single tube reaction.

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MATERIALS AND METHODS

Parasite material and DNA isolation

Metacercariae of *P. westermani* (the diploid type) and *P. miyazakii* were harvested from the freshwater crab, *Geothelphusa dehaani*, and used for DNA isolation (Sugiyama *et al*, 2002). DNA was also extracted from the metacercariae of *P. ohirai*, which were isolated from the brackish water crab, *Chiromantes dehaani*, collected in Tokyo, Japan (Sugiyama *et al*, 2004).

Primers and amplification by PCR

The *P. westermani*-specific forward primer (PwF1; 5'-GTTTATGTTGCGCGTGGTCTGCTTTC-3', alignment positions 351 to 376 for *P. westermani* ITS2 region) and *P. miyazakii*-specific forward primer (PmF1; 5'- TTCCCCAACCTGGCCTCGTGG-3', alignment positions 184 to 204 *for P. miyazakii* ITS2 region) were newly designed in this study to target the 3'-terminal and the central portion of the ITS2 sequences of the corresponding species, respectively (*Fig 1*). In combination with the species-specific primers, consensus primers, 3S: 5'-GGTACCGG TGGATCACTCGGCTCGTG-3' (forward) and/or A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTC CGC-3' (reverse), which were designed based on the conserved sequences of the 5.8S and 28S genes (Bowles *et al*, 1995), were used.

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Pw   1:TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACA
Pm   1:.....T.....

Pw  61:TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG
Pm  61:.....

Pw 121:TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT
Pm 121:.....

Pw 181:GATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACAT
Pm 181:...T.....C...C..C...G.....
          PmF1—>

Pw 241:ACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTG
Pm 241:.TC.....T.....T....T.....T.....

                                                        PwF1-->
Pw 301:GCTCAGTGAATGATTTATGTGCGCGTTCCGCTGTCCTGTCTTCATCTGTGGTTTATGTTG
Pm 301:.....T.....--.....A....A..A..G...A.....G......

Pw 361:CGCGTGGTCTGCTTTCGATGCTGACCTACGTATGTGCCATGTGGTTCATTCTCCTGACCT
Pm 359:.....G..TGCC.....T.....C.....

Pw 421:CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA  :463
Pm 419:.....  :461
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Fig 1- Aligned nucleotide sequences of an ITS2 region from *P. westermani* (Pw) and *P. miyazakii* (Pm) metacercariae. A hyphen indicates an alignment gap. A dot in the *P. miyazakii* sequence indicates a nucleotide identical to that in *P. westermani*. The 5' and 3' ends of the sequences include 5.8S rDNA and 28S rDNA, respectively. The locations of the *P. westermani*-specific forward primer (PwF1; 5'-GTTTATGTTGCGCGTGGTCTGCTTTC-3') and *P. miyazakii*-specific forward primer (PmF1; 5'-TTCCCCAACCTGGCCTCGTGG-3') are underlined. The numbers refer to alignment positions.

PCR amplification was performed as described previously (Sugiyama *et al*, 2002) using 0.25 μm of each primer and 2.5 units of Taq polymerase (Invitrogen, USA). In the present study, 1 ng of the DNA was added to each PCR reaction (final reaction volume, 100 μl); 1 ng of the DNA was equivalent to about 1/250, 1/400 and 1/100 of the DNA isolated from a single *P. westermani, P. miyazakii* and *P. ohirai* metacercaria, respectively. The resultant PCR products were separated by electrophoresis through 2% (w/v) agarose gels. the amplified PCR products were also excised from agarose gels and sequenced using the corresponding primers and the BigDye Terminator Cycle Sequencing Kit (Applied biosystems, USA) on an automated sequencer (ABI310, Applied Biosystems).

RESULTS

In the first step, the species-specificity of the newly designed forward primers, PwF1 and PmF1, was evaluated as to whether they could amplify different sized species-specific fragments from metacercarial DNA by PCR in combination with the consensus reverse primer A28. As was expected, the primer set PwF1-A28 amplified a PCR product of about 140 bp from *P. westermani* DNA, but not from *P. miyazakii* DNA (Fig 2). In contrast, PmF1-A28 amplified a product of about 300 bp from *P. miyazakii* DNA, but not from *P. westermani* DNA (Fig 2).

Having demonstrated the species-specificity of the primers PwF1 and PmF1, these two primers were mixed and incorporated into single tubes with the consensus primer A28 for the PCR reaction. As a result, a PCR product of about 140 bp was amplified from *P. westermani* DNA and that of about 300 bp was from *P. miyazakii* DNA (Fig 3). The same PCR amplification was carried out using DNA extracted from *P. ohirai* metacercariae but no product was amplified (Fig 3).

We previously reported that PCR products of the same size (520 bp) were amplified from *P. westermani* and *P. miyazakii* metacercarial DNAs using the

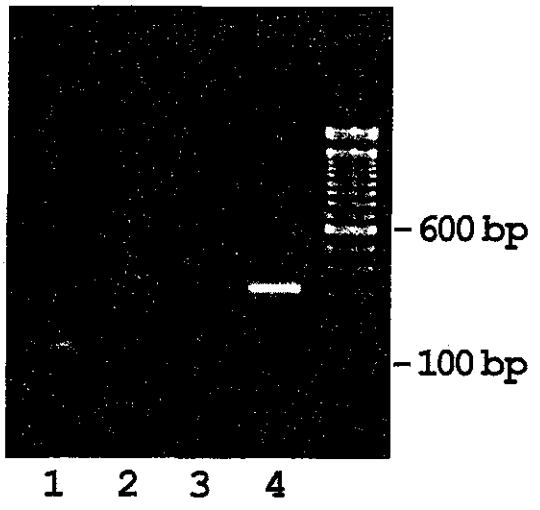

Fig 2- Results of PCR amplification of DNA from *P. westermani* (lanes 1 and 2) or *P. miyazakii* (lanes 3 and 4) metacercariae using the *P. westermani*-specific PwF1 (lanes 1 and 3) and *P. miyazakii*-specific PmF1 (lanes 2 and 4) primers. A 100-bp DNA ladder was used to estimate the size of the fragments.

consensus primer set 3S-A28 (Sugiyama *et al*, 2002). These two consensus primers as well as the two species-specific primers, PwF1 and PmF1, were all incorporated into single tubes, and PCR amplification was then carried out. As a result, two PCR products of about 520 bp and 140 bp were amplified from *P. westermani* DNA and products of about 520 bp and 300 bp were amplified from *P. miyazakii* DNA (Fig 4). In addition, we found that only the 520-bp product was amplified from *P. ohirai* DNA (Fig 4). The amplified PCR products were sequenced, and we confirmed that they corresponded to the ITS2 regions of rDNA from the respective species.

The sensitivity of the established multiplex PCR system was tentatively determined by titration of the DNA. The lowest concentration of *P. westermani*, *P. miyazakii* and *P. ohirai* DNAs that produced PCR products was estimated to be 0.001 ng (figure not shown). This is equivalent to approximately 10^{-5} of the total DNA isolated from a single metacercaria.

DISCUSSION

In the present study, we developed a multiplex PCR system that allowed us to identify *P. westermani* and *P. miyazakii*, and discriminate them from other *Paragonimus* species at the metacercarial stage in a single tube reaction. By this system, species-specific bands of different sizes were produced from *P. westermani* DNA (140 bp) and *P. miyazakii* DNA (300 bp). At the same time, interspecies-conserved bands (520 bp) were generated from DNAs prepared from

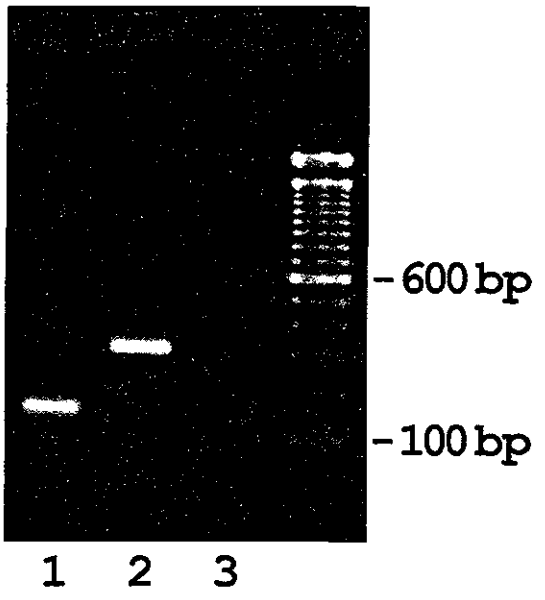

Fig 3- Results of multiplex PCR amplification of DNA from *P. westermani* (lane 1), *P. miyazakii* (lane 2) or *P. ohirai* (lane 3) metacercariae using the two species-specific primers, PwF1 and PmF1, in combination with the consensus reverse primer, A28. A 100-bp DNA ladder was used to estimate the size of the fragments.

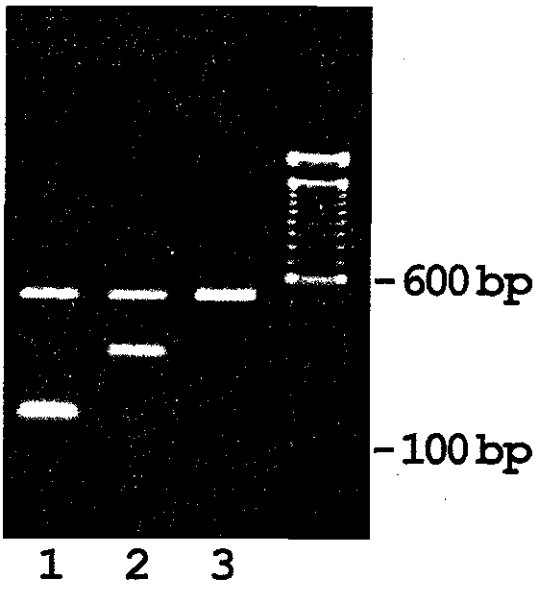

Fig 4- Results of multiplex PCR amplification of DNA from *P. westermani* (lane 1), *P. miyazakii* (lane 2) or *P. ohirai* (lane 3) metacercariae using the two species-specific primers, PwF1 and PmF1, combined with the consensus primer set, 3S and A28. A 100-bp DNA ladder was used to estimate the size of the fragments.

metacercariae of all the three species examined, *P. westermani*, *P. miyazakii* and *P. ohirai*. The PCR products of about 520 bp could serve as an internal control for the integrity of the PCR reaction and might

function as a diagnostic reagent for genus identification. The consensus primers, 3S-A28, were previously used to amplify the ITS2 region from *P. ohirai* adult DNA (Blair *et al*, 1997).

From the high sensitivity of the multiplex PCR system, we speculated that it has the potential to be used for species identification and discrimination of eggs of lung flukes, the life cycle stages with particularly small sizes. In addition, the eggs of *P. westermani* (the diploid type) and *P. miyazakii* are quite similar in size and shape (Miyazaki, 1991), and therefore it is quite difficult to differentiate the species of individual eggs using morphological characters. As the eggs in the feces or sputa from patients represent the parasite stage for which diagnostic tests are most often requested, studies are underway to elucidate the established multiplex PCR system for this purpose.

REFERENCES

Blair D, Agatsuma T, Watanobe T. Molecular evidence for the synonymy of three species of *Paragonimus, P. ohirai* Miyazaki, 1939, *P. iloktsuenensis* Chen, 1940 and *P. sadoensis* Miyazaki *et al*, 1968. *J Helminthol* 1997;71:305-10.

Bowles J, Blair D, McManus DP. A molecular phylogeny of the human schistosomes. *Mol Phylog Evol* 1995;4:103-9.

Miyazaki I. Paragonimiasis. In: Miyazaki I, ed. An illustrated book of helminthic zoonoses. Tokyo: International Medical Foundation of Japan, 1991:76-146.

Sugiyama H, Morishima Y, Kameoka Y, Kawanaka M. Polymerase chain reaction (PCR)-based molecular discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. *Mol Cell Probes* 2002;16:231-6.

Sugiyama H, Morishima Y, Kameoka Y, Arakawa K, Kawanaka M. *Paragonimus ohirai* metacercariae in crabs collected along the Arakawa River in Tokyo, Japan. *J Vet Med Sci* 2004;66: 927-31.

FULL PAPER *Parasitology*

Paragonimus ohirai Metacercariae in Crabs Collected along the Arakawa River in Tokyo, Japan

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ABSTRACT. Brackish water crabs infected with *Paragonimus ohirai* metacercariae have been reported in various regions in Japan. However, infected crabs have not been identified in Tokyo. We therefore collected the crab, *Chiromantes dehaani*, between August 2002 and July 2003 from 12 locations along the Arakawa River that flows through Tokyo. Of the 922 captured crabs, 177 (19%) from 6 locations were infected with *Paragonimus* metacercariae. The prevalence of metacercariae at these 6 locations ranged from 5 to 89%. The number of metacercariae per infected crab ranged from 1 to 190, with an average of 13.1. The morphological features of the metacercariae and of adult worms recovered from test rats infected with metacercariae showed that the metacercariae in the infected crabs were *P. ohirai* Miyazaki, 1939. The ITS2 sequence data support this conclusion. This paper is the first description of *P. ohirai* infection of crabs in Tokyo.

KEY WORDS: brackish water crab, *Chiromantes dehaani*, geographical distribution, lung fluke, *Paragonimus ohirai*.

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Paragonimus metacercariae of an unknown species were isolated from brackish water crabs in Kumamoto Prefecture, Japan [5]. The novel species was subsequently named *P. ohirai* Miyazaki, 1939 according to the morphological features of metacercariae and adult worms from infected test mammals [6]. Since this discovery, brackish water crabs infected with *P. ohirai* metacercariae have been found in various regions of Japan [7, 8]. However, with respect to the Kanto district, *P. ohirai* metacercariae have been confirmed only in crabs found in the Boso area of Chiba Prefecture [3, 13].

Although crabs infected with *P. ohirai* have not been identified in other areas of the Kanto district, adult *P. ohirai* worms were detected in a stray dog that died in Bunkyo Ward, Tokyo, about 50 years ago [4]. The source of this canine infection remains unknown. We extensively surveyed the distribution of *P. ohirai* metacercariae in brackish water crabs at several locations along the Arakawa River that flows through metropolitan Tokyo.

MATERIALS AND METHODS

Isolation and examination of Paragonimus metacercariae: Between August 2002 and July 2003, we collected 922 brackish water crabs (*Chiromantes dehaani*, the second intermediate host of *P. ohirai*; formerly referred to as *Sesarma dehaani* or *Holometopus dehaani* [9]) from 12 locations along the banks of the Arakawa River in Tokyo. To examine crabs for *Paragonimus* metacercariae, we removed the carapace and then excised and compressed the midgut gland between two 10×6 cm glass plates. The presence of metacercariae was examined under a stereoscopic microscope. The compressed tissues were recovered using teasing needles or a spatula and then suspended in artificial gastric juice consisting of 1 g of pepsin (Sigma Aldrich Japan, Tokyo, Japan) in 1 *l* of 0.7% hydrochloric acid. The digest was filtered through 60-mesh wire sieves with a little water. The sediment in the filtrate was repeatedly washed with 0.4% NaCl and poured onto a watch glass. Metacercariae in the sediment were placed on glass slides and pressed slightly under a coverslip for morphological observations and measurements.

Adult worms from test rats: We infected 4 male Wistar rats with metacercariae to raise them to adult worms. The rats were necropsied 42 to 70 days after infection to recover worms from the lungs and pleural cavities. Recovered worms were compressed between two glass slides, fixed in 70% alcohol, stained with borax carmine and mounted with Canada balsam for morphological observations and measurements.

DNA amplification and sequencing of the ITS2 region: The ribosomal DNA ITS2 region from two metacercariae isolated from each of the 6 positive locations was amplified by PCR and sequenced as described [10]. The primers used were 3S: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' (forward) and A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3' (reverse). We aligned and compared sequences using the GENETYX-WIN (ver. 4.0, Software Development Co., Tokyo, Japan) program.

RESULTS

Prevalence, amount and infection intensity of P. ohirai metacercariae in crabs: We identified *Paragonimus* metacercariae in 177 (19%) of the 922 crabs collected at the following locations: Senju in Adachi Ward, Yahiro in

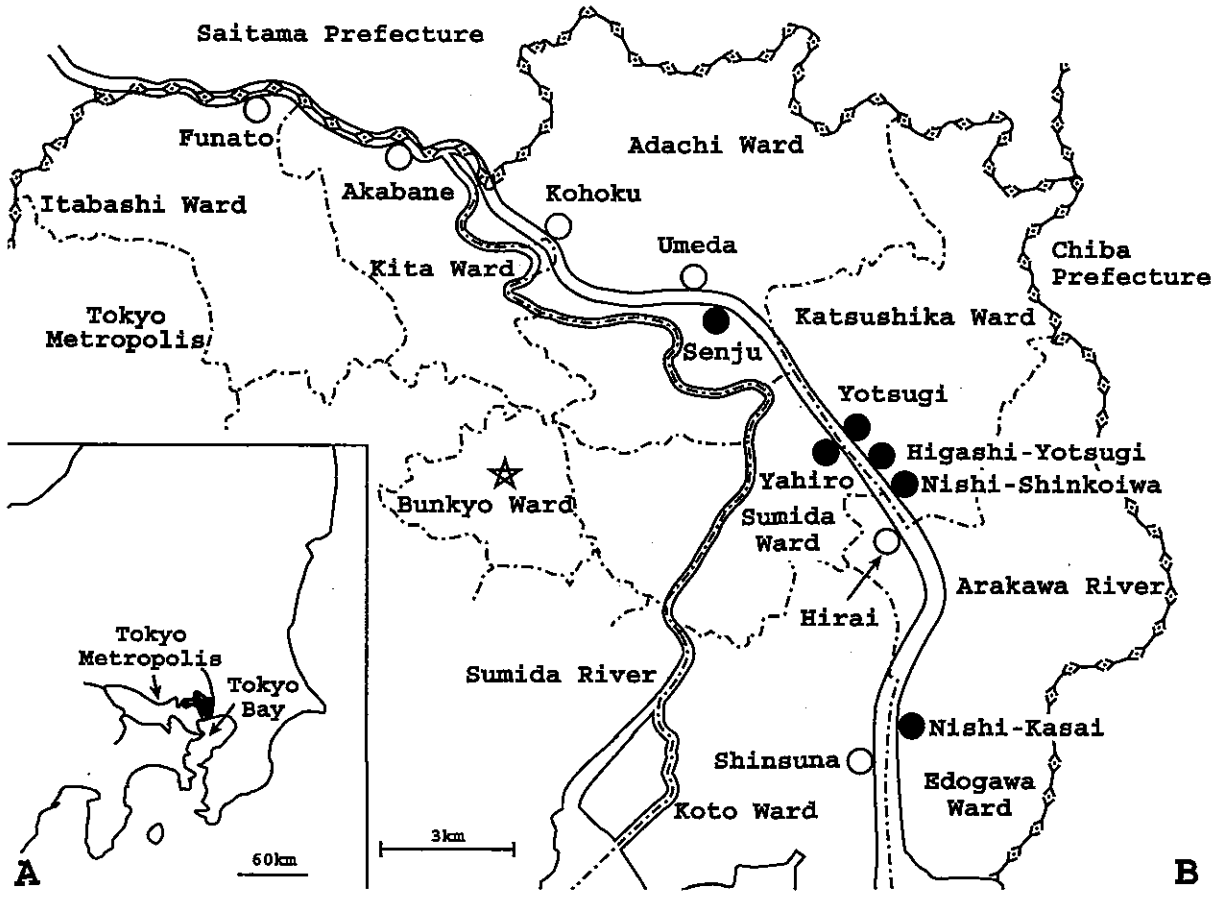

Fig. 1. Map of central Japan. (A) Area examined in this study is eastern Tokyo. (B) Details of study area showing locations where brackish water crabs, *C. dehaani*, were collected. Closed circles, locations of crabs infected with *P. ohirai*; open circles, locations of uninfected crabs; star, Hakusan (formerly Koishikawa-Hara-machi) in Bunkyo Ward, where a stray dog infected with *P. ohirai* died about 50 years ago.

Table 1. Prevalence, amount and infection intensity of *P. ohirai* metacercariae in *C. dehaani* crabs from locations along the Arakawa River in Tokyo

Location		No. of crabs		% infected	Total no. of mc[a] detected	No. of mc per infected crab	
		Examined	Infected			Range	Average
Itabashi Ward	Funato	139	0	–	–	–	–
Kita Ward	Akabane	27	0	–	–	–	–
Adachi Ward	Kohoku	75	0	–	–	–	–
	Umeda	93	0	–	–	–	–
	Senju	36	3	8	3	1	1.0
Sumida Ward	Yahiro	110	86	78	858	1–64	10.0
Katsushika Ward	Yotsugi	37	33	89	1,085	1–190	32.9
	Higashi-Yotsugi	50	35	70	310	1–159	8.9
	Nishi-Shinkoiwa	60	3	5	13	1–11	4.3
Edogawa Ward	Nishi-Kasai	58	17	29	42	1–10	2.5
	Hirai	76	0	–	–	–	–
Koto Ward	Shinsuna	161	0	–	–	–	–
Total		922	177	19	2,311	–	13.1

a) Metacercariae.

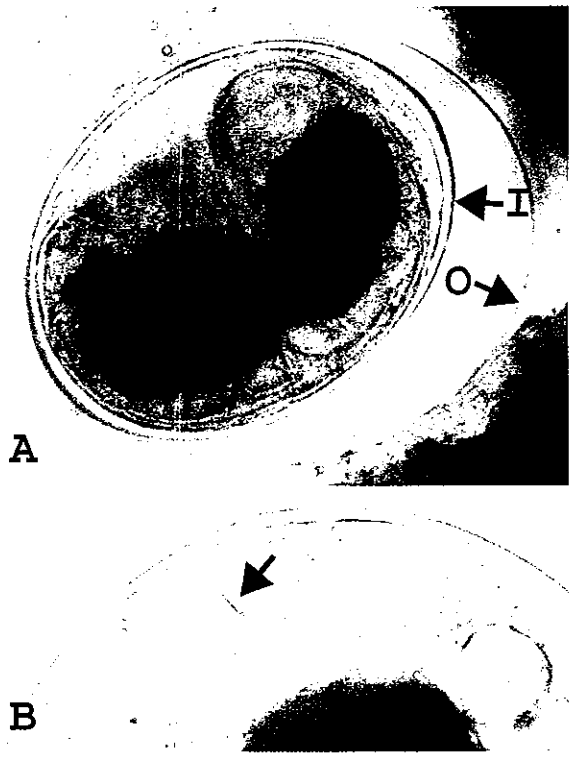

Fig. 2. Morphology of a metacercaria. (A) Photomicrograph of a fresh *P. ohirai* metacercaria encysted with a thin outer (O) and a thick inner (I) cyst wall, attached to the liver of the crab host. (B) Photomicrograph of the oral sucker of encysted metacercaria showing a stylet (arrow) dorsally embedded in the oral sucker.

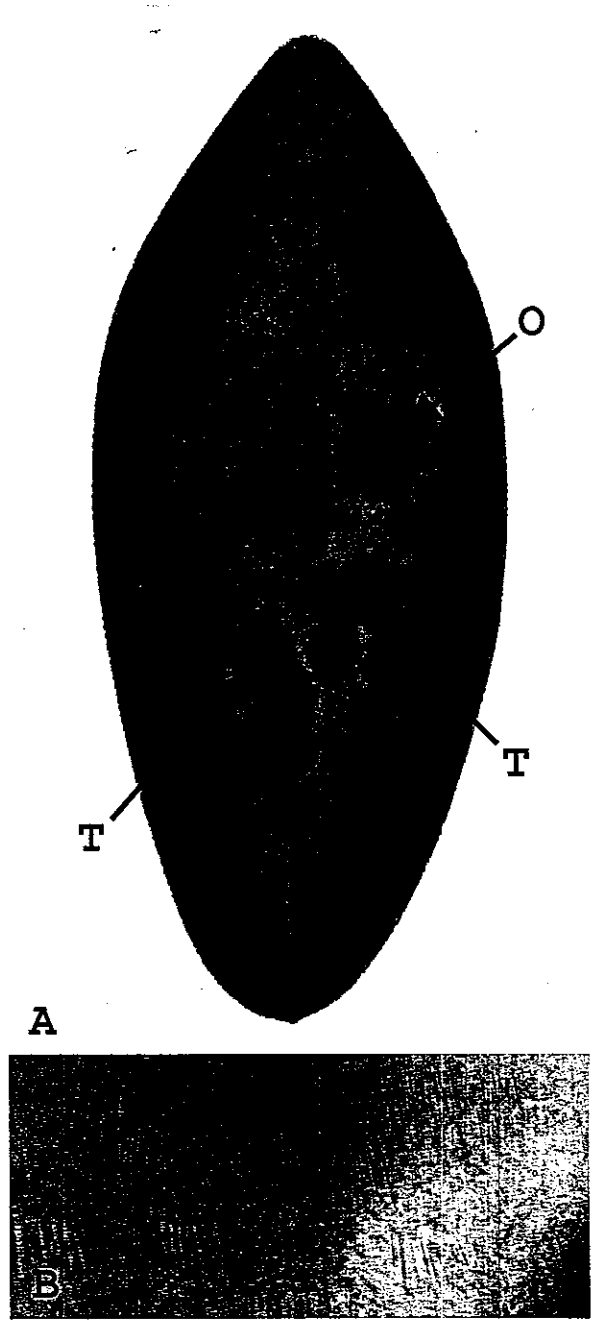

Fig. 3. Morphology of an adult worm. (A) Dorsal view of a mounted adult worm from the lung of the rat 70 days after infection, stained with borax carmine, showing the testes (T) and ovary (O). (B) Photomicrograph of cuticular spines arranged in groups.

Katsushika Ward, Yotsugi, Higashi-Yotsugi and Nishi-Shinkoiwa in Katsushika Ward, and Nishi-Kasai in Edogawa Ward (Fig. 1). The prevalence of metacercariae at these 6 locations varied from 5 to 89% (Table 1). We isolated 2,311 metacercariae from these 177 infected crabs. The number of metacercariae collected from an infected crab ranged from 1 to 190, with an average of 13.1. The average was 2.5 per crab for all crabs examined. The highest incidence (89%) of metacercarial infection and the largest number (32.9) of the mean burden per infected crab was found in Yotsugi in Katsushika Ward. We did not find *Paragonimus* metacercaria in 745 crabs collected from Funato in Itabashi Ward, Akabane in Kita Ward, Kohoku and Umeda in Adachi Ward, Hirai in Edogawa Ward and Shinsuna in Koto Ward.

We also attempted to collect crabs from the banks of the Sumida River, which diverges from the Arakawa River at Akabane in Kita Ward and flows into Tokyo Bay as one of its tributaries (Fig. 1). However, we did not capture any crabs from this river, probably because most of the banks have been sealed in concrete.

Morphology of metacercariae from crabs: *Paragonimus* metacercariae obtained from crabs were spherical, with thin outer and thick inner walls (Fig. 2A). The thickness of the inner cyst wall of the 50 specimens from Yotsugi ranged from 2.9 to 5.2 μm, with an average of 4.1 μm. The longitudinal and transverse diameter of the inner cyst freed from the outer cyst ranged from 262 to 331 μm and from 196 to