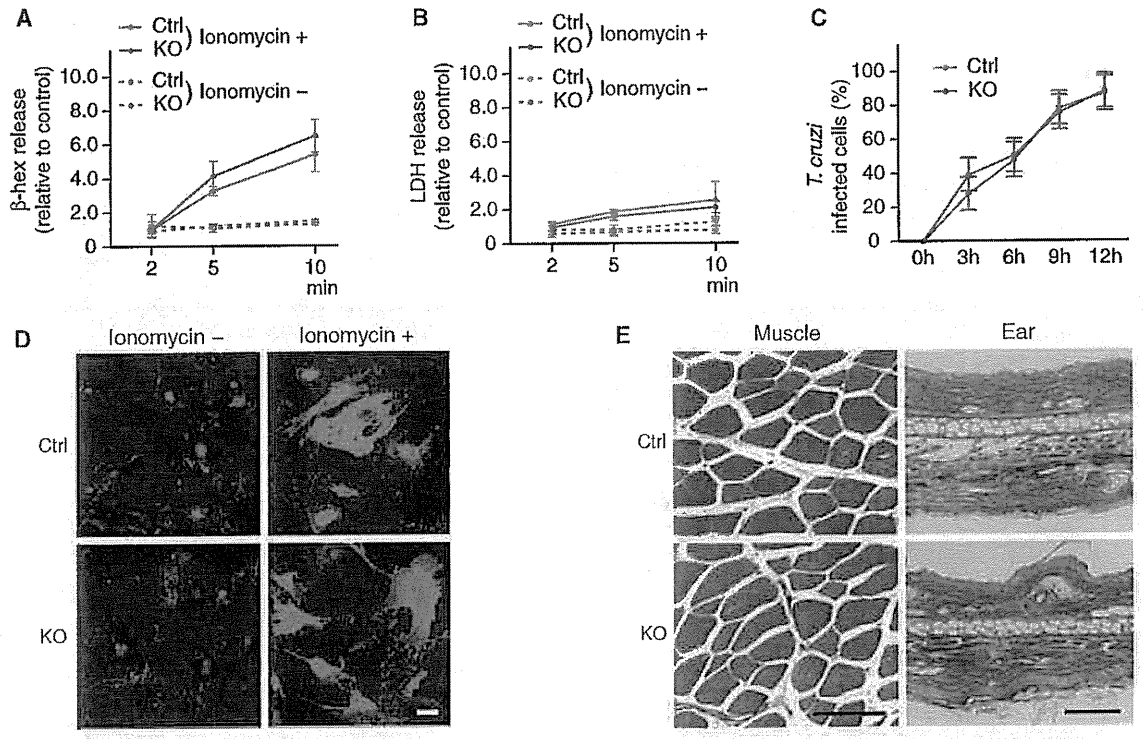

Figure 7: VAMP7 is not essential for lysosomal exocytosis. A and B) β-Hexosaminidase (A) and LDH (B) activity in the culture supernatant of MEFs after ionomycin treatment for 2, 5 and 10 min, presented relative to the total activity of β-hexosaminidase (A) and LDH (B) of MEFs. The data represent the average ± SD. C) Invasion of *T. cruzi* into MEFs, expressed as 'infected cells/total cells'. MEFs were exposed to trypomastigotes for the indicated periods of time, washed and stained with Giemsa. The data represent the average ± SD. D) MEFs from control (Ctrl) and KO embryos stimulated with or without ionomycin were stained with anti-lamp1 antibody without permeabilization to observe exposed lamp1 (green) by lysosomal exocytosis. Nuclei were stained by DAPI (blue). E) Masson trichrome staining of muscle and ear from control and KO mice. Bars, 50 μm.

exocytosis and decreased susceptibility to *Trypanosoma cruzi* invasion (14,15). To assess whether VAMP7 deficiency also affects lysosomal exocytosis, we first used cell scraping assays to injure the plasma membrane to induce lysosomal exocytosis. Though we observed a similar increase in the amount of β-hexosaminidase (β-hex) in both control and KO MEFs, we also observed increased amount of LDH (lactate dehydrogenase), an indicator of cell death, at the same time in the supernatant of both control and KO MEFs. Therefore, we used ionomycin treatment, a milder method to induce lysosomal exocytosis by calcium influx (Figure 7A,B). After 2, 5 and 10 min ionomycin treatment we found an increased concentration of β-hex in the culture supernatant from KO and control MEFs. However, we did not find a significant difference in the concentration of β-hex between KO and control culture supernatant (Figure 7A) (16).

To know whether we could distinguish lysosomal exocytosis in control and KO MEFs morphologically, we stained the cells with an antibody, which recognizes the luminal region of lamp1. However, we were not able to detect a clear difference between KO and control MEFs treated with ionomycin (Figure 7D).

To know the difference in lysosomal exocytosis further, we measured the infection rate of *T. cruzi*, which uses lysosomal exocytosis for invasion (17). As shown in Figure 7C, the infection rate of *T. cruzi* in KO MEFs was similar to that in control MEFs.

In synaptotagmin VII KO mice, the defect in lysosomal exocytosis causes autoimmunity, resulting in fibrosis of connective tissues and muscles as the animals age (14). However, this phenotype was not observed in KO mice (Figure 7E). These results collectively suggest that VAMP7 deficiency does not affect lysosomal exocytosis.

VAMP7 is essential for axonal elongation to the full extent as expected from previous results (8). We did not detect difference in the localization of NgCAM-GFP and L1 between control and KO neurons in our experiments. We cannot completely exclude the possibility that VAMP7 is involved in NgCAM or L1 transport within neurons

because it was very hard for us to detect differences due to the variable expression and localization of NgCAM-GFP and L1.

We did not detect significant differences in lysosomal exocytosis using a number of methods (immunolocalization of lysosomal proteins, staining with LysoTracker/LysoSensor and morphological observation of surface lamp1 and biochemical measurement of β-hex released by ionomycin). Therefore, it is likely that the absence of VAMP7 does not affect the localization/acidification of lysosomes and lysosomal exocytosis in embryonic fibroblast cells.

The simplest explanation for not detecting overt phenotypes in lysosomal exocytosis and epithelial polarity would be that the function of VAMP7 is compensated for by other VAMPs. Although we were not able to show increases or decreases in other SNAREs using the antibodies that were available to us, other SNAREs may be up or downregulated in the absence of VAMP7.

This suggests complex redundancy among v-SNAREs involved in lysosomal functions and polarized transport.

To clarify the functional significance of VAMP7, we will cross VAMP7 KO mice with other KO mice deficient in other VAMPs and/or perform other tests that can detect subtle differences in the absence of VAMP7 in the future.

Materials and Methods

Generation of VAMP7 knockout mice

All animal procedures were performed with the guidelines of the Animal Care and Experimentation Committee of Gunma University, and all animals were bred in the Institute of Animal Experience Research of Gunma University. VAMP7 KO mice were generated largely according to a previous work (18). By mating the chimeric mice with C57Bl/6 mice, we obtained heterozygous female (VAMP7 neo/+) mice. To generate null mice, we crossed VAMP7 neo/+ mice with Cytomegalovirus promoter (CMV)-cre transgenic mice from Jackson Laboratory. For genotyping by PCR analysis, we used the following primers: 5′-GCATTACCTGCCCCAGGCAAAACTG-3′ (primer a), 5′-GGGACACAGAGGAAGCAGGTAACGG-3′ (primer b1) and 5′-GAGAGATCAGGGAATTGGTACCGGA-3′ (primer b2). Primers a and b1 detected the wild-type allele. Primers a and b2 detected the Cre-recombined allele.

cDNA and plasmids

cDNA encoding the NgCAM fragment was generated by PCR using an NgCAM cDNA in the plasmid generously provided from Dr Sonderegger (19).

The primers used were as follows: NgCAM sense 5′-GAATTCATGCGTGAA AGTGCGGATCC-3′, NgCAM antisense 5′-GTCGACATGCGTGAAAGTGC GGATCC-3′.

Amplified cDNA fragments were subcloned into pEGFP vectors (Clontech). NgCAM-GFP was integrated into the adenovirus genome by Gateway systems (Invitrogen). Adenovirus was expanded in 293 cells and cell extracts were filter-sterilized and used for infection into neurons. Infection was carried out by adding the cell extract diluted 100-fold to the medium of neuronal culture overnight.

Antibodies

The following antibodies were used: anti-VAMP2, 3, 4 and 8 (Synaptic Systems); anti-DPPIV (R&D Systems); anti-SGLT and lamin B (Santa Cruz); anti-E-cadherin (TAKARA); anti-alkaline phosphatase (Rockland); anti-phosphorylated neurofilament (clone SMI-31; Covance); anti-GFP (Nacalai Tesque); anti-LAMP1 and anti-LAMP2 (clone 1D4B and Abl-93; Developmental Studies Hybridoma Bank); anti-cathepsin D (R&D Systems), anti-GS28, anti-PDI and anti-KDEL (BD Biosciences). A polyclonal antibody against VAMP7 (peptides C-LKGIMVRNIDLVAQ) was raised in rabbits and affinity-purified. A monoclonal antibody against MAP1A was previously described (20). Anti-syntaxin7 and anti-rab7 rabbit antisera were provided by Dr Wada (Osaka University) (21). Anti-L1 antibody was provided by Dr Miura (University of Tokyo) (22).

Histology and western blot analysis

Eight- to nine-week-old mice were used for histology and immunofluorescence microscopy. They were fixed by perfusion of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and processed as previously described (18,20). We performed HE and Masson trichrome staining according to standard histological procedures. Western blot analysis was performed as previously described (18), and the following amounts of protein were loaded per lane: VAMP2, 3, 4 and 8:20 μg, VAMP7:50 μg.

Primary culture of neurons and MEFs

VAMP7-/Y or −/− embryos generated by matings between VAMP7-/Y and +/− mice were dissected on embryonic day 16.5 (E16.5) for hippocampal neuronal culture and for MEF culture. Neurons were dissociated and cultured as previously described (23). In brief, the hippocampi of E16.5 mice were cut into pieces and incubated for 30 min at 30°C in the presence of 0.15% trypsin (type XIII; Sigma) and 0.1% collagenase (Wako). After enzyme inactivation with soybean trypsin inhibitor (600 U/mL; Wako), the tissue fragments were gently triturated with a pipette. Dissociated cells were plated at a density of 2×10^4/mL on 35-mm dishes that had been pretreated with poly-L-lysine and fetal bovine serum. Cells were cultured in DMEM (Gibco-BRL) supplemented with insulin (5 μg/mL), 13 μM ascorbate, 45 nM selenite, 1 nM 3,5,3′-triiodothyronine, 10 pM estradiol, 160 mM glutathione, 150 mM *N*-acetylcysteine, 50 μg/mL transferrin, 10 U/mL superoxide dismutase and 0.3% bovine serum albumin. The cells were fixed and analyzed as described in the next section.

Measurement of the length of axons

Cultured hippocampal neurons were fixed in 3% paraformaldehyde in PBS for 15 min at room temperature, at 12, 24, 36, 48, 60 and 72 h after plating. They were stained with Alexa-488 phalloidin (Invitrogen) to visualize neurites. We also stained neurons 48, 60 and 72 h after plating with an antibody against phosphorylated neurofilament (SMI-31) as described below to identify axons. We observed that many neurons were stained with this antibody 72 h after plating.

The length of axons was measured as follows. From hippocampal neurons, 72 h after plating from three embryos, areas of 317 × 317 μm were photographed at ×40 magnification, and the length of one longest axon in every six areas was measured. The data represent the average ± SD (the total numbers of control and KO neurons were more than 16; $p = 0.019$).

Measurement of axonal markers

As the intensity of GFP was not high in the infected neurons, we used anti-GFP antibody to know the localization of NgCAM-GFP more clearly. Briefly, we fixed the neurons in 3% paraformaldehyde in PBS for 15 min at room temperature. Then, neurons were incubated with anti-GFP antibody in combination with anti-phosphorylated neurofilament antibody in 0.05%

saponin, 5% normal donkey serum in PBS for 1 h at 37°C, washed, and then incubated in Alexa-488 anti-rat immunoglobulin G (IgG) and Alexa-594 anti-mouse IgG in 0.05% saponin, 5% normal donkey serum in PBS for 1 h at 37°C.

The total intensity (amount) of NgCAM-GFP was quite variable from neuron to neuron. Thus, it was very difficult to compare the absolute amount of this marker in the axons between KO and control neurons. Therefore, we counted (1) the number of neurons positive for NgCAM-GFP similarly in axons and dendrites and (2) the number of neurons which had NgCAM-GFP predominantly in axons. We calculated the ratio of 2/(1+2) in control (from three control embryos) and KO (from three KO embryos) neurons and used this ratio as an index of axonal localization of NgCAM.

To know the localization of L1, we incubated the neurons in anti-L1 antisera (22) at 4°C overnight. We used tyramide signal enhance system (Invitrogen) to visualize L1 staining clearly.

Staining of organelles in MEFs

MEFs were isolated from the bodies of embryos by 0.125% trypsin and cultured in 20% fetal calf serum (FCS) in DMEM.

MEFs were fixed in 3% paraformaldehyde in PBS for 15 min at room temperature. For lysosome staining, they were incubated with anti-lamp2 in combination with anti-cathepsin D, Rab7 or syntaxin7 antibodies in 0.05% saponin, 5% normal donkey serum in PBS for 1 h at 37°C, washed, and then incubated in Alexa-488 anti-rat IgG and Alexa-594 anti-goat IgG (or Alexa-568 anti-rabbit IgG) in 0.05% saponin, 5% normal donkey serum in PBS for 1 h at room temperature. For Golgi and ER staining, anti-GS28, or anti-PDI (or KDEL) antibodies were used, respectively.

To know the acidification of lysosomes, we used LysoSensor combined with LysoTracker probes; 50 nM LysoTracker Red DND-99 (Invitrogen) and 1 μM LysoSensor Green (Invitrogen) were added in the culture medium simultaneously and incubated for 3 min at 37°C. The images of MEFs were photographed while the MEFs were alive because after fixation, the punctate lysosomal pattern of LysoTracker and LysoSensor became diffuse and the fluorescence intensity became greatly diminished.

Lysosomes were observed and photographed in live cells under a fluorescence microscope (Olympus). Immunostaining of MEFs and neurons was photographed using confocal microscopy (FV-1000, Olympus or TCS SP5 MP, Leica Japan). Images were processed using Adobe Photoshop (Adobe Systems).

Induction of lysosomal exocytosis (cell scraping assay)

We used cell scraping or ionomycin to induce lysosomal exocytosis. To determine the concentration of β-hex in culture supernatants by cell scraping, MEFs were plated at a density of 1.0×10^5 cells/well in 6-well plates. The 6-well plates were washed by PBS twice and were added 800 μL of PBS per well. Then, the cells were scraped by cell scraper and pipetted 10 times by 1 cc pipetteman (Gilson). Cellular suspensions were collected and centrifuged at 150 × ***g*** for 5 min at room temperature to collect supernatants. The remaining cell pellets were lysed in 0.5% Triton-X-100 in deionized distilled water (DDW) to make cell lysates.

Induction of lysosomal exocytosis (ionomycin treatment)

Ionomycin treatment was performed largely according to the previous reports (17). MEFs were plated in 12-well plates and were cultured to confluency. Before measurement, we washed the cells with PBS twice and then added 300 μL of PBS (+1.2 mM $CaCl_2$) alone or PBS (+1.2 mM $CaCl_2$) with 5 μM ionomycin. After 2, 5 or 10 min, culture supernatants were collected, and the remaining cells were incubated in 300 μL of 0.5% Triton-X-100 in DDW for 15 min at 37°C. After incubation, total cell lysates were collected for measuring the total amount of β-hexosaminidase and LDH.

N-Acetyl-β-D-glucosaminidase (β-hexosaminidase) activity assay

For each sample, 175 μL of the incubation buffer was incubated for 15 min at 37°C with 25 μL of 4 mM 4-methyl-umbellyferyl-*N*-acetyl-β-D-glucosaminide in 20 mM sodium citrate phosphate buffer, pH 4.5. The reaction was stopped by the addition of 50 μL of 2 M Na_2CO_3 and 1.1 M glycine, and the fluorescence was measured in an SH9000 spectrofluorometer (Corona Electric Co. Inc.) at an excitation wavelength of 365 nm/emission wavelength of 450 nm. To determine the total cellular content of β-hex, cell extracts were diluted 1:10 and 175 μL was used for enzyme detection. β-Hex activities were normalized by dividing the value (=activities in the supernatant/total cellular activities) at each time-point by the control value, which is the value at 2 min after stimulation in the presence of ionomycin (=0.335).

LDH activity assay

We used Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Diagnostics GmbH) to measure the amount of LDH. Briefly, for each point, 50 μL of the incubation buffer or 1:10 dilutions of Triton-X-100 extracts prepared as described above were incubated with 100 μL of reaction buffer, incubated for 30 min at room temperature, and terminated by adding 50 μL of stop solution. LDH activities were normalized by dividing the value (=activities in the supernatant/total cellular activities) at each time-point by the control value, which is the value at 2 min after stimulation in the presence of ionomycin (=0.816).

Staining of exposed lamp1 on the plasma membrane

MEFs treated with PBS (+1.2 mM $CaCl_2$) alone or PBS (+1.2 mM $CaCl_2$) with 5 μM ionomycin for 10 min at 37°C were fixed in 3% paraformaldehyde in PBS. They were incubated with anti-lamp1 antibody in 5% normal donkey serum in PBS for 1 h at 37°C, washed, and then incubated in Alexa-488 anti-rat IgG in 5% normal donkey serum in PBS at 37°C with DAPI (4′,6-diamidino-2-phenylindole).

T. cruzi *and host cells*

HT 1080 cells, a human fibrosarcoma cell line obtained from Japan Health Sciences Foundation, were used as an *in vitro* host. The Tulahuene strain of *T. cruzi* (24) and HT1080 cells were maintained and passaged in culture as previously described (25,26).

In vitro T. cruzi *infection and determination of infection rate*

MEFs from control and KO mice were plated at a density of 10 000 cells in a single well of a 24 well-plate. After overnight incubation, the cells were infected with *T. cruzi* trypomastigotes (2.5×10^5 parasites/well) from the culture medium of HT1080 subcultures, as previously described (27). Infected MEFs were fixed and stained with Diff-Quik (Sysmex). The cells were embedded in HSR solution for observation under a microscope. The percentage of infected host cells, including those containing more than one amastigote, was determined by analyzing more than 200 host cells.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: An enlarged and merged figure of Figure 6C. This figure is presented to better examine the staining of lysosomes at higher magnification because VAMP7 has been believed to be important in lysosomal function.

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SHORT COMMUNICATION

A novel C-type lectin identified by EST analysis in tissue migratory larvae of *Ascaris suum*

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Abstract C-type lectins (CTLs) are a group of proteins which bind to carbohydrate epitopes in the presence of Ca^{2+}, which have been described in a wide range of species. In this study, a cDNA sequence coding a putative CTL has been identified from the cDNA library constructed from the pig round worm *Ascaris suum* lung L3 (LL3) larvae, which was designated as *A. suum* C-type lectin-1 (As-CTL-1). The 510 nucleotide open reading frame of As-CTL-1 cDNA encoded the predicted 169 amino acid protein including a putative signal peptide of 23 residues and C-type lectin/C-type lectin-like domain (CLECT) at residue 26 to 167. As-CTL-1 was most similar to *Toxocara canis* C-type lectin-1 and 4 (*Tc*-CTL-1 and 4), and highly homologous to namatode CTLs and mammalian CTLs as well, such as human C-type lectin domain family 4 member G (CLECG4). In addition, As-CTL-1 was strongly expressed in tissue migrating LL3 and the L4 larvae, which were developmental larvae stages within the mammalian host. These results suggest that *A. suum* larvae might utilize As-CTL-1 to avoid pathogen recognition mechanisms in mammalian hosts due to it is similarity to host immune cell receptors.

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Introduction

C-type lectins (CTLs) constitute a large family of proteins that binds carbohydrate moieties in a Ca^{2+}-dependent manner (Drickamer 1988, 1996). They are characterized by a conserved C-type lectin/C-type lectin-like domain (CLECT) which shares Ca^{2+}- and carbohydrate-binding motifs. CLECT also contains at least four critical cysteine residues which form a two-loop structure by disulphide bounds. It is well known that CTLs are widely expressed among metazoan organisms (Drickamer and Fadden 2002; Zelensky and Gready 2005). In vertebrates, CTL represents a very large family that is classified into 17 groups (Drickamer and Fadden 2002), many of which are known as pattern-recognition receptors implicated in the recognition of pathogens by innate immunity (Weis et al. 1998). In addition, some evidence have indicated that CTLs play an important role in immune homeostasis by endogenous 'self' ligand recognition (García-Vallejo and van Kooyk 2009), and they themselves have a bactericidal activation (Cash et al. 2006).

Ascaris suum, a common round worm in pigs, is infective to a wide range of hosts, including humans, mice, cattle and chickens. When embryonated eggs are ingested by a definitive swine host, larvae hatch in the small intestine, penetrate the intestinal mucosa, and migrate through the liver and lungs, before finally reaching the intestine, where they sexually mature and produce eggs (Dold and Holland 2011). In contrast, it is generally considered that larvae, which are reached the lungs following liver migration, disperse into various tissues and

organs without further development in non-swine host (Slotved et al. 1998; Crompton 2001), although *A. suum* has been reported to develop into adult stage infrequently in human hosts (Anderson 1995; Nejsum et al. 2005; Arizono et al. 2010). However, it has not been fully explained how they discriminate pigs and other animals and what kind of interaction is involved between host and parasite during the lung phase of migration.

Expressed sequencing tag (EST) analysis is a powerful tool for profiling the gene expression pattern in a particular parasite population. Although publicly available EST databases of *A. suum* already exist in NAMBASE4 (http://www.nematodes.org/nembase4/index.shtml), they were constructed from adult worm, intestinal L4 larvae, newly hatched infective larvae (iL3) and egg embryos. EST analysis of tissue-migrating larvae has not yet been performed. Therefore, we explored cDNA of *A. suum* lung L3 (LL3) collected from infected rabbit lungs in order to examine what kind of biological processes were activated in tissue-migrating larvae. As one of the most frequently occurring clones, we identified a cDNA sequence for a putative *A. suum* CTL that showed specific expression during internal larval stages in mammalian hosts.

Materials and methods

Parasites and infection

Adult *A. suum* were collected from infected pigs at a local abattoir in Japan. Eggs were freed from the uterine tissue by incubating uteri in 0.1 N NaOH. After washing with distilled water, eggs were suspended and stirred in 0.1 N H_2SO_4 and cultured at 27°C for 3–4 weeks. Infective L3 larvae (iL3) were mechanically hatched from eggs and isolated free from egg shell contaminants (Takamiya et al. 1993). For the preparation of lung L3 larvae, male Japanese white rabbits (Kyudo, Kumamoto, Japan) were orally inoculated with 1.5×10^5 embryonated eggs. Six days after infection, the lungs were removed and cut into 5-mm cubes using scissors. The cubes were wrapped with Kimwipe papers and incubated in phosphate-buffered saline (PBS) at 37°C for 1.5 h, and then emerging worms were collected. Culture driven-L4 larvae (cL4) were obtained from cultures of LL3 in vitro (Islam et al. 2006).

RNA isolation and cDNA library construction

Total RNA of LL3 was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA), followed by purification of poly $(A)^+$ RNA with GenElute™ mRNA Miniprep Kit (Sigma, St. Louis, MO). A cDNA library was constructed using the SMART cDNA Library Construction Kit (Clontech, Mountain View, CA). The reverse transcription step was performed using MMLV Reverse Transcriptase with the SMART IV oligonucleotide primer and the CDS III/3′ PCR primer provided in the kit. The double-stranded cDNA (ds-cDNA) was synthesized by long distance PCR with the 5′ PCR primer and the CDS III/3′ PCR primer using the Advantage 2 PCR kit (Clontech). The ds-cDNA was treated with proteinase K and then digested by *Sfi*I. After size fractionation, cDNA was cloned into pDNR-LIB vector, and transformed into *Escherichia coli* ElectroMAX™ DH10B™ cells (Invitrogen, Carlsbad, CA).

EST sequencing, processing and analysis

Plasmid DNA of the 2,024 randomly selected clones was extracted and single-pass sequenced from the 5′-end using sequencing primer (5′-GCATACATTATACGAAGTTAT CAGTCG-3′). The sequencing was conducted on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA), using ABI Prism Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). EST sequences were clustered using SEQUENCHER (Gene Codes Corporation, Ann Arbor, MI), with a minimum sequence overlap length cut-off of 30 bases and an identity threshold of 90%, for the removal of flaking vector and adaptor sequences, followed by assembly. These contigs and singletons were subjected to BLASTN and BLASTX programs (E value of $\leq1\times10^{-5}$) at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A protein sequence motif was identified using the InterProScan at The European Bioinformatics Institute (Zdobnov and Apweiler 2001). Alignment of ESTs was conducted by using GENETYX-WIN software (Genetyx Corporation, Tokyo, Japan).

Real-time PCR analysis

Total RNA from iL3, LL3, cL4 and adult worm tissues (head, muscle, intestine, uterus, ovary and testis) was extracted with TRIzol reagent. After treatment with DNaseI (Ambion Inc., Austin, TX), cDNA was generated from 250 ng of total RNA using PrimeScript® 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan). Primer sets for amplification were as follows: As CTL-1 (sense, 5′-CCACCATGTTCTCGACCGTTGCT-3′; antisense, 5′-ATTTCCTCCTACTGGCGCTCCT-3′) and 18S ribosomal RNA gene (sense, 5′-ATCGGTCGCGTAGGGTGGCT-3′; antisense, 5′-AAGCCGCAGGCTCCACTCCT-3′). Real-time PCR was then performed with an ABI Prism 7000 Sequence Detection Systems (Applied Biosystems) and a GoTaq® qPCR Master Mix (Promega, Madison, WI). Relative quantification was assessed by normalizing the amount of the target transcript to the 18S ribosomal RNA gene.

Results and discussion

In paratenic hosts such as humans, larvae of *A. suum* penetrate the mucosal epithelium but thereafter remain developmentally arrested in the migratory tissue phase (Crompton 2001). To understand biological events taking place in the arrested larvae, we carried out EST analysis in a cDNA library of migrating L3 larvae (LL3) collected from infected rabbit lungs (LL3). As a result from 5′ ends single-pass sequencing of 2,024 clones, 1,650 ESTs were yielded. Upon clustering, these ESTs were represented by 279 distinct gene products, which consist of 78 contigs and 201 singletons.

The consensus sequences of contigs and singletons were compared against NCBI BLAST databases in BLASTX analysis, revealing a novel CTL sequence referred to as *A. suum* C-type lectin-1 (As-CTL-1). We focused on this molecule, because CTL might contribute to the establishment of successful parasitism in nematodes (Loukas et al. 1999; Urwin et al. 2002). Using RT-PCR, the full-length cDNA corresponding to As-CTL-1 was successfully amplified from LL3 RNA (data not shown). As-CTL-1 is 710 nucleotides (GenBank accession no. HQ025087), which encoded the protein of 169 amino acids including the putative signal peptide of 23 residues and C-type lectin/C-type lectin-like domain (CLECT) at residue 26 to 167. The four cysteine residues at positions 62, 136, 154 and 166, which are required to form the CLECT internal disulfide bridge formations (Zelensky and Gready 2003), were completely conserved in As-CTL-1, but the WIGL and WND motifs conserved in the classical CTLs (Zelensky and Gready 2003) were replaced by WLAL and WDD. According to carbohydrate specificity, CTLs are categorized into mannose/GlcNAc - or galactose/GalNAc -recognizing lectins (Weis et al. 1992; Iobst and Drickamer 1994). These differences suggest substitutions at the key substrate binding residues. As-CTL-1 had QPD, which was found in galactose/GalNAc-binding CTLs. Thus, although the Ca^{2+}-dependent carbohydrate binding activity of As-CTL-1 was not assessed in this study, it is most likely a galactose-binding CTL from its amino acid motifs.

Subsequent sequence analysis showed that the amino acid sequence of CLECT in As-CTL-1 was found to have 33% identity to canine roundworm *Toxocara canis* C-type lectin-1 (*Tc*-CTL-1) and 38% identity to *Tc*-CTL-4. It has been reported that the free-living nematode *Caenorhabditis elegans* has more than 270 genes encoding CLECT of CTLs in their genome (Schulenburg et al. 2008). Interestingly, As-CTL-1 showed greater identity with human CTL domain family 4 member G (CLECG4; 28% identity) than with the *C. elegans* homologue (clec-149; 24% identity). Considering the eukaryotic phylogeny, it is reasonable that several CTLs from parasitic nematodes, such as *Ancylostoma ceylanicum* (AceCTL-1), *Necator americanus* (NaCTL-2), *Heligmosomoides polygyrus* (*Hp*-CTL-1) and *Nippostrongylus brasiliensis* (*Nb*-CTL-1 and 2) CTLs, share greater identity with *C. elegans* CTLs than mammalian CTLs (Brown et al. 2006; Daub et al. 2000; Harcus et al. 2009). On the other hand, *Tc*-CTL-1, *Tc*-CTL-4 and NaCTL-1, as well as As-CTL-1, appear to be much closer to homologues in mammalian CTLs than those in *C. elegans* (Loukas et al. 1999, 2000; Daub et al. 2000), raising speculations about the role they might play in the adaptation to parasitism.

The expression of As-CTL-1 was evaluated by real-time PCR in different developmental stages, including mechanically hatched iL3, LL3, cL4 and adult (Fig. 1). The mRNA for As-CTL-1 was scarcely detected in iL3 and tissues from adult worms, including the head, muscle, intestine, uterus, ovary and testis, whereas LL3 and cL4 larvae showed strong expression of As-CTL-1 transcript. After arriving at the jejunum, L3 larvae developed to L4 stage larvae in definitive swine host in vivo. These results indicate that the expression of As-CTL-1 is up-regulated through the tissue migrating stage and intestinal larval stage.

Although the physiological function of CTLs remains unclear, a number of nematode CTLs identified so far act as a pathogen recognition molecule or an antibacterial protein in immune responses to protect the worm itself against microbial infection (O'Rourke et al. 2006; Schulenburg et al. 2008). However, considering that the expression of As-CTL-1 is confined during tissue migration, it would not be very likely that As-CTL-1 is employed for the recognition of microbes in *A. suum*, because the worms may not encounter hazardous bacteria on the migrating route, which would be maintained clean by the host immunity. Instead, greater sequence identity that As-CTL-1 shares with mammalian CTLs than *C. elegans* proteins, seems to suggest that As-CTL-1 acts as a

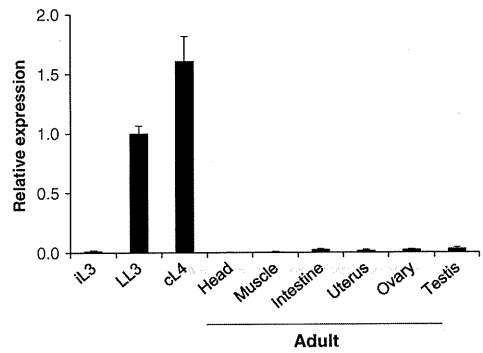

Fig. 1 Comparison of As-CTL-1 mRNA expression in developmental stages. Real-time PCR was performed with mechanically hatched iL3, LL3, cL4 and various adult worm tissues (head, muscle, intestine, uterus, ovary and testis). Relative expression of the As-CTL-1 mRNA was assessed by normalizing to 18S rRNA expression. Data were expressed as a ratio to As-CTL-1 gene expression in LL3

competitor for host cell receptors, possibly interfering host immune response to the worms (Loukas and Maizels 2000; Loukas and Prociv 2001). As-CTL-1 might be able to bind to ligands for mammalian CTL, such as NK receptors and/or macrophage/dendritic cell receptors (Osorio and Reis e Sousa 2010; Sun and Lanier 2011). In the present study, As-CTL-1 showed high similarity to *Tc*-CTLs and specific expression in LL3 and cL4, both of which were exposed to attack by host immune responses. Considering these findings, *A. suum* larvae might interfere with host inflammation processes by As-CTL-1 to avoid protective immune responses in infected animals during tissue migration. Further study on functional aspects of this molecule will identify novel dimensions of the host–parasite relationship and the significance of tissue migration in ascarid infection.

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Short Communication

Eosinophilic Pneumonia Due to Visceral Larva Migrans Possibly Caused by *Ascaris suum*: a Case Report and Review of Recent Literatures

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SUMMARY: We report the case of a 62-year-old man who developed eosinophilic pneumonia due to visceral larva migrans (VLM) that was possibly caused by *Ascaris suum*. The patient, a resident of the middle Kyushu area who was fond of eating raw porcine liver, complained of dry cough without dyspnea. The chest radiography showed a migration of infiltrative shadow. Transbronchial lung biopsy of the right middle lobe revealed massive infiltration of eosinophils. The multi-dot enzyme-linked immunosorbent assay (ELISA) and microtiter plate ELISA showed positive results for *A. suum*; therefore, the patient was diagnosed with VLM caused by *A. suum*. The patient was administered albendazole (600 mg/day) for 28 days; he recovered successfully with no adverse effects except mild liver dysfunction. Several cases of VLM caused by *A. suum* have been reported in Japan, with a majority of the cases being reported in Kyushu. Careful history taking of the patient's area of residence and dietary habit is essential for the diagnosis of this parasitic disease with underestimated prevalence.

Visceral larva migrans (VLM) caused by *Ascaris suum* is a major parasitic infection that especially affects people living in southern Kyushu, Japan, which has a prominent livestock industry (1). *A. suum* infects pigs, and 30% of all the pigs in southern Kyushu are infected (2). Humans are usually infected when they eat the raw liver or meat of infected cattle or chicken or fresh vegetables grown in soil fertilized with porcine excrement contaminated with *A. suum* eggs. In humans, the larvae of *A. suum* migrate to various organs and cause a wide variety of nonspecific symptoms such as general malaise, cough, liver dysfunction, hypereosinophilia with hepatomegaly and/or pneumonia (3,4). Here, we report a case of eosinophilic pneumonia resulting from VLM that could have been possibly caused by *A. suum*, and present a review of the recent literature on VLM.

A 62-year-old man living in Shimabara, Nagasaki Prefecture, Kyushu, Japan, was referred and admitted to Izumikawa Hospital because he had dry cough and chest radiography had shown an infiltration shadow in both lungs. All in one cold and flu capsules prescribed in a previous clinic had not been effective. The patient complained of dry cough without dyspnea at the time of admission. He had no remarkable underlying diseases, although he possessed a unique dietary habit such as eating raw porcine, chicken, and cattle livers.

At the time of admission, the vital signs of the patient were as follows: body temperature, 36.8°C; heart rate, 72 beats/min (regular rhythm); respiratory rate, 16 breaths/min; and blood pressure, 110/60 mmHg. Auscultation revealed no abnormal pulmonary crackles or heart sounds. The patient showed no clinical signs of lymphadenopathy, hepatosplenomegaly, and pretibial edema.

Chest radiography showed an infiltrative shadow in both the middle and lower lung fields (Fig. 1A), and computed tomography (CT) showed consolidation with ground-glass opacity in both lung fields (Fig. 1B and 1C). The laboratory test results were as follows: leukocytes count, 9.5 × $10^3/\mu L$; eosinophil count, 2,175/μL (22.9%); C-reactive protein (CRP) concentration, 0.4 mg/dL; and IgE level, 333.2 IU/mL. All other results were within the normal range. The results of the arterial blood gas (ABG) analysis at room air were as follows; pH, 7.404; PaO_2, 72.6 Torr; and $PaCO_2$, 40.8 Torr. Routine microbiological tests revealed no causative bacteria. Bronchoscopy was performed and the bronchoalveolar lavage (BAL) fluid was analyzed; the results of the cell count analysis were as follows: alveolar macrophages, 2%; eosinophils, 93%; lymphocytes, 3%; and basophils, 2%. No microorganisms, including fungi and mycobacteria, could be isolated from the BAL fluid in routine microbiological tests. Although the polymerase

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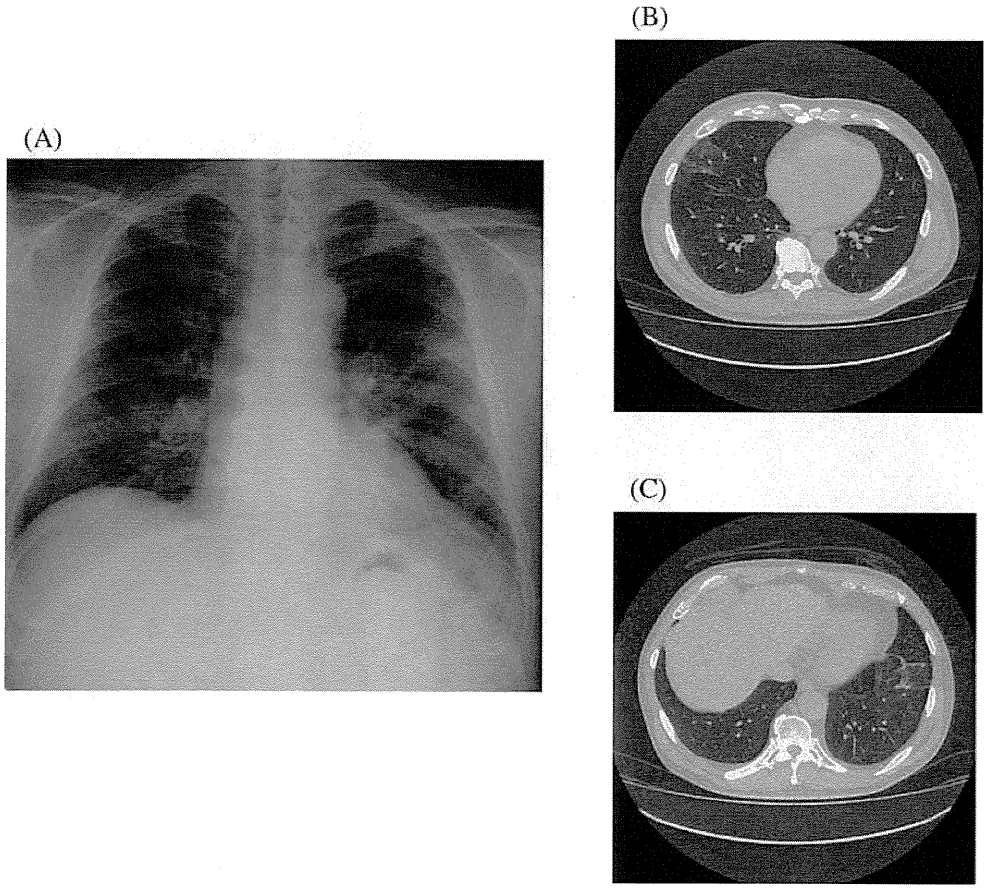

Fig. 1. Chest X-ray films on admission. (A) Chest X-ray film showing infiltrative shadow in both middle and lower lung fields. (B) and (C) CT scan images showing consolidation with ground-glass opacity at right upper lobe and left lower lobe.

chain reaction test for mycobacteria showed positive results for *Mycobacterium intracellulare*, an 8-week culture of the BAL fluid sample showed negative growth. Transbronchial lung biopsy of the right middle lobe (B_4) revealed massive infiltration of eosinophils in the parenchyma and that of alveolar macrophages in the alveoli. Eosinophilic pneumonia was diagnosed on the basis of the results of this pathological analysis (Fig. 2). Multi-dot enzyme-linked immunosorbent assay (multi-dot ELISA) was performed for detecting anti-parasitic antibodies in the patient's serum (5). A serum sample of the patient showed positive results for *Dirofilaria immitis, A. suum*, and *Gnathostoma doloresi* but negative results for *Toxocara canis* (the test was not performed for *T. cati*). A microtiter plate-ELISA for the semi-quantitative measurement of the antibodies for the three parasites (6) was performed, and the strongest reaction was observed for *A. suum* antibodies. Since the patient had a history of eating raw porcine liver, we diagnosed his condition as eosinophilic pneumonia due to VLM that was possibly caused by *A. suum*.

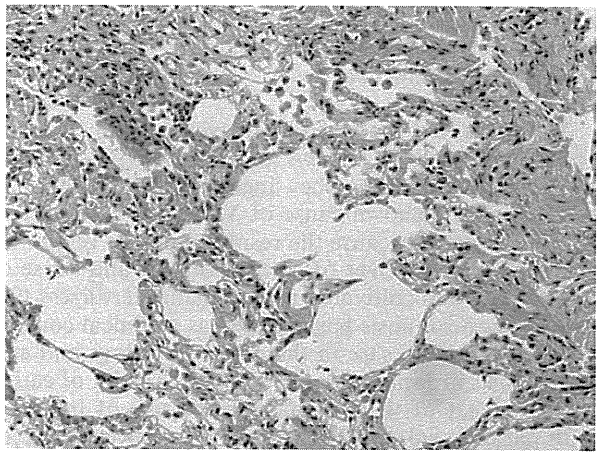

Fig. 2. Pathology of transbronchial lung biopsy from the right middle lobe (right B_4) demonstrates severe eosinophil infiltrations in lung parenchyma. HE stain, ×40.

The patient received no treatment during an observation period of 17 days after the diagnosis of VLM, and the clinical symptoms and signs such as cough and hypereosinophilia persisted. Chest radiography performed on day 17 after the admission showed that the infiltrative shadow in the right middle and lower lung fields had migrated, and CT showed new consolidation with ground-glass opacity in the right upper and lower lobes of the lungs (Fig. 3A, 3B, and 3C). The patient was administered albendazole (600 mg/day) for 28 days. The clinical symptoms resolved completely, and the eosinophil count decreased to 390/μL. The infiltra-

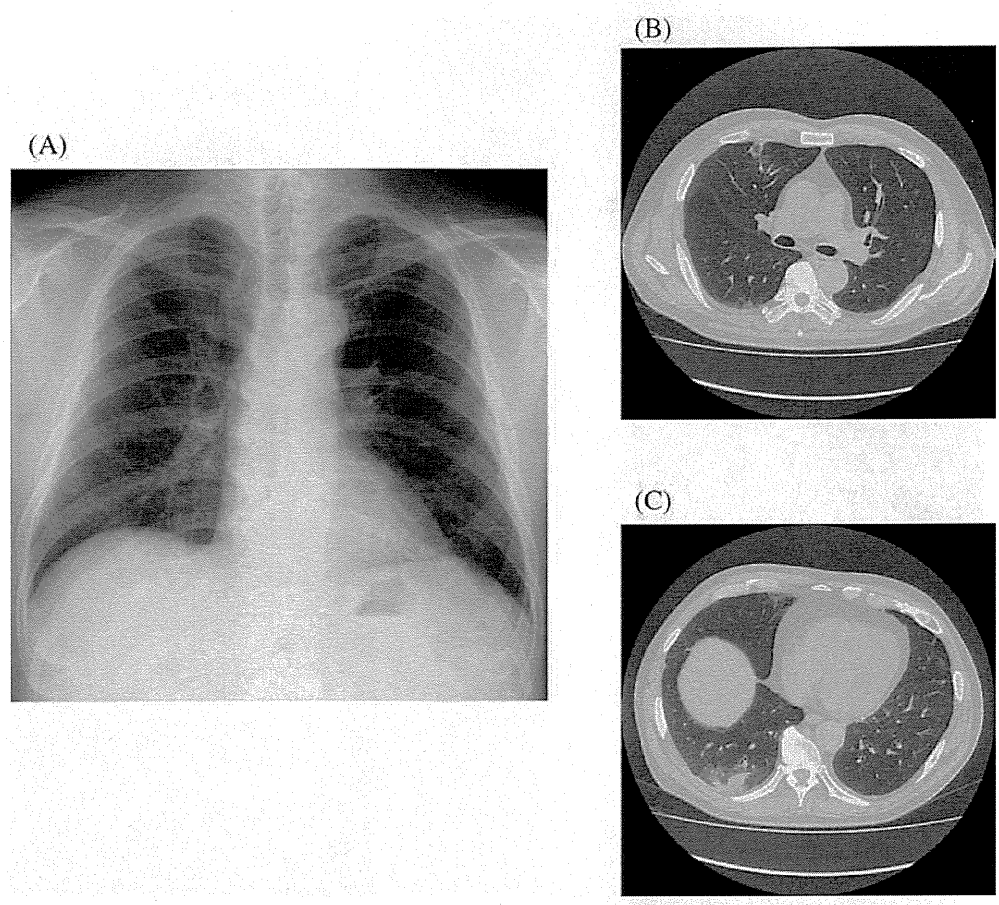

Fig. 3. Chest X-ray films before albendazole treatment (17 days after admission). (A) Chest X-ray film showing infiltrative shadow in right middle and lower lung fields and migrated from the time of admission. (B) and (C) CT scan images showing new consolidation with ground-glass opacity at right upper and lower lobes.

tive shadow disappeared completely in 28 days. No adverse effects except mild liver dysfunction were noted during the 28 days. No recurrence was observed after discharge.

VLM was first described by Beaver et al. in 1952 and is mainly caused by *T. canis* and *T. cati* (3,7). Humans become infected when they ingest *Toxocara* eggs. *A. suum* is also a known cause of VLM (8), especially in Kyushu, Japan, because the residents of this region eat the raw meat and liver of cattle, poultry, and horse or fresh vegetables cultivated using organic fertilizers (9).

VLM as a zoonosis has emerged as a clinical concern because of an increase in the number of dogs and cats kept as pets in Japan. People who have a habit of eating the raw meat of wild animals are at risk of infection with parasitic worms. The current trend of eating fresh vegetables as a part of a healthy lifestyle also increases the risk of infection with parasitic worms since fresh vegetables may be contaminated with them.

A definitive diagnosis of VLM is possible only if the larvae of *Toxocara* or *Ascaris* are found in the patient's body; however, detecting these larvae is quite difficult and not practical. To date, no suitable or applicable molecular methods are available for accurately detecting the genomic DNA of parasites. The multi-dot ELISA method (5), performed using the patient's serum sample, is a useful and convenient tool for diagnosing VLM. Although it is a simple method, cross-reactions among the parasite antigens have been observed. Therefore, a definite diagnosis of VLM cannot be made unless the larvae or DNA of the causative organism, such as *Toxocara* or *Ascaris*, is detected in the patient's body. Information such as the patients' area of residence and their dietary habits should be obtained and carefully evaluated by attending physicians.

An outbreak of VLM caused by *A. suum* in Japan was reported by Maruyama et al. (6) in 1996, with a total of 17 patients with pronounced eosinophilia and high antibody titers against the *A. suum* antigen. A review of recent literature on *A. suum* cases showed that at least 9 cases of VLM in Japan were reported in various journals after 1996 (Table 1). All the patients, except 2, were infected in the Kyushu island, and possible sources of infection were the raw meat of chicken, boar, deer, and cattle (5 patients); vegetables (2 patients); raw meat of poultry or horse, raw liver of cattle or vegetables cultivated using organic fertilizer (1 patient); and unknown (1 patient). Because the eggs of *A. suum* could not be detected in these possible sources, the apparent source and route of infection were not confirmed. Almost all the patients showed high levels of serum IgE and hypereosinophilia. Albendazole and ivermectin were administered and were effective in 7 patients and 1 patient, respectively. Only one patient was diagnosed

Table 1. Summary of cases of visceral larva migrans due to *Ascaris suum* in Japan

Study (ref. no.)	Year	Age (y)	Sex	Place	Case	Possible source of *A. suum*	Eosinophil (/μl)	Serum IgE (IU/ml)	Treatment
Matsushita et al. (12)	1997	70	Female	Miyazaki, Kyushu	Eosinophilic pneumonia, + intrahepatic lesion	Raw chicken	9,440	7,022	Albendazole
Takeyama et al. (13)	1997	56	Female	Kyushu	Eosinophilic colitis	N.A.	7,872	10,960	Predonisolone
Matsuyama et al. (14)	1998	46	Male	Kagoshima, Kyushu	Eosinophilic pneumonia	Fresh vegetables cultivated using pig manure	9,188	3,190	Ivermectin
Arimura et al. (15)	2001	26	Male	Miyazaki, Kyushu	Pulmonary nodule	Raw boar, deer meat	750	926	Albendazole
Arimura et al. (15)	2001	57	Male	Miyazaki, Kyushu	Pulmonary nodule	Raw chicken, turkey	342	832	Albendazole
Sakakibara at al. (16)	2002	32	Male	Aichi, Honsyu	Eosinophilic pneumonia, + intrahepatic lesion	Fresh vegetables cultivated using organic fertilizer, raw meat of cattle liver, poultry meat, horsemeat	10,773	20,284	Albendazole
Sakurai et al. (17)	2003	25	Female	Tokyo, Honsyu	Eosinophilic pneumonia	Raw liver of caw	7,290	98	Albendazole
Tokojima et al. (18)	2004	50	Male	Kagoshima, Kyushu	Eosinophilic pneumonia	Vegetables	445	1,208	Albendazole
Hirakawa et al. (19)	2009	64	Male	Kagoshima, Kyushu	Eosinophilic pneumonia	Raw chicken liver	4,223	279	Albendazole

All cases are diagnosed with multi-dot enzyme-linked immunosorbent assay.
Outcome of all cases are cured.
N.A., not available.

with eosinophilic colitis and was administered predonisolone but not albendazole.

In the present case, the follwoing clinical signs were consistent with those of VLM: (i) remarkable eosinophilia and high IgE levels, (ii) positive results in the multi-dot ELISA and the strongest reaction for the antibody for *A. suum*, in microtiter plate-ELISA, (iii) migration of the pulmonary infiltrates, and (iv) eosinophilic pneumonia, as diagnosed on the basis of the results of BAL fluid analysis and transbronchial lung biopsy. Although there is little evidence in favor of any treatment modality for VLM caused by *A. suum*, administration of albendazole or ivermectin for 2 to 3 weeks is recommended (10,11). The patient was administered albendazole (600 mg/day) for 28 days, and he showed no remarkable adverse effects except mild impairment of liver function (a common adverse effect of albendazole). Although most of the cases of VLM caued by *A. suum* are not fatal, VLM could sometimes become life threatening if a large number of *A. suum* eggs are ingested (8). It is important for clinicians to consider VLM caused by *A. suum* in case a patient presents hypereosinophilia, high IgE levels, and a migrating pneumonia shadow in addition to various nonspecific symptoms. Careful history taking of the patients' area of residence and dietary habit is essential for the diagnosis of this parasitic disease with underestimated prevalence. Furthermore, although VLM caused by *A. suum* is most prevalent in Kyushu, a couple of VLM cases have been reported in the Honshu region as well (Table 1). Owing to advances in mass-transportation of fresh vegetables and meat and improvement of the related logistics, cases of such originally localized parasitic infections are now being detected in other areas of Japan and even in other countries.

Conflict of interest None to declare.

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Case Report

A patient who developed toe necrosis due to poor blood circulation after an interdigital tick bite

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KEYWORDS
Tick bite;
Toe necrosis;
Thrombogenic vasculopathy

Summary A 71-year-old female had worked on a farm in the mountains and noticed itching of the left 3rd toe. She visited a local hospital due to a color change to purple in this area. Attachment of a tick was observed between the left 2nd and 3rd toes, and it was extracted. However, due to persistent pain, she was referred to our department of cardiovascular medicine for close examination and treatment. Lower extremity angiography showed that vascular visualization was poor in the area supplied by the arteries distal to the tick bite site, but the other blood vessels of the toe were clearly visualized. Toe amputation was performed and pathological examination of a surgical specimen revealed that most blood vessels near the necrosis were occluded by thrombi. We speculated that tick bite reactions were associated with thrombogenic vasculopathy. This report shows a patient who developed toe necrosis due to poor blood circulation after an interdigital tick bite.

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Introduction

Tick bites are relatively frequently encountered in daily clinical practice. Most patients with tick bites develop dermatitis, but some develop Japanese spotted fever or Lyme disease via the bite. However, there have been no reports of peripheral necrosis due to poor blood circulation following tick bites. We report a patient who developed toe necrosis due to poor blood circulation after an interdigital tick bite.

Case report

A 71-year-old female had previously been healthy, and, although she had annually undergone a human dry dock, no abnormalities had been detected. She had worked on a farm in the mountains on June 7, 2009, noticed itching of the left 3rd toe on June 12, and visited a local hospital due to a color change to purple in this area on June 16. Attachment of a tick was observed between the left 2nd and 3rd toes. The tick was extracted, and minocycline was prescribed. However, due to persistent pain, she was referred to our department of cardiovascular medicine for close examination and treatment on June 23. She was obese, but had no history of smoking, hypertension, diabetes mellitus, or lipid abnormalities. The bilateral dorsalis pedis and posterior tibial arteries were palpable, and the skin temperature of the foot was normal. There was a decrease in the skin temperature, loss of sensation, and a color change to black distal to the middle phalanx of the left 3rd toe (Fig. 1A). The area between the 2nd and 3rd toes as the site of the tick bite showed erosion, but not redness or swelling (Fig. 1B). No eruptions were observed in the other areas. The thermography (Fig. 1C) showed an increase in the skin temperature in the left compared with the right foot. In the portion peripheral to the proximal interphalangeal joint of the left 3rd toe, the skin temperature showed a decrease in the area corresponding to that showing black necrosis. Lower extremity angiography (Fig. 1D) was performed with selective enhancement of the left external iliac artery. The area extending to the plantar artery and arch was clearly visualized, and no atherosclerotic changes were identified. Vascular visualization was poor in the area supplied by the arteries distal to the tick bite site, but the other blood vessels of the toe were clearly visualized. She was referred to the department of plastic surgery of our hospital on July 3, and toe amputation was considered to be indicated. On July 24, toe amputation was performed (Fig. 2A and B). Pathological examination of a surgical specimen revealed necrosis of the toe tip, and most blood vessels near the necrosis were occluded by thrombi. Between fat tissues, foam cells aggregated, and marked eosinophil infiltration was observed. There were no findings of angiitis (Fig. 2C and D). Her postoperative course was favorable. After walking became possible, she was discharged on August 4.

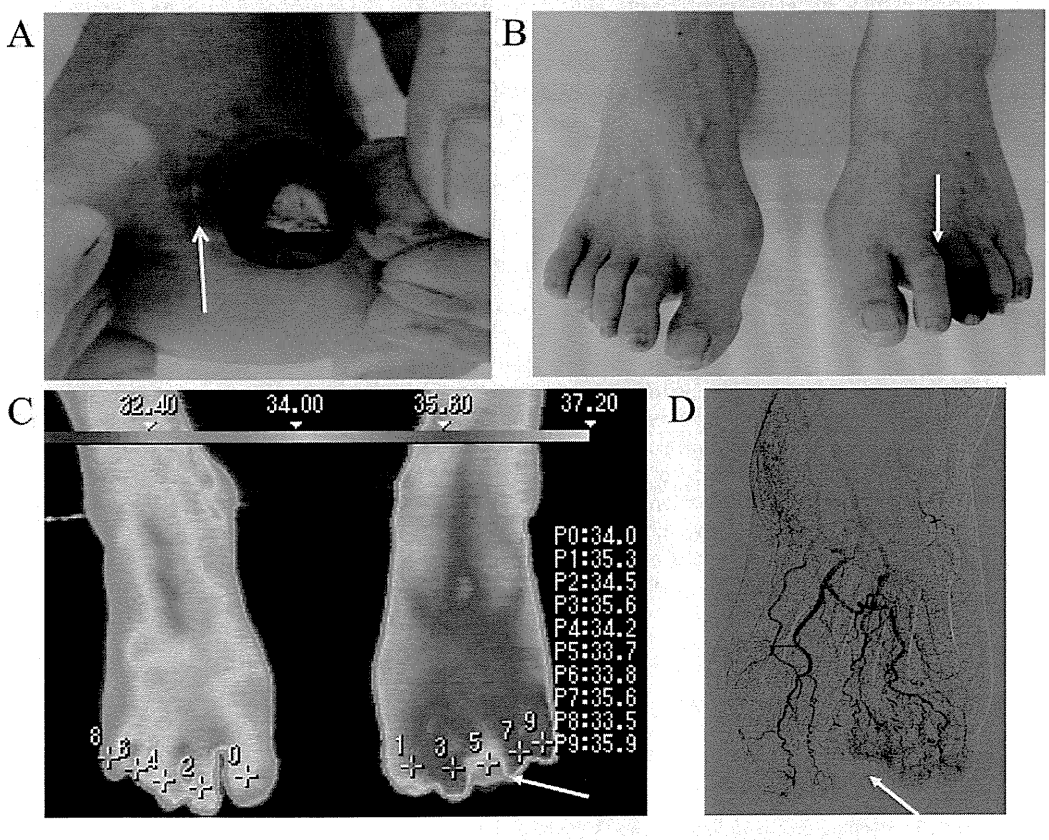

Figure 1 (A and B) Photo of the left toes (white arrow: tick bite site). (C) Thermography (white arrow: a decrease in the skin temperature was observed in the area corresponding to the site of black necrosis). (D) Lower extremity angiography (white arrow: blood flow interruption was confirmed in the area corresponding to the site of black necrosis).

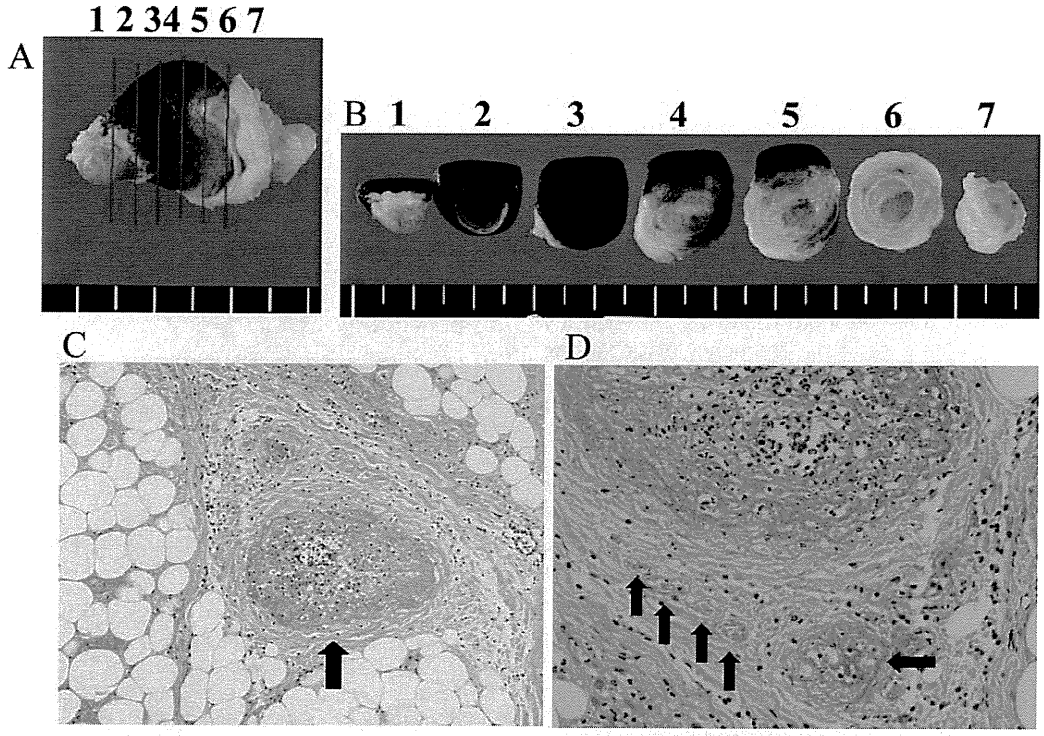

Figure 2 (A) Surgical specimen. (B) Cross-section of the surgical specimen. (C) Image of the pathological specimen (Cross-section 4, black arrow: blood vessels near the necrosis were occluded by thrombi. In the vascular lumens, granulomatous tissue formed, but reperfusion was observed in some parts. Hematoxylin-eosin staining). (D) Image of the pathological specimen (Cross-section 4, black arrows: microscopic vessels occluded by thrombus, hematoxylin-eosin staining).

Discussion

When this patient visited our department, about 2 weeks had passed since the tick bite. Since she had already been treated by a local doctor, and the tick had been extracted, we could not directly confirm the tick. However, in the southern part of Tokushima Prefecture where she lives, tick bites are often observed, and so the diagnosis of a tick bite made by the previous doctor may be accurate. There has been a report [1] of patients with tissue necrosis in a tick bite area, but no report of patients with necrosis distance from the bite site. Previous studies have shown blood coagulation responses as biological responses to ticks [2] and pathologically confirmed thrombi in about 66% of tick bite areas [3]. Histopathological examination in our patient showed the occlusion of many blood vessels by thrombi in the area near necrosis and the presence of necrosis peripheral to these thrombi. In general, tick bites lead to reactions such as the extravascular leakage of dermal erythrocytes, hardening of collagen fibers, epidermal necrosis, ulceration, dermal neutrophil infiltration, and thrombosis [3]. However, the saliva of ticks contains anti-hemostatic factors and immunosuppressive and anti-inflammatory components, which make tick removal difficult, facilitating long-term blood-feeding [4]. The intractability of areas bitten has been suggested to be associated with the formation of a foreign body granuloma due to remnant tick mouth parts, impairment of the wound healing process by fibrosis-promoting factors contained in the saliva, and ischemia in the wound area due to various types of local microvasculitis [5]. Since no pathological examination of the tick bite area was performed, detailed findings of this area such as the possible presence of thrombi could not be obtained. However, we speculated that thrombi induced by the tick bite caused peripheral embolism, and impaired peripheral blood circulation resulted in necrosis. We supposed that the involvement of angiitis was probably denied because the value of myeloperoxidase – antineutrophil cytoplasmic antibody was very small. Additionally, we denied the presence of shaggy aorta which was associated with blue toe syndrome by use of contrast-enhanced computed tomography. Coronary angiography was also performed in this patient, but showed no findings of stenosis. Lower extremity angiography also revealed no atherosclerotic findings anywhere except the occluded areas, most strongly suggesting the involvement of thromboembolism. Therefore, tick bites in areas with a few collateral circulation routes are associated with a risk of peripheral necrosis, and require caution. The thermography showed an increase in the foot skin temperature on the affected side. This may be an immune response or biological response such as vascular dilation in the central area due to impaired peripheral blood circulation, but the details were unclear. Dermatitis due to tick bites is often encountered in clinical practice. The most interesting point is the difference between patients with dermatitis not inducing serious conditions and those such as in the present patient who develop marked thrombotic circulatory impairment. Although an intractable case showing

local periarteritis nodosa after a tick bite to the thigh has been reported [6], peripheral necrosis was not induced in this case. We speculate that the degree of a host's immune tissue responses to ticks and the bite site are important factors associated with whether necrosis is induced. Tick bites in the peripheral areas of the four limbs, as in this patient, have the potential to induce necrosis because there are only a few collateral circulation routes, and so require caution. The characteristics of cases that show marked immunohistological responses at the tick bite site are unclear. A previous study showed that inflammatory reactions became marked, and severe inflammatory cell infiltration was histologically confirmed with an increase in the frequency of tick biting [7]. Our patient had no history of treatment for tick bites, but was a farmer, and so it is likely that she had a history of untreated asymptomatic tick bites. Concerning the association with background factors such as the residence, sex, and age, we have encountered no patient with tick bites leading to peripheral necrosis, and, therefore, could not perform statistical analysis. In the future, the accumulation of data on patients with similar necrosis may allow the clarification of more detailed mechanisms of peripheral necrosis.

We encountered a patient who developed toe necrosis due to poor blood circulation after a tick bite. We speculated that tick bite reactions were associated with thrombogenic vasculopathy. Tick bites in peripheral areas of the four extremities with only a few collateral circulation routes are associated with a risk of peripheral necrosis, and careful observation of the course is necessary.

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寄生虫疾患の各種診断法と漏らさないための検査システムの提案

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Key Words: 好酸球増多，便検査，抗体スクリーニング，遺伝子スクリーニング

はじめに

宮崎大学医学部寄生虫学教室では，全国の医療機関からの血清診断依頼に対応しており，毎年新規症例の診断に関与している（表 1）．「寄生虫症免疫診断検査申込書」は，（株）エスアールエルを通して，あるいは当研究室のサイトからダウンロードできるようになっているが(http://www.miyazaki-med.ac.jp/parasitology/detail.htm)，この申込書には現病歴を簡単に記述するスペースがある．したがってわれわれは，それぞれの症例について，どのような経緯で主治医あるいは医療チームが寄生虫疾患を疑い，宮崎大学へ検査を依頼したのかをある程度知ることができる．その中のほとんどは合理的な検査依頼だが，中にははなはだ理解が難しい例もある．

ここでは，これまでに経験した寄生虫疾患の検査依頼や経過の具体例を通して，わが国における寄生虫疾患診断の問題点を明らかにし，その対策にどのようなものがあり得るのかを提案する．なお，検査診断業務にともなって得られる臨床データの分析と発表については，宮崎大学医学部医の倫理委員会の承認を得ている．

検査依頼理由

1．好酸球増多

もっとも多い検査依頼の理由が好酸球増多である．末梢血の好酸球増多はもちろん，胸水や腹水中，心嚢液中や脳脊髄液に多くの好酸球がみとめられた，気管支肺胞洗浄液中に好酸球多数がみとめられた，あるいは生検組織中への好酸球浸潤が著明であった場合などもある．

末梢血好酸球増多以外に異常所見がない例もあるが，好酸球の出現と同時に現れる所見で多いのは肺炎，とくに「抗菌薬を投与したが軽快しない肺炎」である．胸部 X 線所見はさまざまだが，肺野の異常陰影が多発性であり出没を繰り返し

Proposal of systematic screening for the diagnosis of parasitic diseases.

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表1　宮崎大学医学部寄生虫学教室における寄生虫症診断実績

寄生虫	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
イヌ回虫・ブタ回虫	68	67	77	100	103	82	101	78	49	48
アニサキス	6	6	6	0	4	4	6	3	2	2
イヌ糸状虫	6	4	4	7	1	5	1	1	0	0
顎口虫	13	10	11	11	0	0	6	7	9	3
鉤虫	6	2	3	0	1	0	1	0	1	1
マンソン孤虫	4	8	6	5	4	3	6	4	5	2
嚢虫	2	4	2	4	0	0	0	0	1	0
肺吸虫	37	36	32	45	30	37	46	38	38	45
肝蛭	1	1	8	5	6	2	3	1	1	3
住血吸虫	0	0	1	5	5	6	6	4	4	3
肝吸虫	1	3	1	1	0	0	0	0	0	1
糞線虫	8	21	11	11	2	1	1	2	0	2
回虫	3	3	4	0	1	1	1	2	0	0
広節・日本海裂頭条虫	0	0	2	1	0	2	0	1	0	4
計	155	165	168	195	157	143	178	141	110	114

たり移動する時には，原因が寄生虫である可能性がきわめて高い．申込書によると，たいてい他の原因検索も実行されており，各種細菌陰性，アスペルギルス陰性，細胞診陰性などの記述がみとめられる．マイコプラズマ肺炎と思われる症例で，回復期に好酸球が上昇してきたので検査を依頼する例もある．

呼吸器症状以外には，肝臓の異常陰影をはじめとしてさまざまな症状，所見が付随している．好酸球性髄膜炎では髄膜刺激症状がある．当然のことながら，これらの症例で抗体が陽性なのか陰性なのかは検査してみないと予想はつかない．しかしながら，透析患者にみとめられる好酸球増多症では，抗寄生虫抗体が陽性になることはまれである．

2. 脊髄炎，ぶどう膜炎

特徴的な臨床所見から好酸球増多の有無に関わらず寄生虫感染を疑う場合があり，特に目立つのは脊髄炎とぶどう膜炎である．これはトキソカラなどの回虫類幼虫による幼虫移行症を疑ったもので，脊髄炎では陽性の場合には比較的高い抗体濃度が得られる．血清と髄液を同時に測定できれば診断の精度を上げることができる．ぶどう膜炎では通常血清抗体濃度は境界値前後であり，診断では眼科医の判断が最も重要である．硝子体液などの局所液が得られれば診断精度は大幅に上昇する．

好酸球性肺炎でも成り立つが，とくに脊髄炎とぶどう膜炎症例では，同じクリニックあるいは医療機関の診療科（それぞれ神経内科と眼科）からの依頼が多い．これは，一度寄生虫症例を経験した医療施設では，同様の症状に対して再び寄生虫感染の可能性を考えるからであろう．逆に言うと，一度も寄生虫疾患に遭遇したことのない，あるいは寄生虫疾患の存在を思いつかない医師しかいない医療施設では，いつまで経っても寄生虫感染を疑うことはないということになる．

3. 慢性の消化器症状

原因不明の下痢で寄生虫検査依頼を受けることがあるが，多くの場合，送付されてくる検体は血清である．好酸球性胃腸炎であれば抗体陽性の結果が得られることもあるが，好酸球増多が見ら

れない場合にはほぼ抗体陰性であり，仮に陽性反応が得られたとしても消化器症状との関連ははっきりしない（抗アニサキス抗体陽性など）．ただし，これらには報告書に便検査を実施するように促すコメントを付けるようにしているので，便検査を実行してもらえれば，検査依頼自体は必ずしも無駄ではない．

4. 炎症所見

頻度は多くはないが，何らかの炎症所見（発熱や CRP 値の上昇など）があるにも関わらず，通常の感染症や自己免疫疾患，悪性疾患が否定的である場合も，診断に苦慮して血清が送付されることがある．検査結果は陰性で寄生虫感染は否定的，との報告を返すことが多いが，寄生虫が鑑別に上がった点で評価すべきであると考えている．

5. その他

皮膚爬行疹や移動性皮下腫瘤も寄生虫感染を疑う所見で，実際にほとんどの場合感染ありであろうと判断される．これらの症候は比較的わかりやすく見逃すことは少ないと考えられる．また，途上国への渡航歴があったり，患者が欧米以外，とくに東から東南アジアの出身者である場合には，寄生虫疾患が鑑別の対象になりやすい傾向があるように思われる．

寄生虫疾患のサインに気づかなかった症例

以上のように，いくつかの所見を契機にして寄生虫疾患の診断依頼が出されるわけだが，もちろんこの場合医師は寄生虫の存在に考えが及んでいる．問題なのは，寄生虫疾患を示すサインがあるにも関わらず寄生虫感染が全く鑑別に上らず，検査を依頼しない医師である．どのような場面かというと，それは上述した検査依頼理由の裏返しであり，① 好酸球増多があるにも関わらず寄生虫抗体検査をしなかった，② 慢性の下痢があるにも関わらず便検査をしなかった，というふた通りに大別される．

1. 好酸球増多に注意を払わなかった肺吸虫症例

症例は男性会社員で，ある年の 11 月初旬から咳と血痰あり，翌年 1 月に近医を受診したところ胸部 X 線で左胸水と右上肺野に空洞性陰影をみとめた．末梢血好酸球増多（好酸球 18%）があり，ツベルクリン反応も強陽性であった．抗菌剤による治療を受けていたが血痰が持続して胸水も増加したため 2 月中旬に大規模総合病院を受診した．

総合病院への転院時検査成績では末梢血好酸球数 30 %であり，一般細菌検査は常在菌のみ，抗酸菌培養陰性，結核菌に対する PCR は陰性であった．診断がつかないまま退院となったが胸水が増量し，4 月初旬に再入院，4 月 10 日の気管支鏡検査で喀痰中に肺吸虫卵が見いだされて診断がついた．

好酸球増多に対して早い段階で寄生虫抗体検査を実施していれば，初診から診断確定まで 3 ヶ月も要することはなかったであろうと考えられるが，この症例で示唆的と思われるのは，虫卵による診断がついた後に，血清が抗体検査のために送付されてきたことである．形態学的診断に対する評価が低いのか，あるいは抗体による診断が一般的とされるから機械的に検体を送付したのかは不明だが，いずれにしても，検査から得られる情報の診断的価値に対する順位付けに疑問を感じざるを得ない．

同様に当初から好酸球増多がありながら肺結核の治療がおこなわれた症例や，あるいは肺癌を疑って切除術を実施された例がいくつも報告されている[1),2)]．