



Detection of α -defensin in eosinophils in helminth-infected mouse model

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ABSTRACT. α -defensin is a potent antimicrobial peptide secreted from intestinal mucosal epithelial cells, such as Paneth cells, and affects not only bacteria but also parasites and fungi. Recently, human eosinophils have also been shown to produce α -defensin, but no studies have been done on other animals. In this study, we attempted to detect α -defensin protein in mouse eosinophils infiltrating the intestinal mucosa during a helminth infection using Zamboni fixation and immunohistochemistry. Most of the eosinophils infiltrating the intestinal mucosa during helminth infection were positive for α -defensin. The expression level of α -defensin mRNA was 50 fold that in the control. Meanwhile, the number of Paneth cells was doubled, and their α -defensin fluorescence intensity was increased. These results suggested that eosinophils are also important producers of α -defensin, such as Paneth cells in mice, and that α -defensin produced from eosinophils might be involved in defensive mechanisms against helminths. Moreover, the experimental system used in this study is a good model to study the generation of α -defensin by eosinophils.

KEY WORDS: α -defensin, eosinophil, helminth, murine

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Eosinophilia can be caused by a wide variety of diseases, including parasite infection, allergy, tumor, autoimmune disease, and is known to be induced by Th2-mediated immune reactions [11, 21, 22, 37, 41, 42]. Usually, bacterial infection is associated with Th1 immune responses as protective immunity, is characterized by production of gamma interferon, and exhibits mutual inhibitory effects against Th2 [3, 5, 16]. However, unlike other bacterial infections, mycobacterial infection is known to induce eosinophilia that is usually associated with Th2 immunity [20]. A recent study reported that, in humans, eosinophils, activated against mycobacteria, produced α -defensin [8] and antibacterial cationic peptides, which are most strong inhibitors for mycobacteria [7]. However, it is still unknown whether eosinophils produced α -defensin in other animals.

Several types of defensins are found both in human and mouse; human defensins are released from neutrophils and epithelial cells during bacterial infection [7, 24]. Human α -defensin homologue has also been identified in mice (cryptdin 4) [34]. It has already been shown that mice, transfected with human α -defensin, have acquired resistance to *Salmonella* spp. [32, 38, 39]. In addition, murine α -defensin has also been shown to exhibit resistance to *Escherichia coli* [35], *Staphylococcus aureus*, *Listeria monocytogenes*, etc. [28]. α -defensin acts not only against bacteria but also against fungi, protozoa, and viruses [24, 25, 32, 46]; furthermore, it has also been shown to act against helminths [12, 15, 19, 27].

It has been previously reported that Paneth cells are crucial for mouse α -defensin production [15, 19, 24, 38], because unlike human neutrophils, murine neutrophils do not express α -defensin [9, 33]. Therefore, most of the researches related to α -defensin are limited to Paneth cells in mice. Paneth cells are predominantly increased during helminth infection [18, 40] and have been speculated to contribute to elimination of parasites via α -defensin production [12, 27]. Many previous researches have studied the direct action of defensin against helminths [12, 27] and there have been few studies on *in vivo* kinetics and activation mechanisms. In the previous studies related to α -defensin production by Paneth cells, researchers used neutral buffered formalin for sample fixation for immunohistochemistry [4, 34, 44]. However, Zamboni fixative solution is more suitable for fixing small size molecules and soluble substances, including α -defensin, compared to buffered formalin, because of rapid penetration property [1]. If we can detect α -defensin production by murine eosinophils (similar to human eosinophils) [8], eosinophils might be recognized as important cells, similar to Paneth cells, producing α -defensin in mice.

Using *Nippostrongylus brasiliensis* (Nb) infected mouse models, we examined α -defensin expression using immunofluorescent analysis and real-time PCR in the duodenum, which was the infection site of Nb.

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MATERIALS AND METHODS

Animal

Pathogen free female ICR mice (Clea Japan, Tokyo, Japan) were fed with autoclaved food (MF; Oriental Yeast, Tokyo, Japan) and tap water *ad libitum*. All animals were handled according to the regulations for animal welfare of Yamaguchi University (Permit number: 223).

Parasitological technique

Nb maintained by serial passage in Sprague Dawley rats as previously described [30] and infective stage larvae were recovered from feces by using a modified Baermann apparatus, and were washed [30]. Mice were infected by subcutaneous injection of 800 infective larvae.

Blood eosinophil count and intestinal tissue preparation

Mice were anesthetized with mixture of xylazine and ketamine, and killed by exsanguination at day 9 after nematode infection. Blood was drawn from the heart using a heparinized syringe. The number of eosinophils in blood was counted after staining with Hinkelman's solution, as reported previously [29].

Duodenum were removed as it was main infection site for Nb and fixed in Zamboni's solution [36] at 4°C overnight.

Histopathology and immunofluorescence

Fixed duodenal samples were routinely processed and embedded in paraffin. Then, they were cut into 2 μ m-thick sections. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin. Paneth cell number was counted in hematoxylin and eosin stained duodenal sections (characterized by pyramidal shape with apical located eosinophilic granules at crypt base) and expressed as number per 10 villus crypt units (VCU). The sections were examined using a microscope (BX53 microscope with DP73 camera, Olympus Corp., Tokyo, Japan).

For double immunofluorescence staining assay, sections were deparaffinized and subjected to antigen retrieval by treating with 0.5% trypsin for 30 min. Then, the sections were blocked by 5% skim milk with albumin, obtained from Bovine Serum Cohn Fraction V, pH 7.0, (Wako Pure chemical Industries Ltd., Osaka, Japan) in PBS for 30 min at room temperature to avoid non-specific reactions. For detection of α -defensin production by eosinophils, sections were incubated with rabbit anti-mouse eosinophil cationic protein (ECP) IgG antibody (1:400, Aviscera bioscience, Inc., Santa Clara, CA, U.S.A.) for 1 hr at room temperature and washed, followed by incubation with Alexa Flour 488 conjugated goat anti-rabbit IgG (1:200, Life technologies, Eugene, OR, U.S.A.). After washing, sections were incubated with α -defensin 4 (R-19) polyclonal antibody [4, 26] (1:50, Santa Cruz Biotechnology, Inc., Dallas, TX, U.S.A.) over night at 4°C and washed, followed by incubation with Alexa Fluor 555 donkey anti goat IgG (H&L) antibody (1:200, Abcam, Cambridge, U.K.) for 1 hr at room temperature. After washing, specimens were mounted with glycerol. Among all mice α -defensin, α -defensin 4 is unique and the most potent one [17, 34], so we chose it for our study. For detection of α -defensin-positive enteroendocrine cells, sections were incubated with α -defensin 4 (R-19) polyclonal antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C, followed by incubation with Alexa Fluor 488 donkey anti goat IgG (H&L) antibody (1:200, Abcam) for 1 hr at room temperature. Then, after washing, the sections were incubated with sheep anti-human chromogranin A antibody (1:50, Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) for 1 hr at room temperature followed by incubation with Alexa Fluor 555 donkey Anti sheep IgG H&L (Abcam) for 1 hr at room temperature. After washing, the specimens were mounted. All observations were performed using a fluorescence microscope (BX53 fluorescence microscope with DP73 camera, Olympus Corporation) equipped with suitable filter set (red filter with excitation range of 530–550 nm and an emission range of 575 nm, and green filter with excitation range of 470–495 nm and an emission range of 510 nm). Images were analyzed using GIMP software (version 2.8).

RNA extraction and reverse-transcription (RT)

Thirty milligrams of duodenum of the control group and infected group were used for RNA extraction according to the manual of RNeasy plus mini kit (Qiagen, Tokyo, Japan). Next, 12.5 μ l of total isolated RNA was mixed with 5x RT buffer, dNTP, 0.1 M DTT, random primers (Invitrogen, Thermo Fisher Scientific, Waltham, MA, U.S.A.) and RNasin (Promega, Madison, WI, U.S.A.), and total volume was kept at 24.5 μ l. Samples were incubated at 70°C for 5 min. Then, 0.5 μ l SuperScript III (Invitrogen) was added to reach total volume at 25 μ l and incubated at 37°C for 60 min, followed by incubation at 95°C for 5 min, and on ice for 5 min to generate cDNA.

Real time PCR

One microgram of cDNA sample was amplified by TaqMan[®] Gene expression assay for murine α -defensin (Mm00651736_g1 Defa4, Applied Biosystems, Tokyo, Japan) using a Step One[™] Real-time PCR System (Applied Biosystems). For amplification, the protocol followed was: 50°C for 2 min; 95°C for 10 min; 95°C for 15 sec, 60°C for 1 min cycle was repeated for 50 times. For the quantification of the α -defensin 4 mRNA, 18s rRNA (Mm03928990_g1 18S, Applied Biosystems, Tokyo, Japan) was used as housekeeping gene and α -defensin 4 expression was normalized against the value of 18s rRNA.

Fluorescent intensity analysis

Using fluorescent image for cell counting (Fig. 6), fluorescent intensity of α -defensin was quantified for eosinophils, Paneth cells, and enteroendocrine cells. The images were analyzed in the software BZ-II analyzer (Keyence, Osaka, Japan). For each cell type, 20 cells were randomly selected per mice and maximum fluorescent intensity was measured.

Statistical analysis

All data were expressed as mean \pm standard error (SEM). Statistical significance was determined at $P < 0.05$ following the Student's *t* test. For the comparison of the α -defensin 4 mRNA expressions, Mann-Whitney *U* test was used, because the expression was represented in logarithmic number and there was no normal distribution for it.

RESULTS

Eosinophil reaction during *Nb* infection in mice

The number of eosinophils in the blood was significantly higher in infected group (91.5 ± 24.4) than in control group (11.0 ± 2.5) (Fig. 1). In the intestine, infiltration of eosinophils in the mucosal lamina propria around the epithelial cells of crypts lined in tubular form was observed by immunofluorescence in the infected group, and it was significantly increased compared to the control group (Fig. 2, Table 1).

Epithelial cell reaction during *Nb* infection in mice

In control group, several Paneth cells existed in the bottom of crypts and they had small number of eosinophilic granules in the cytoplasm (Fig. 3a). On the other hand, the Paneth cells became filled with more prominent eosinophilic granules in the infected group (Fig. 3b). The number of Paneth cells (number/10 villus crypt units (VCU)) for control and infected group were 15.4 ± 1.6 and 35.3 ± 2.1 , respectively; that is, a significant increase in the number of Paneth cells was observed (Table 1).

Production of α -defensin by infiltrated eosinophils

To investigate whether eosinophil produced α -defensin, we conducted a double immunofluorescence staining using antibodies specific for eosinophil cationic protein (ECP), a matrix protein associated with specific granules in the eosinophils [43], and α -defensin 4 protein. Immunoreactivity against ECP and α -defensin 4 protein almost co-localized, and more than 82% of increased eosinophils were positive for α -defensin in the *Nb* infected group (Fig. 5). However, the α -defensin-positive eosinophils were not found in the specimens in the control group; in addition, no co-localization was found in the control group (Fig. 4, Table 1).

Production of α -defensin by epithelial cells

To detect α -defensin-positive epithelial cells, we conducted immunofluorescence staining using antibodies specific for α -defensin 4 protein and chromogranin A, marker of enteroendocrine cells. Paneth cells were identified from their site of existence and morphological characteristics. Besides eosinophils, some percentages of Paneth cells and chromogranin A-positive enteroendocrine cells also showed positivity for α -defensin (Figs. 6 and 7, Table 1). The number of α -defensin-positive Paneth cells doubled after infection, and thus, an increase in the fluorescence intensity was observed (Fig. 6, Table 1). On the other hand, there was no change in the number of α -defensin-positive enteroendocrine cells or their fluorescence intensity before and after infection (Tables 1 and 2).

Kinetics of α -defensin 4 mRNA expression in duodenum

Using comparative Ct method, the values of α -defensin 4 mRNA expression level in infected group relative to control were calculated. The expression level of α -defensin 4 mRNA in *Nb* infected mice was significantly higher than that in control mice (50-fold increase) (Fig. 8).

Fluorescent intensity analysis of α -defensin

The fluorescent intensity of α -defensin was not detected in eosinophils in control group. However, high fluorescent intensities of α -defensin were observed in Paneth cells and enteroendocrine cells (Tables 1 and 2). In addition, high fluorescent intensity of α -defensin was detected in infiltrating eosinophils in *Nb* infected group. The fluorescent intensity of α -defensin in Paneth cells was higher in *Nb* infected group compared with control group, and intensity of α -defensin was stronger compared with that of eosinophils. However, the fluorescent intensity of α -defensin in enteroendocrine cells did not change significantly compared with the control group (Table 2).

DISCUSSION

The results of the immunohistochemical analysis done in this study showed, for the first time, that the tissue infiltrating

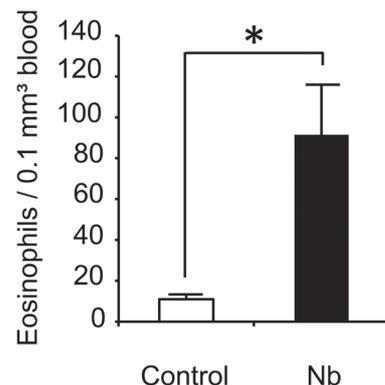


Fig. 1. Blood eosinophil count in control and infected mice. Each group consisted of 5 mice. Data are shown as mean \pm SEM. *Significantly different values from those of control mice ($P < 0.05$).

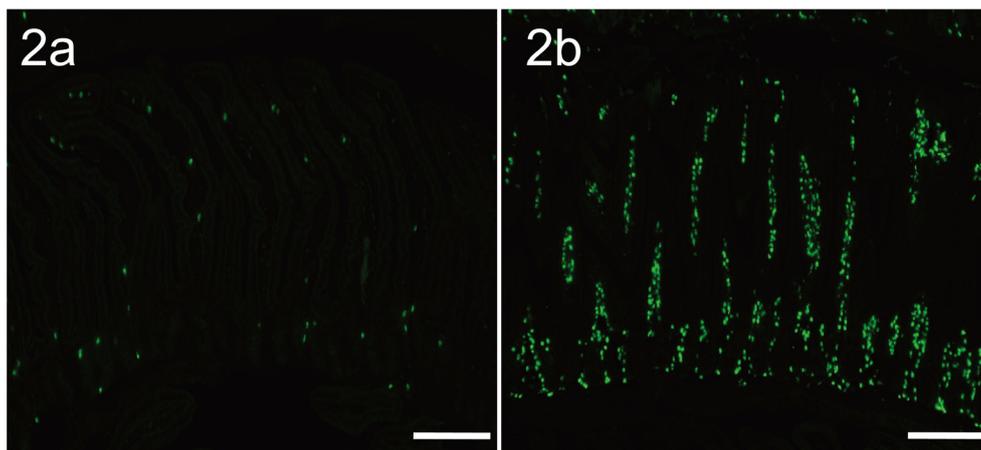


Fig. 2. Immunofluorescence analysis for ECP. (a) A few ECP-positive eosinophils in duodenum of control mice. (b) Numerous ECP-positive eosinophils in duodenum of infected mice. Scale bar=200 μm .

Table 1. Number of duodenal α -defensin-positive cells in control and *Nippostrongylus brasiliensis*-infected group

Cell type	Control			Nb		
	Cell number	α -Defensin positive cell number	Percentage of α -defensin positive cells number	Cell number	α -Defensin positive cell number	Percentage of α -defensin positive cells number
Eosinophil	28.0 \pm 0.6	0 \pm 0	0 \pm 0	532.0 \pm 12.0 ^{a)}	450.6 \pm 13.3 ^{a)}	81.7 \pm 9.4
Paneth cell	15.4 \pm 1.6	7.6 \pm 0.9	49.1 \pm 1.2	35.3 \pm 2.1 ^{a)}	17.8 \pm 2.7 ^{b)}	48.1 \pm 1.2
Enteroendocrine cell	36.2 \pm 2.8	25.4 \pm 3.0	72.2 \pm 9.4	45.6 \pm 7.1	29.6 \pm 1.9	68.7 \pm 6.2

Data are presented as the mean \pm SEM of 5 mice in each group. Significantly different from the values of control mice. a) $P < 0.05$, b) $P < 0.01$. Cell number expressed as number per 10 VCU. (VCU: villus crypt units).

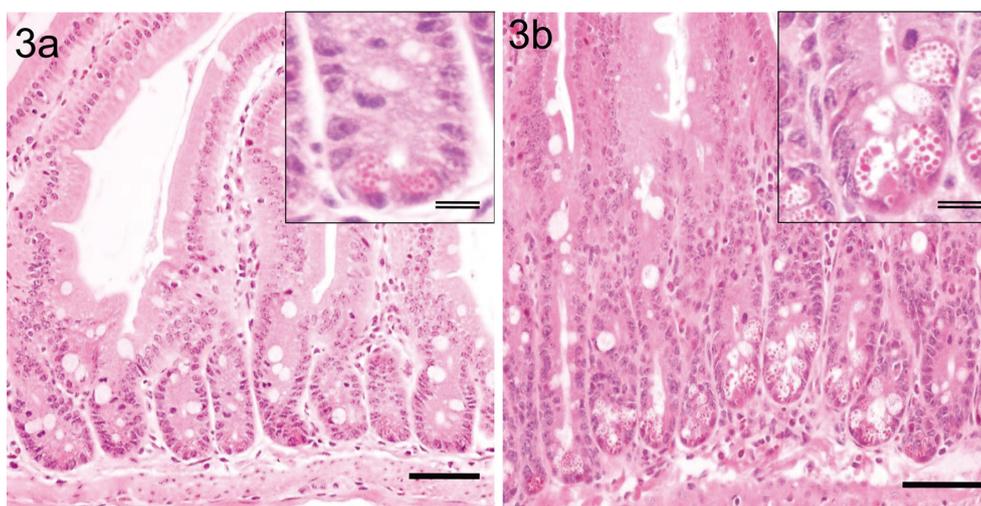


Fig. 3. Histological detection of Paneth cell. Hematoxylin and eosin (HE) staining (a) A couple of Paneth cells in crypt bottom of duodenum of control mice. (b) Higher number of Paneth cells in infected duodenum compared to control duodenum. Cytoplasm of Paneth cells increased in volume with prominent eosinophilic granules in infected duodenum. Scale bar (single line)=100 μm , scale bar (double lines)=20 μm .

eosinophils produce α -defensin (Fig. 5), and that these eosinophils reacted with host defense immune Th2 responses against helminth Nb in murine model [13, 23].

Several studies have shown that number of Paneth cells increased significantly during intestinal helminth infection [18, 40] and α -defensin production by activated Paneth cells has been detected using immunohistochemical methods [4, 34, 44]. In this study, we also observed increase in the number of α -defensin-positive Paneth cells, and increase in the fluorescent intensity of the

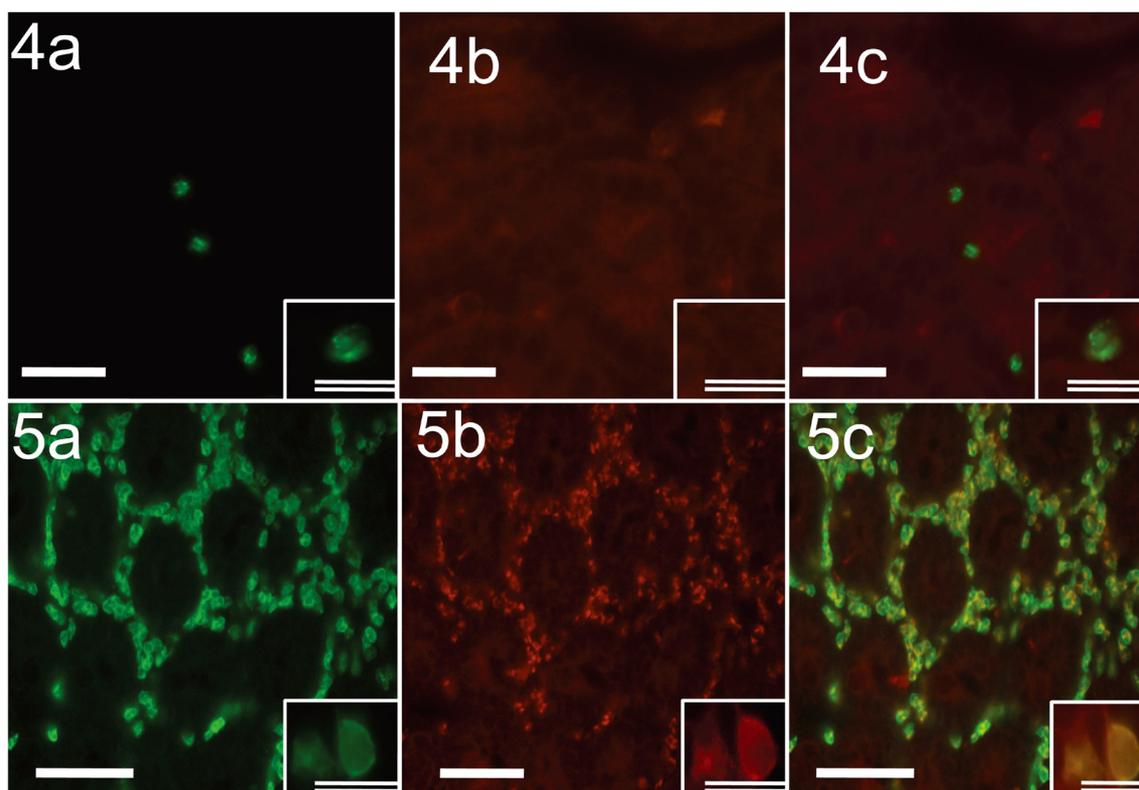


Fig. 4. Double immunofluorescence analysis for ECP (a) and α -defensin 4 (b) in control mice. (c, merged) A few ECP-positive eosinophils in duodenum without α -defensin 4 expression. Scale bar (single line)=50 μ m, scale bar (double lines)=20 μ m.

Fig. 5. Double immunofluorescence analysis for ECP (a) and α -defensin 4 (b) in infected mice. (c, merged) Almost all ECP-positive eosinophils in duodenum expressed α -defensin 4. Scale bar (single line)=50 μ m, scale bar (double lines)=20 μ m.

α -defensin. However, detection of eosinophil-derived α -defensin has not been reported, and murine neutrophils do not express α -defensin [9, 33], therefore, Paneth cells were speculated to be the only important cells which produce α -defensin in mice against helminth infection. However, previous studies used ordinary fixing solutions, such as neutral buffered formalin, for the samples subjected to immunohistochemistry [4, 34, 44]. We also tried the ordinary fixation methods using formalin and paraformaldehyde, but we could not detect α -defensin in eosinophils (data not shown). Finally, further experiments revealed that Zamboni fixation made it possible to detect α -defensin in eosinophils. Zamboni fixative contains phosphate buffered picric acid and formaldehyde, is very stable, and provides good general fixation with rapid penetration and optimal preservation and stabilization of cellular proteins [1]. These facts suggested that the eosinophil secretory granules are easier to degranulate than the Paneth cell granules, and the ordinary fixation methods were not enough to keep α -defensin within eosinophils. Therefore, Zamboni fixation is recommended for future α -defensin research in the intestine rather than using ordinary formalin fixation.

In this study, α -defensin mRNA expression in the duodenum of helminth-infected mice upregulated significantly and showed 50-fold increase compared to mice without helminth infection (Fig. 8). Infiltration of α -defensin-positive eosinophils into the submucosal tissue was prominent (Figs. 2b and 5); however, there was only a 2-fold increase in the number of Paneth cells and the α -defensin-positive fluorescence intensity strengthened too (Tables 1 and 2). There were no significant changes in enteroendocrine cell number and their fluorescence before and after infection (Tables 1 and 2). These facts indicated that eosinophil-derived α -defensin contributed a considerable part in the increased α -defensin production due to infection.

Marked eosinophilia is well documented in Nb-infected mice associated with nematode expulsion [18, 40]. Previous evidence indicated that eosinophil and its granule proteins participated in host resistance to helminths; *in vitro* experiments showed that eosinophils can kill a wide range of helminth species and are considered highly toxic for schistosomula, *Trichinella spiralis*, and *Trypanosoma cruzi* [6]. Secreted proteins have been speculated to be important in mediating the anti-helminthic action of eosinophils, and these proteins include major basic protein-1, major basic protein-2, eosinophil peroxidase, ECP, and eosinophil-derived neurotoxin [2, 14]. Major basic protein-1 is highly toxic to mammalian cells *in vitro*, and can damage helminths by disrupting the lipid bilayer membrane or altering the activity of enzymes within tissues [2, 14]. ECP caused membrane disruption of helminth parasites by non-ion selective membrane pore formation [2]. Eosinophil peroxidase exerted cytotoxic effects as a cationic toxin, being able to eliminate parasites by lipid peroxidation [2]. In addition to these eosinophil-secreted proteins, α -defensin also has been reported to have anti-helminthic effects [12, 15, 19]. Therefore, eosinophils, similar to Paneth cells, may play an important role in helminth exclusion

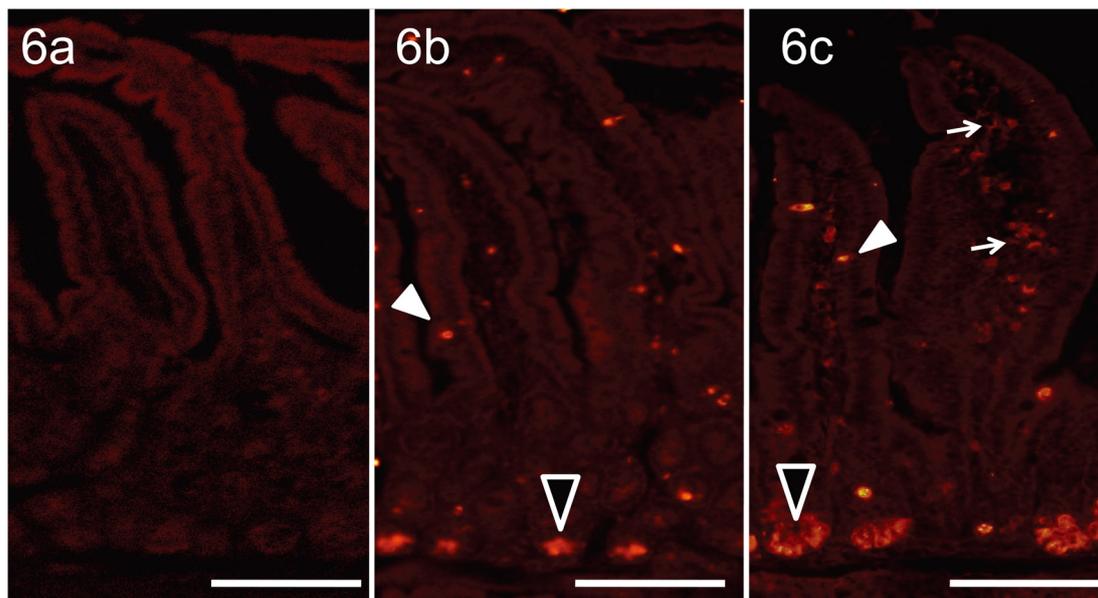


Fig. 6. Immunofluorescence analysis for α -defensin 4. (a) Isotype control. (b) A couple of α -defensin 4-positive Paneth cells (black arrowhead) and enteroendocrine cells (white arrowhead) in duodenum of control mice. (c) Numerous α -defensin 4-positive eosinophils (arrows) with a couple of α -defensin 4-positive Paneth cells (black arrowhead) and enteroendocrine cells (white arrowhead) in duodenum of infected mice. Scale bar=200 μ m.

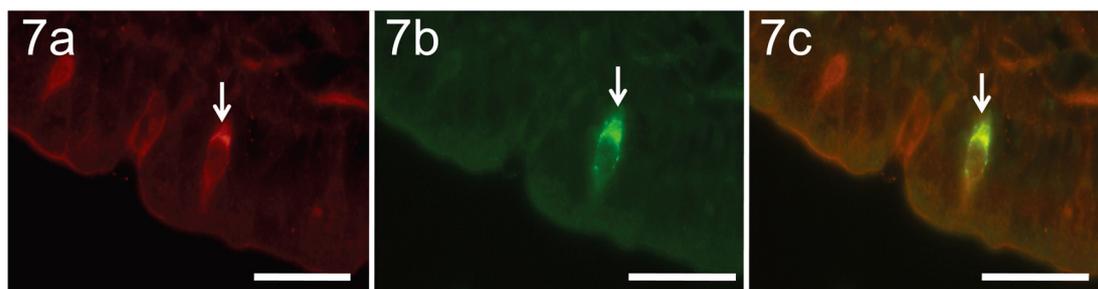


Fig. 7. Double immunofluorescence analysis for chromogranin A (a) and α -defensin 4 (b) in infected mice. (c, merged) A part of chromogranin A-positive enteroendocrine cells in duodenum expressed α -defensin 4 (arrow). Scale bar=20 μ m.

Table 2. Measurement of intensity level of α -defensin-positive signals in duodenal tissue for control and *Nippostrongylus brasiliensis*-infected group

Cell type	Control	Nb
Eosinophil	0	171.1 \pm 10.60 ^{b)}
Paneth cell	141.57 \pm 22.84	219.72 \pm 17.12 ^{a)}
Enteroendocrine cell	220.3 \pm 2.98	223.26 \pm 7.02

Data are presented as mean \pm SEM of 5 mice in each group. Significantly different from the values of control mice. a) $P < 0.05$, b) $P < 0.01$.

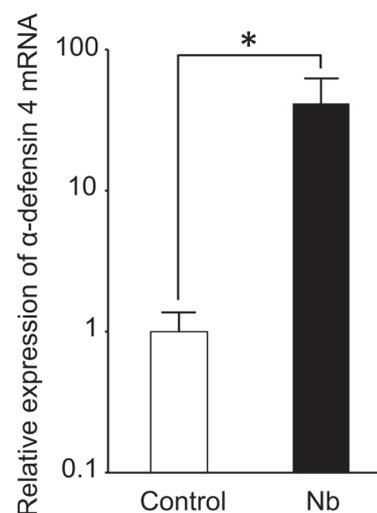


Fig. 8. The effect of Nb infection on α -defensin 4 mRNA expression levels in duodenum. Each group consisted of 5 mice. Data was expressed relative to control non-infected group. *Significantly different values from those of control mice ($P < 0.05$).

via α -defensin [10]. Because several proteins in the eosinophil granules have anti-helminthic action, it is difficult to compare their activities. Moreover, the anti-helminthic effects of these proteins may differ depending on the type of helminth [2, 14, 21].

In humans, the α -defensins are classified into human neutrophil proteins (HNP) and human defensin (HD) which are produced by neutrophils and Paneth cells, respectively [34]. However, Paneth cells were considered to be the major α -defensin producing cells before this study [9, 33], and α -defensin, as detected with the R-19 antibody (cryptdin 4) in Paneth cells, was considered to be the most potent defensin in mice [17, 34]. In addition, R-19 antibody did not cross-react with other murine α -defensins [34]. α -defensin detected in Paneth cells and eosinophils in this study were considered to be similar. In addition, since the number of Paneth cells and eosinophils and their fluorescence intensity increased after parasite stimulation (Tables 1 and 2), it was speculated that the increased α -defensin, both in Paneth cells and eosinophils, has the same antiparasitic effect [10].

In this study, α -defensin was detected in Paneth cells, eosinophils, and endocrine cells, but their fluorescence intensities were different (Table 2). Since, each cell produces different class of proteins [2, 14, 32, 33], it might be possible that they may differ in their ability to produce α -defensin. In addition, α -defensin was detected in eosinophils for the first time in this study using Zamboni fixative solution; however, it may still have been insufficient to fix all the α -defensin in eosinophils. In addition, Paneth cells and enteroendocrine cells produced α -defensin in non-infected control groups. α -defensin eliminated the pathogen but did not kill commensal bacteria [31]; α -defensin derived from Paneth cells and endocrine cells may keep enteric microbiota in health. However, more research is needed to further elucidate the role of α -defensin.

We also showed that enteroendocrine cells produced α -defensin. However, α -defensin from enteroendocrine cells seemed to have no anti-helminthic role, because the α -defensin-positive cell number and the fluorescence intensity of α -defensin of enteroendocrine cells did not change after the helminth infection. Therefore, α -defensin produced by enteroendocrine cell may not be important for the antiparasitic effect. Enteroendocrine cells are known as cells secreting more than 20 peptide hormones for digestion [45]. α -defensin also plays a role in regulating the immune system [24, 32, 33]. Thus, these data suggested that enteroendocrine cells may be involved in the regulation of the intestinal environment.

In the current study, we have shown, for the first time, that eosinophils produce α -defensin, so future research is expected to elucidate the anti-helminthic action of eosinophils via α -defensin production. In addition, Nb infection is well known to activate Th2 immune response [13, 23] and as Nb infection increased α -defensin production, we speculated that Th2 upregulation was associated with the production of antimicrobial peptides in mice.

In conclusion, this was the first study which revealed the production of α -defensin in murine eosinophils. α -defensin expressing eosinophils played an important role in exclusion similar to Paneth cells in Nb-infected mouse model. This animal model is well known as a model to activate Th2 immune response [13, 23]. Therefore, mice infected with Nb could be a useful animal model for the study of α -defensin mechanisms with respect to helminth expulsion and to elucidate the relationship between eosinophilia and α -defensin production.

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