

The effectors responsible for gastrointestinal nematode parasites, *Trichinella spiralis*, expulsion in rats

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Abstract Helper T (Th2) cells type 2 have a central role in host protective responses to gastrointestinal nematode parasite, *Trichinella spiralis* infection, but the actual effector mechanisms involved in parasite expulsion are still uncertain. Recent evidences suggest that mast cell recruitment and activation may associate with parasite elimination from host intestines in mice. On the other hand, IgE production may induce defensive responses to primary infection with the helminth in rats. The differences of host effector mechanisms to the same experimental infections might disturb our understanding of the host protective mechanisms to gastrointestinal nematode parasite infection. In order to redefine these differences, we investigated in detail the relationship between intestinal immune responses and worm expulsion following *T. spiralis* infection among several rat strains including mutants. As a result, there were significant correlations of parasite expulsion with mast cell hyperplasia in addition to serum IgE level. Moreover, mast cell-deficient and dysfunction rats showed delayed worm elimination from their gut. Therefore, the present study suggests that mast cells should also be one of the prominent

effector cells involved in *T. spiralis* parasite expulsion in rats as well as mice.

Introduction

Immune expulsion of gastrointestinal helminthes is strongly associated with helper T cell (Th) 2 responses, and the immune responses induced mainly by interleukin (IL)-4 regulate antibody production and switching to immunoglobulin G1 (IgG1) and immunoglobulin E (IgE) isotypes and induction of inflammation including mast cells and eosinophils activation (Abbas et al. 1996). Therefore, Th2-regulated immune responses are considered to play a central role in protection against helminthic infections, but the effectors directly responsible for the parasite expulsion have not been elucidated. It is confusing to clarify the precise mechanisms of experimental infections with different species of gastrointestinal nematode parasites and/or different species of experimental hosts, which indicated that different effector cells could be responsible for worm expulsion in each model.

In mice, eosinophilia, IgE production, and mast cell hyperplasia are well-known as the three hallmarks of the immune response triggered by gastrointestinal nematode parasites, in particular *Trichinella spiralis* infection (Else and Finkelman 1998). Firstly, a significant eosinophilic inflammation is induced during *T. spiralis* nematode infection, and there is a marked increase in the levels of IL-5, a Th2-type cytokine responsible for the production of eosinophils. Mice treated with anti-IL-5 antibody to ablate eosinophils could not block the elimination of *T. spiralis* worms; hence, it strongly suggested that eosinophils might have a little effect on protective mechanism against the adult helminthes in the gut (Herndon and Kayes 1992). In

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contrast, eosinophils are shown to have a toxic effect to larvae but not to adult worms of *T. spiralis* using mice with disruption of CCR3 (Gurish et al. 2002). Secondly, IgE response is strongly associated with not only helminthic infections but also allergic diseases. Recently, Gurish et al. (2004) reported that the IgE antibody was responsible for the protection of muscle larvae during *T. spiralis* infection using IgE gene knockout mice, but a role of the IgE antibody in protective immunity against primary infections has not been elucidated. Finally, mast cell hyperplasia is known to create an unsuitable environment for the worm in infection with *T. spiralis*, and mast cells are thought to be a crucial effector in expulsion of the parasites in mice (Lawrence et al. 2004). Depletion of mast cells with anti-c-kit and anti-stem cell factor antibodies or infections of mast cell-deficient W/W^v or IL-9 transgenic mice have demonstrated a role for mast cells in the rejection of this parasite (Ha et al. 1983; Alizadeh and Murrell 1984; Donaldson et al. 1996; Faulkner et al. 1997; Grecis et al. 1993).

In rats, it is likely that the IgE antibody but not mast cells would be involved in host defensive responses to infection with *T. spiralis* nematode, as the mechanism is still being investigated. Rapid expulsion of larvae is considered to be IgE-mediated and T-cell-mediated protection against *T. spiralis* infection (Ramaswamy et al. 1994; Bell 1998). In consequence of a more detailed discussion about IgE involvement in protective immunity against this parasite infection, it is hypothesized that specific IgE can also block adult worm invasion into gut epithelial cells by activating enterocytes (Negrão-Corrêa et al. 1999; Negrão-Corrêa 2001). Thus, IgE has protective roles against both *T. spiralis* larvae and adults in rats.

These findings as described above suggest that the effector mechanism of protection in mice is different from that in rats regardless of the same parasite infection, which might lead to difficulty in our understanding of host immune responses against gastrointestinal nematode parasite infection. In the present study, we would reconsider the correlation of *T. spiralis* elimination with host effector responses based on histological and immunological analyses using several strains of rats, thereby attempting to refine candidate effectors responsible for the worm expulsion in rats. Especially, in order to focus on the protective role of mast cells for worm expulsion, we included mutant strain with mast cell dysfunction.

Materials and methods

Rats and parasites

F344/N Slc (F344), DA/Slc (DA), and BN/SsN Slc (BN) inbred strains and Slc:WsRc-Ws/Ws (*Ws/Ws*)

outbred strain of rats were supplied by Japan Slc Inc. (Hamamatsu, Japan). *Ws* (*white spotting*) mutant was originally derived from an inbred colony of BN strain maintained at the Laboratory of Experimental Animals, Yagi Memorial Park (Gifu, Japan), and thereafter defined as a deficiency of mast cells, caused by a small deletion in tyrosine kinase domain of *c-kit* gene at the W locus (Niwa et al. 1991; Tsujimura et al. 1991). All experiments were performed according to the guidelines of Hamamatsu University School of Medicine for Animal Care. *T. spiralis* (Polish strain) was kindly supplied by the Department of Parasitology, Gifu University (Gifu, Japan) and maintained in our laboratory by periodical passages through Wistar rats.

Parasite infection

The fresh infective larvae of *T. spiralis* were collected by muscle digestion of the Wistar rats, according to the Wassom method (Wassom et al. 1983) with slight modification. All rats were orally inoculated with 2,000 larvae via a stomach tube under light anesthesia with ether. To count adult worms, ten to 16 rats from each of the four strains were killed and their small intestines were removed at 7, 10, and 14 days after infection when a clear-cut strain difference is displayed as described previously (Suzuki et al. 2006). Then, the guts were immediately transferred to a phosphate-buffered saline solution and incubated for 2 h at 37°C, and the number of intestinal worms was counted under stereoscopic observation and transformed logarithmically to normalize the variance. The intestines and sera of five rats from each of the three inbred and one outbred strains were sampled to assess quantitation of gut immunity on days 0, 5, 7, 10, and 14 post-infection.

Histology and IgE enzyme-linked immunosorbent assay (ELISA)

Small pieces of the jejunum taken at 10 cm distal from the pylorus were fixed in buffered 4% paraformaldehyde solution for 12 h and embedded in paraffin wax. The sections at 4 µm thickness were stained with toluidine blue (0.5% eluted in 0.5 M HCl) overnight to visualize mast cells (Pennock and Grecis 2004) and counterstained with eosin. Eosinophils in the tissue were stained 10 min in 1% chromatrope 2R (Gauvreau et al. 1998). The number of mast cells and eosinophils was counted in 20 randomly chosen villi and expressed as the number of cells per one villus. Total IgE level in serum was measured with Rat IgE ELISA Quantitation and ELISA Accessory Starter Kit (Bethyl Laboratories, Inc., Montgomery, TX, USA), according to the manufacturer's protocol.

Results

Total worm count

The immune expulsion against 2,000 *T. spiralis* larvae infections of four rat strains, three inbred and one mutant strain, were investigated and compared at days 7, 10, and 14 post-infection. The mean number of total adult worms among strains was analyzed with analysis of variance (ANOVA) and Scheffe's *F* test for post hoc test. There were clear-cut strain differences ($P<0.0001$) in the mean number of intestinal worms throughout the observation period (Fig. 1). On day 7 post-infection, BN strain had significantly ($P<0.05$) reduced worm burdens as compared with the three other strains. The mean number of intestinal worms of BN and DA rats was significantly ($P<0.05$) lower than those of F344 rats and *Ws/Ws* rats on day 10 post-infection. At 2 weeks after infection, all BN and DA rats had completely expelled worms from the gut, whereas F344 rats had still retained considerable worms in their gut. Moreover, *Ws/Ws* rats had sustained over 20% of the infected larvae through the observation period.

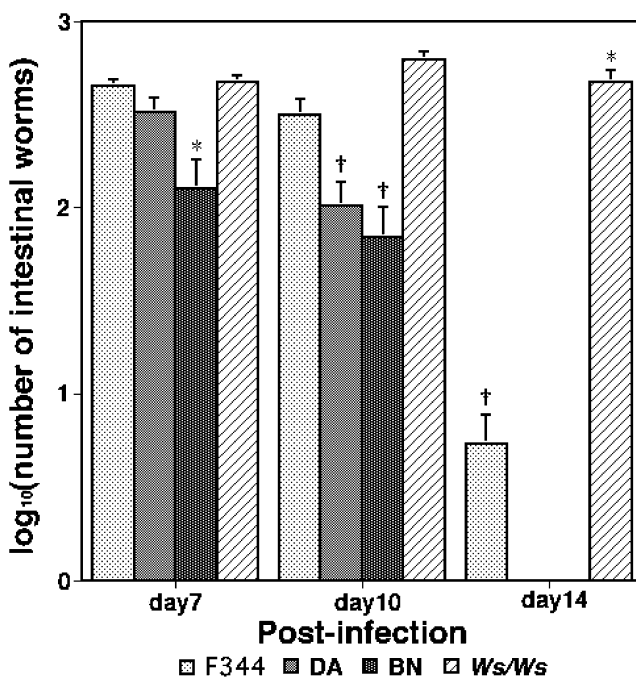


Fig. 1 Comparison of mean intestinal worm count among three (F344, DA, and BN) inbred strains and one (*Ws/Ws*) mutant strains on days 7, 10, and 14 post-infection with 2,000 *T. spiralis* larvae. Data were shown as the mean of logarithmic total worm count $\pm$ SE ($n=10$ to 16 each). *, $\dagger P<0.05$, significant difference from the three other strains

Mast cell count

The kinetics of mast cells in three inbred and one outbred strains after infection with 2,000 *T. spiralis* infective larvae was examined and compared. The mean number of mast cells per one villus among strains was analyzed with ANOVA and Scheffe's *F* test for post hoc test. The representative photomicrographs for each strain are shown in Fig. 2. In *Ws/Ws* rats, none of mast cells was detected throughout the observation period as shown in Fig. 2g. Without infection of the larvae, there was no clear difference in the number of mast cells among the three remaining strains (Fig. 3). The number of mast cells in BN rats reached the maximal value 7 days post-infection and sustained plateau of the value until day 14 post-infection. In DA rats, the number of mast cells was peaked on 10 days after infection, 3 days behind that of BN rats, and then kept invariable until day 14 post-infection. The mast cell hyperplasia in F344 strain was seen from day 5 post-infection and finally attained maximal level 14 days after infection. These results demonstrated that the patterns of mast cell hyperplasia during infection were significantly ($P<0.005$) different among the four strains. To determine whether mast cells have a role as an effector involved in worm expulsion, we analyzed the correlation between the patterns of mast cell recruitment from 5 to 10 days post-infection and the parasite expulsion following the responses of three inbred strains with Pearson's correlation coefficient. As a result, there was a significant correlation ($R^2=0.667$, $P<0.01$) between both specific immune responses. Thus, the finding suggests that mast cells play an important role in immune expulsion of the gastrointestinal helminthes in rats, which is additionally supported by the fact that mast cell mutant-type rats displayed delayed worm expulsion than wild-type rats (Fig. 1).

Eosinophil count

The inflammation response of eosinophil in three inbred and one outbred strains to infection with 2,000 *T. spiralis* larvae was investigated and compared. The mean eosinophil count per one villus among strains was analyzed with ANOVA and Scheffe's *F* test for post hoc test. The patterns of eosinophilia for each strain are shown in Fig. 4. With no infection of the larvae, the number of eosinophils in the BN strain was significantly ($P<0.001$) different from those in the three other strains. The eosinophilia in BN rats was peaked on day 5 post-infection, and then retained the plateau of high value throughout the observation period. In contrast, the eosinophil count of DA and F344 strains showed a gradual increase until 10 days after infection, and came to the level of the BN strain. In the *Ws/Ws* strain, the number of eosinophil showed the peak value at day 10 post-infection, and then signifi-

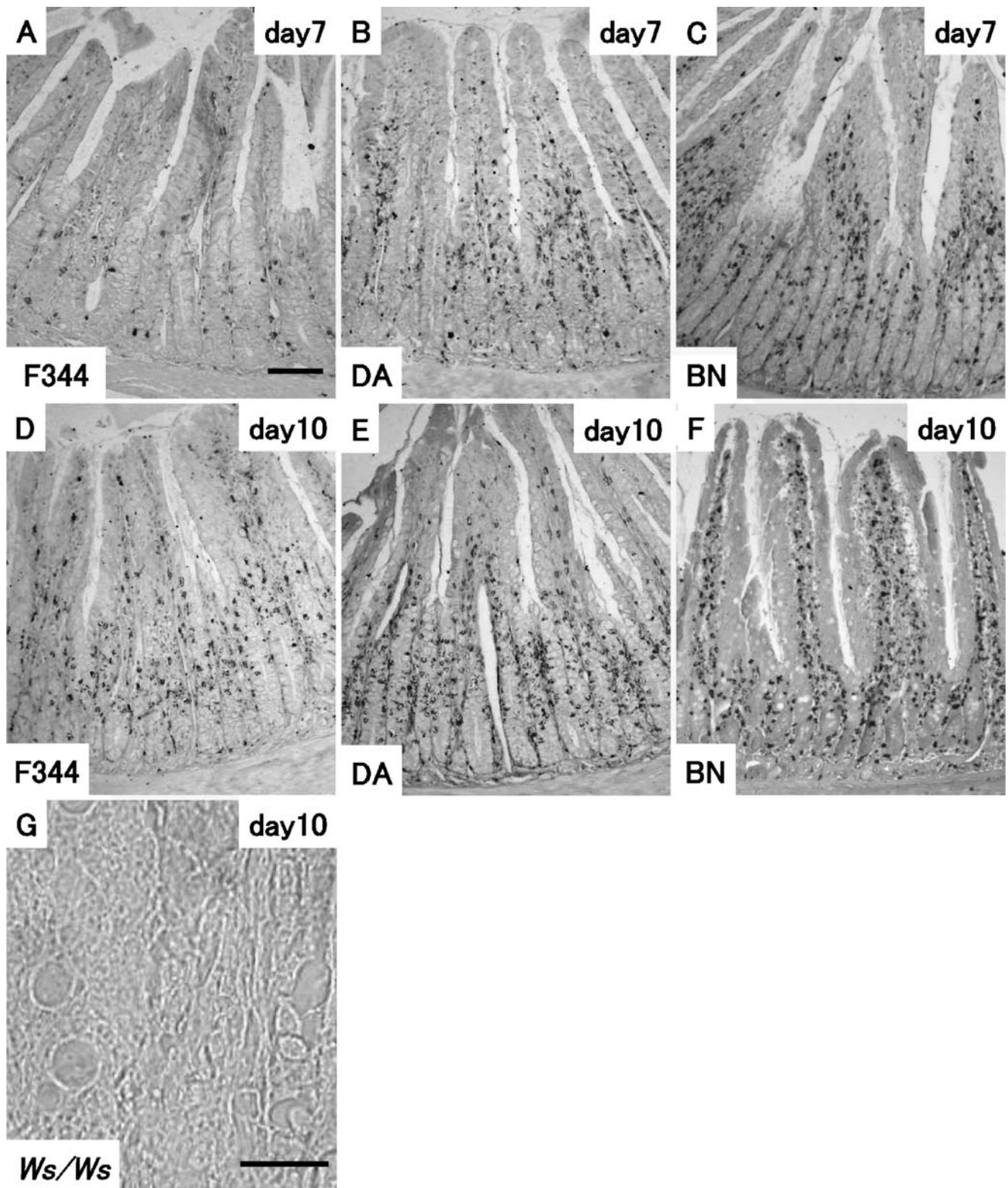


Fig. 2 Representative photomicrographs of toluidine blue and eosin-stained intestinal tissue from F344 (**a**, **d**), DA (**b**, **e**), BN (**c**, **f**), and *Ws/Ws* (**g**) strains on day 7 (**a–c**) and day 10 (**d–g**) post-infection with

Trichinella spiralis nematodes. Scale bar 25 μm (**a–f**) and 10 μm (**g**). As shown in **g**, no signals were detected in *Ws/Ws* rats throughout the observation period

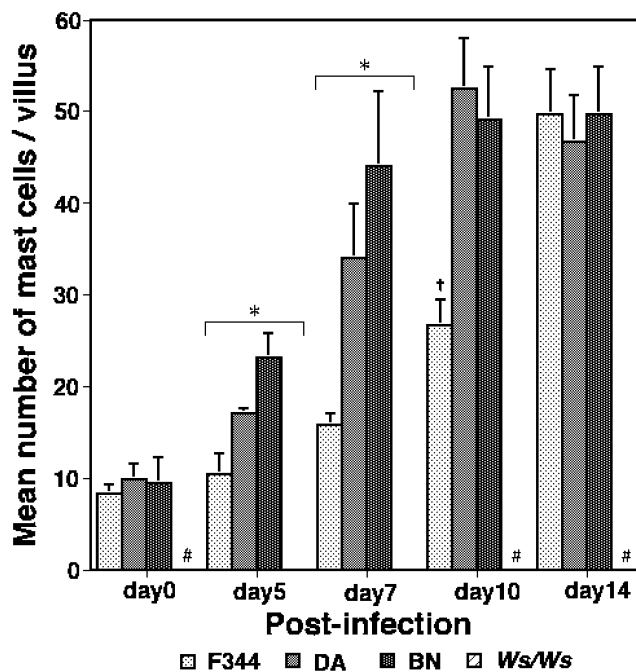


Fig. 3 Kinetics of mean mast cell count in intestines of F344, DA, BN, and Ws/Ws rats following infection with 2,000 *T. spiralis* larvae. Data were expressed as the mean number of mast cells per one villus $\pm$ SD ($n=5$ each). * $P<0.05$, significant difference among the four strains. †, # $P<0.05$, significant difference from the three other strains

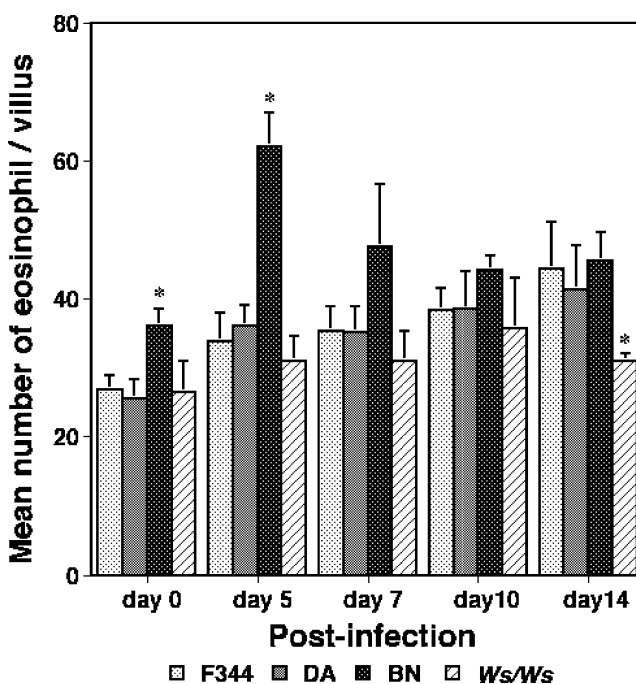


Fig. 4 Kinetics of average eosinophil count in guts of F344, DA, BN, and Ws/Ws rats following infection with 2,000 *T. spiralis* larvae. Data were shown as the mean number of eosinophils per one villus $\pm$ SD ($n=5$ each). * $P<0.05$, significant difference from the three other strains

cantly ($P<0.05$) decreased as compared with those of the three other strains. Subsequently, to test whether eosinophils play a role in parasite expulsion or not, an analysis on the relationship between the patterns of eosinophilia from 5 to 10 days post-infection and immune expulsion of three inbred strains was performed with Pearson's correlation coefficient. There was no significant correlation ($R^2=0.006$, $P>0.05$) between the two immune responses. Therefore, it suggests that eosinophils have a little effect on protective immunity against the helminth infection in rats.

Total serum IgE level

The level of total serum IgE in three inbred and one outbred strains during infection with 2,000 larvae of *T. spiralis* was measured and compared. The means of total IgE in the serum among strains were analyzed with ANOVA and Scheffé's *F* test for post hoc test. Regardless of no infection with larvae, the level of total IgE in the BN strain showed much higher value (>13 $\mu\text{g/ml}$) than those in the three other strains (Fig. 5). Thereafter, the total serum IgE in BN rats kept at high level and gradually increased until 14 days after infection. The total serum IgE levels of the three remaining strains were lower than 1 $\mu\text{g/ml}$ until day 5 post-infection. Subsequently, the IgE production in F344 as well as in Ws/Ws strains gradually increased during the observation period. In contrast, the IgE response of the

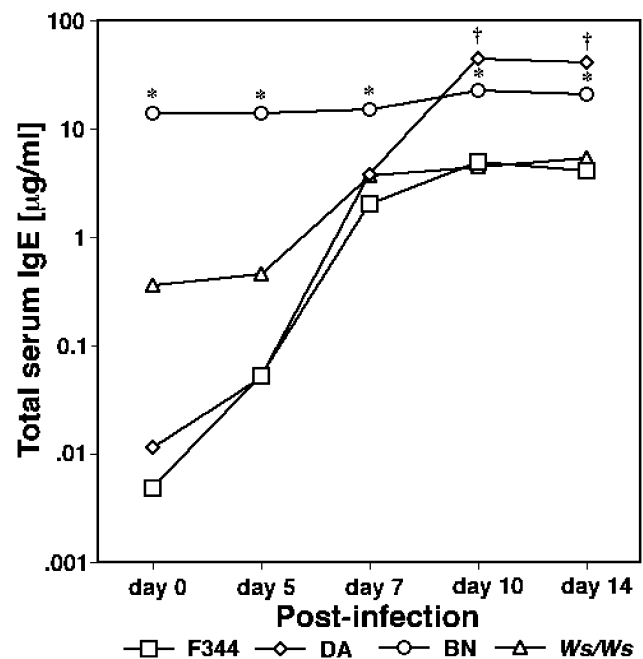


Fig. 5 Fluctuation of the mean total IgE level in sera of F344, DA, BN, and Ws/Ws strains following infection with 2,000 *T. spiralis* larvae. Data were represented as the means $\pm$ SE ($n=5$ each). The vertical axis was transformed into logarithmic scale. *, † $P<0.05$, significant difference from the three other strains

DA strain markedly increased 10 days after infection, which was significantly ($P < 0.0001$) higher than the three other strains. To examine whether total serum IgE level correlates closely with worm expulsion, Pearson's correlation coefficient was calculated for the relationship between the patterns of IgE response from 5 to 10 days post-infection and immune expulsion of the three inbred strains. There was a significant correlation ($R^2 = 0.501$, $P < 0.05$) between both immune responses, which was slightly lower than that calculated for the relationship between mast cell hyperplasia and helminth expulsion as described above. Thus, the fact suggests that IgE is also involved in host defensive response to infection with the nematode in rats.

Discussion

BN rats exhibited faster responses of worm expulsion, mast cell recruitment, and IgE production, whereas F344 rats displayed slower responses of worm rejection, mast cell hyperplasia, and IgE production. These findings coincided with the previous report that BN rats were mast cell and IgE high responders, while F344 rats were low responders in *Nippostrongylus brasiliensis*, gastrointestinal nematode parasite infections (Uchikawa et al. 1996). The DA strain showed intermediate response compared to the BN and F344 strains in both worm expulsion and mast cell recruitment, but strikingly higher response in IgE production than those strains. Moreover, there were significant correlations of mast cell hyperplasia and total serum IgE level with immune expulsion among those strains, respectively. The present study cannot determine whether the parasite specific IgE antibody participates in worm expulsion by directly activating enterocytes as described by Negrão-Corrêa (2001) or by indirectly regulating mast cell responses through the high affinity receptor, FcεR for IgE, which is expressed at high density in mast cells and basophils (Corry and Kheradmand 1994), or if the mast cell itself has a central role in worm expulsion as reported in mice (Lawrence et al. 2004). However, it was reported that IgE was not involved in the recruitment and expansion of mucosal mast cells in the gut following *T. spiralis* infection from the experiment using IgE knockout mice (Gurish et al. 2004). Moreover, the mast cell hyperplasia correlated closely with parasite expulsion among the three strains as compared with IgE response. During *N. brasiliensis* infection, a similar correlation between mast cell recruitment in guts and worm rejection among three rat strains including the DA strain was reported by Nawa and Miller (1979). Therefore, those data suggest that mast cells rather than the IgE antibody might be responsible for the gastrointestinal helminth expulsion in rats as well as in mice.

Mast cell-deficient *Ws/Ws* rats had retained more worms and longer than the other strains during the observation. In addition, total serum IgE level and eosinophilia in these rats did not show a noteworthy kinetics following the helminth infection. The host responses to the intestinal nematode infection in these rats were thought to be similar to those in mast cell deletion strain of mice (Alizadeh and Murrell 1984); hence, it strongly suggested that the effector mechanisms for *T. spiralis* expulsion are common to both mice and rats, and the prominent role of the effectors were assumed by mast cells.

DA-*bg/bg* rats, a model for the Chediak–Higashi syndrome, have partially amorphous and/or giant granules in mast cells, eosinophils, and neutrophils from histological observations (Nishimura et al. 1989; Ozaki et al. 1994). Thus, it is possible that effectors responsible for host protective immunity against gastrointestinal nematode infection have no function or impaired function in these rats. Interestingly, DA-*bg/bg* rats represented delayed and incomplete helminth expulsion compared to wild-type DA rats (data not shown). Moreover, DA-*bg/bg* rats were observed to have morphologic abnormalities of mast cells within their guts to some extent. These findings suggest that delayed worm expulsions shown in these rats might be caused by the dysfunction of mast cells in their guts, and therefore, the activation of mast cells might affect worm rejection from the gut.

Goblet cell hyperplasia other than IgE production, mast cell hyperplasia, and eosinophilia is suspected to be a potential candidate effector in resistance to *T. spiralis* infection (Khan and Collins 2004). Although the number of goblet cells in the gut was compared between DA and F344 rats on days 0, 7, 10, and 14 after *T. spiralis* infection, no significant differences in both strains were detected during the observation period (data not shown). Thus, the finding suggests that goblet cells have a little role in the worm rejection from these rats.

Taken together, the present study strongly demonstrates that mast cells other than the IgE antibody are an important effector for worm expulsion following *T. spiralis* infection in rats as well as in mice. At the same time, this study suggests that eosinophils and goblet cells have little effects on parasite elimination. However, the effector mechanisms may be more complex because host protective responses to gastrointestinal nematode infections would have different effector mechanisms at different parasite species and stages and sites of infection (Negrão-Corrêa 2001). To dissect the precise mechanisms for worm expulsion in rats, further analyses will be required.

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