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A Role of Mast Cell Glycosaminoglycans for the Immunological Expulsion of Intestinal Nematode, *Strongyloides venezuelensis*¹

Haruhiko Maruyama,²,* Yoshisada Yabu,* Ayako Yoshida,* Yukifumi Nawa,† and Nobuo Ohta*²

We examined effects of mast cell glycosaminoglycans on the establishment of the intestinal nematode, *Strongyloides venezuelensis*, in the mouse small intestine. When intestinal mastocytosis occurred, surgically implanted adult worms could not invade and establish in the intestinal mucosa. In mast cell-deficient W/W* mice, inhibition of adult worm invasion was not evident as compared with littermate +/+ control mice. Mucosal mastocytosis and inhibition of *S. venezuelensis* adult worm mucosal invasion was tightly correlated. To determine effector molecules for the invasion inhibition, adult worms were implanted with various sulfated carbohydrates including mast cell glycosaminoglycans. Among sulfated carbohydrates tested, chondroitin sulfate (ChS)-A, ChS-E, heparin, and dextran sulfate inhibited invasion of adult worms into intestinal mucosa in vivo. No significant inhibition was observed with ChS-C, desulfated chondroitin, and dextran. ChS-E, heparin, and dextran sulfate inhibited adhesion of *S. venezuelensis* adult worms to plastic surfaces in vitro. Furthermore, binding of intestinal epithelial cells to adhesion substances of *S. venezuelensis*, which have been implicated in mucosal invasion, was inhibited by ChS-E, heparin, and dextran sulfate. Because adult worms of *S. venezuelensis* were actively moving in the intestinal mucosa, probably exiting and reentering during infection, the possible expulsion mechanism for *S. venezuelensis* is inhibition by mast cell glycosaminoglycans of attachment and subsequent invasion of adult worms into intestinal epithelium. *The Journal of Immunology*, 2000, 164: 3749–3754.

Expulsion of parasites from the host intestine is the most dramatic form of immunity in intestinal nematode infections (1). Studies with rodents have provided considerable information about cytokine regulation of host defense against intestinal nematodes (2, 3); however, little is known as to how effector cells expel intestinal nematodes, and no molecules so far are known that are directly responsible for worm expulsion (4, 5). The role of mast cells in worm expulsion has been studied extensively in various experimental host-parasite systems (6–8). In infection of mice with *Strongyloides ratti* and *Strongyloides venezuelensis*, worm expulsion completes within 2 wk, which is closely associated with intestinal mastocytosis. In nude mice, neither intestinal mastocytosis nor worm expulsion occur, and repeated injection of IL-3 restore the ability of expelling S. ratti as well as intestinal mastocytosis (9, 10). When mast cell-deficient W/W* mice are infected with *S. ratti* or *S. venezuelensis*, expulsion is delayed significantly as compared with littermate control mice (11, 12). Delay in the worm expulsion as well as mast cell response is restored by bone marrow reconstitution with cells from littermate +/+ mice (11, 12). Worm expulsion is more severely impaired in WW* mice deficient for IL-3 gene (13). In these mice, mucosal mast cell responses are almost completely absent, and *S. venezuelensis* continues to parasitize in the intestine for >50 days.

Recent studies with hamsters and rats suggest that sulfated carbohydrates seem to play a certain role in expulsion of *S. venezuelensis*. The production of large quantity of intestinal mucin is associated with *S. venezuelensis* expulsion in hamsters. Hamsters that have heavily sulfated goblet cell mucin expel *S. venezuelensis* within 2 wk, whereas those with moderately sulfated goblet cell mucin harbor *S. venezuelensis* for >40 days (14). In rats, reserpinduced sulfated intestinal goblet cell mucin inhibits establishment of implanted adult worms (15). Mast cells glycosaminoglycans are highly sulfated (16–18), and granular contents are released into the intestinal lumen during worm expulsion (8). Thus, glycosaminoglycans in the secretory granules of mucosal mast cells are primary candidates for effector molecules in *S. venezuelensis* expulsion. Here we demonstrate that mast cells contribute to mouse expulsion of *S. venezuelensis* by preventing adult parasites from invading host intestinal mucosa and that glycosaminoglycans of the type secreted by mast cells can inhibit the binding of *S. venezuelensis* adhesion molecules to mucosal epithelium and the invasion of gut mucosa by *S. venezuelensis* adults. Our findings establish a novel role for mast cell glycosaminoglycans in vivo in host defense against intestinal infection.

**Materials and Methods**

**Animals and parasites**

Male Wistar rats, C57BL/6 mice, mast cell-deficient WBB6F₁−/− mice, and littermate control WBB6F₁−/−/+ mice were purchased from SLC Japan, (Hamamatsu, Japan). *S. venezuelensis* was kindly provided by Professor Y. Sato (Department of Parasitology, School of Medicine, University of the Ryukyus) and has been maintained in serial passage in male Wister rats in our laboratory. Third-stage infective larvae were obtained from fecal culture by a filter paper method as previously described (19). Adult worms for intraduodenal implantation were prepared as follows. Wistar rats were inoculated with 20,000 infective larvae, and the upper half...
of the small intestine was removed 8–10 days after infection. The intestine was then cut open longitudinally and washed vigorously with PBS followed by incubation at 37°C for 80 min. Worms that emerged from the intestine were washed with sterile PBS and adjusted to an appropriate concentration. Adult worms suspended in 500 μl of PBS were then injected into the duodenum of recipient mice (1000–1200 worms/mouse) under ether anesthesia using a 1-ml syringe with an 18-gauge needle.

**Invasion of intestinal mucosa by adult worms**

To measure the invasion activity of adult *S. venezuelensis* into mouse intestinal mucosa, adult worms were implanted in the duodenum of recipient C57BL/6 mice, and the mouse intestines were removed at various times after implantation. Intestines were cut open longitudinally and washed lightly to remove worms that were still in the lumen. Then the intestines were incubated at 37°C in PBS for 3 h. The number of worms that emerged from the intestines was counted, and the percentage of recovery was calculated relative to implanted worm number.

**Emergence of adult worms from mouse intestines**

C57BL/6 mice were inoculated with 5000 infective larvae, and the whole small intestine was removed 7 days after infection. The intestine was cut open longitudinally and washed lightly to remove intestinal contents in the lumen. The intestines were then incubated at 37°C in PBS without stirring, and emerging worms were collected and counted under a microscope at various times after incubation. After incubation for 6 h in total, intestinal mucosa were scraped off and the number of adult worms that were still in the mucosa was counted. The total number of adult worms in the intestine was calculated, and the relative number of adult worms emerging at various times after incubation was determined.

**Inhibition of adult worm invasion by mucosal mast cells**

To examine the effects of intestinal mastocytosis on the mucosal invasion of *S. venezuelensis*, C57BL/6 mice were inoculated with 5000 infective larvae, and adult worms were implanted 2, 3, and 8 wk postinfection. Because *S. venezuelensis* that matured in C57BL/6 mice were completely expelled by 12 days postinfection, all adult worms that recovered after intraduodenal adult worm implantation were implanted worms. Four hours after implantation, the whole small intestine was removed, and adult worms that invaded the intestinal mucosa were recovered as described above. The percentage of invading worms was calculated relative to implanted worms. The roles of mast cells were further examined with mast cell-deficient W/Wv mice. W/Wv mice have mutations in c-kit protein, in which W and Wv are a missense mutation in the kinase domain and a deletion mutation in transmembrane domain, respectively (20). Because c-kit protein is the receptor for c-kit ligand/stem cell factor (21), which induces development of both connective tissue type and mucosal mast cells (22, 23), W/Wv mice virtually lack mast cells (24). W/Wv and control +/+ mice were inoculated with 5000 infective larvae, and 2 wk postinfection 20 mg/kg of mebendazole (Sigma, St. Louis, MO) was orally administered for 2 days to remove adult worms that matured in the animals. Three weeks postinfection, adult worms were implanted, and the number of adult worms that invaded the intestinal mucosa was determined.

**Effects of sulfated carbohydrates on the invasion of adult worms**

To test the inhibitory effects of carbohydrates in the mucosal invasion of *S. venezuelensis*, adult worms were implanted in the duodenum of C57BL/6 mice with 20 mg/ml of various carbohydrates, such as dextran (m.w. = 580,000), dextran sulfate (Dxs) (m.w. = 500,000), heparin, chondroitin sulfate (ChS)-E, ChS-A, ChS-C, and desulfated chondroitin. ChS-E and desulfated chondroitin were purchased from Seikagaku (Tokyo, Japan), and others were obtained from Sigma. Worms that invaded the intestine were recovered and counted as described above.

**Inhibition of adhesion of adult worms to culture plates**

*S. venezuelensis* adult worms suspended in PBS were plated in wells of microtiter plates (Nunc, Roskilde, Denmark) at a density of ~120 worms per well with various concentrations of sulfated and nonsulfated carbohydrates. After overnight incubation at 37°C, wells were gently washed with PBS to remove unattached worms, and the number of adherent worms was counted under a dissecting microscope. Percent inhibition in adhesion was calculated relative to PBS control.

3 Abbreviations used in this paper: Dxs, dextran sulfate; ChS, chondroitin sulfate.
womans in the duodenum of recipient mice 2, 3, and 8 wk postinfection with S. venezuelensis and recovered invading worms 4 h after implantation. In mice 2–3 wk after primary infection, right after worm expulsion had taken place, implanted worms were completely rejected. In contrast, a considerable number of implanted worms invaded in mice 8 wk after primary infection, when intestinal mastocytosis had ceased (Fig. 2A). The ability of adult worms to invade intestinal mucosa was inversely correlated with intestinal mastocytosis (Fig. 2B).

To further evaluate the contribution of mucosal mast cells in the worm rejection, similar experiments were conducted with mast cell-deficient W/W\(^{v}\) mice. W/W\(^{v}\) mice, 3 wk after primary infection, allowed significant worm invasion as compared with littermate +/+ control mice, though they accepted less worms than naive W/W\(^{v}\) mice (Fig. 2C). The number of mucosal mast cells in W/W\(^{v}\) mice 3 wk after primary infection indicated that the partial protection was due to weak intestinal mastocytosis in W/W\(^{v}\) mice (Table I). Thus, the inhibition of mucosal invasion by adult worms was tightly associated with intestinal mastocytosis.

**Table I. The number of mucosal mast cells in W/W\(^{v}\) mice and control +/+ mice infected with S. venezuelensis\(^{a}\)**

<table>
<thead>
<tr>
<th>Time After Infection (wk)</th>
<th>W/W(^{v}) Mice</th>
<th>+/+ Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.3 ± 1.2</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>3</td>
<td>19.2 ± 4.0</td>
<td>92.7 ± 8.3</td>
</tr>
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\(^{a}\) All values are expressed as mean ± SD. W/W\(^{v}\) mice as well as +/+ mice showed significant mastocytosis 3 wk postinfection as compared to naive mice (\(p < 0.0001\)). The number of mast cells of naive W/W\(^{v}\) mice is not significantly different from that of naive +/+ mice.

**Establishment of adult worms in the mouse intestine is inhibited by sulfated carbohydrates**

We tested various carbohydrates including mast cell glycosaminoglycans for their ability to inhibit the invasion of adult S. venezuelensis in vivo. When adult worms were implanted in the duodenum with DxS, heparin, ChS-E, and ChS-A, invasion of adult worms was significantly inhibited. Dextran, ChS-C, and desulfated chondroitin did not inhibit significantly (Fig. 3). The invasion inhibition did not seem to be caused by the toxic effects of the carbohydrates added, because worms were totally viable and actively moving after incubation overnight at a higher concentration (100 mg/ml) of these carbohydrates.

**Mechanism for invasion inhibition**

To investigate inhibition mechanisms of sulfated carbohydrates, we first tested inhibitory effects of glycosaminoglycans on the adhesion of adult worms to a plastic surface. Adult worms of S. venezuelensis adhere firmly to plastic surfaces with orally secreted adhesion substances and actively move as if they are trying to burrow into the plastic dishes (25). We hypothesized that adult worms adhere to intestinal cells in vivo upon invasion with adhesion substances, which should be a prerequisite for invasion into host tissues (30). We incubated adult worms with various sulfated carbohydrates in wells of plastic plates. DxS, heparin, and ChS-E inhibited adhesion of adult worms to plastic surfaces in a dose-dependent manner (Fig. 4A). ChS-A had relatively weak inhibitory effects, and ChS-C inhibited only at higher concentrations. Dextran and desulfated chondroitin did not inhibit significantly. These findings agreed well with the results of in vivo implantation experiments.

Next, we attempted to observe attachment of adult worms to intestinal epithelial cells in vitro. For this purpose, adult worms were incubated on a monolayer of CaCo-2 human intestinal epithelial cells. Worms adhered to CaCo-2 cells by orally secreted adhesion substances; however, additional experiments could not be performed because the attached worms tore the monolayer off from the bottom of the wells as they wriggled around. Therefore, we performed because the attached worms tore the monolayer off from the bottom of the wells as they wriggled around.
examined the binding of adhesion substances to CaCo-2 cells. Adhesion substances, or kissing marks, were prepared on the bottom of the wells as previously described (25), and a single-cell suspension of CaCo-2 cells was added to the wells. CaCo-2 cells adhered firmly to the substances within 30 min (Fig. 5), whereas, cells did not attach significantly to noncoated or BSA-coated wells. We found that the binding of parasite-derived adhesion substances to CaCo-2 cells was significantly inhibited by sulfated carbohydrates including DxS, heparin, and ChS-E in a dose-dependent manner (Fig. 4B). However, CaCo-2 cells bound to adhesion substances when cells or adhesion substances were preincubated with DxS or heparin. This is likely to indicate a strong interaction between cells and adhesion substances. Based on the in vitro binding experiments, we concluded that sulfated carbohydrates blocked the attachment of adult worms to intestinal epithelial cells and inhibited subsequent invasion into the epithelial layer.

Next we questioned why invasion inhibition resulted in the worm expulsion. Adult Strongyloides nematodes are believed to move between intestinal cells with a continuous motion of invading and exiting the epithelial layer during infection (29). If this assumption is true, adult worms would be expelled when their reinvasion is unsuccessful. We removed whole small intestines from mice 7 days after infection and incubated them at 37°C in PBS without stirring. Adult worms that resided between intestinal epithelial cells emerged from the intestinal mucosa as early as 10 min after incubation, and about 60% of total worms in the mucosa migrated out within 2 h (Fig. 6). Because adult worms are remarkably active in vitro, and intestinal mucosa did not disintegrate in 2 h of incubation, the quick emergence of adult worms seemed rather an active process than a merely passive one. Although these results did not directly demonstrate that adult S. venezuelensis worms are exiting the mucosa in a natural situation, it seems probable that adult worms were actively moving in the intestinal mucosa. Moreover, scanning electron micrography of the surface of infected intestines revealed adult worms simultaneously embedding their heads and tails in the intestinal mucosa (Fig. 7). The exposure of the worms did not seem to be the result of necrosis of epithelial cells around the worms, because enterocytes around adult Strongyloides worms usually do not show signs of necrosis (29). Based on the present results, we concluded that S. venezuelensis were active in exiting and reentering the intestinal mucosa during infection and that the
inhibition of reinvasion by mast cell glycosaminoglycans caused worm expulsion.

Discussion

Glycosaminoglycans in the secretory granules are the basis of metachromatic staining of mast cells, which is a striking and characteristic property of mast cell cytoplasm (31), though little is known about physiological function of intracellular glycosaminoglycans. A role for mast cell proteoglycans, which consist of numerous glycosaminoglycan side chains linked to serglycin core peptide (32, 33), has been suggested in stabilizing proteases in the granules (34) and control of protease expression through a post-translational mechanism (35). Most studies concerning mast cells in inflammatory processes have focused on low m.w. mediators and granular proteases (36, 37). The present study unraveled a novel function of mast cell glycosaminoglycans in vivo as effector molecules for the expulsion of the intestinal nematode, S. venezuelensis. Glycosaminoglycans of the type secreted by mast cells inhibited the binding of S. venezuelensis adhesion molecules to mucosal epithelial cells and the invasion of intestinal mucosa by S. venezuelensis adults. ChS-E and ChS-A, which were effective in inhibition in vivo, are contained in mucosal mast cells. Biochemical analyses in rat mucosal mast cells reveals that 19-20% of the total disaccharides consist of ChS-E-type structures and 60% consist of ChS-A-type structures (38). Similarly, ChS chains synthesized by mouse cultured mast cells, which phenotypically resemble mucosal mast cells, contain 60-70% of ChS-A-type disaccharides and 30-40% of ChS-E-type disaccharides (39).

The inhibitory effects of glycosaminoglycans and sulfated carbohydrates on the invasion of adult worms seem to reside in their binding activities to adhesion substances. The adhesion substances of S. venezuelensis bind tightly to heparin, which can be inhibited by heparin, DxS, ChS-E, and ChS-A, but not by ChS-C (40). Considering that the invasion inhibition was obvious with heparin, DxS, ChS-E, and ChS-A, but not with ChS-C, the binding affinity between sulfated carbohydrates and adhesion substances is the key in effector function. Charge density seems to be an important factor in the interaction (39). Because adhesion substances are secreted from the mouse (25), the head of the worms would attach to the apical surfaces of intestinal cells, where heparan sulfate proteoglycans or heparin-like molecules are expressed (41, 42). However, at the time of worm expulsion, mast cell glycosaminoglycans would bind to adhesion substances of the worms and inhibit binding of the worms to intestinal epithelial cells. Although the present results did not demonstrate unequivocally that adult worms were exiting the mucosa in a natural situation, it seems probable that adult worms are active in exiting and entering the mucosa during infection. Worms that fail to attach to and invade the intestinal mucosa would eventually be expelled from the intestine.

If mast cell glycosaminoglycans act as effector molecules by preventing worm attachment and subsequent invasion, mast cells have to be close to the surface of the intestinal epithelium and release granular contents. It has been known that intraepithelial mast cells contribute to worm expulsion. When W/Wv mice are challenged with S. ratti after adoptive transfer of cultured mast cells, the mice cannot expel the nematodes despite successful restoration of intestinal mastocytosis (43). The difference between cultured mast cell-transferred W/Wv mice and bone marrow-reconstituted W/Wv mice, which expel the worms normally, is the location of intestinal mast cells. Cultured mast cell-transferred W/Wv have far less intraepithelial mast cells (43). In addition, S. venezuelensis adult worms are not expelled in Mongolian gerbils, in which mucosal mast cells never migrate into the intestinal epithelium (44, 45). Mucosal mast cells release their granular contents upon antigenic stimulation (8, 46), but the mode of degranulation might be different from that in connective tissue mast cells. It is known in mice that mucosal mast cells show characteristic paracrystallization of their granular contents in intestinal nematode infections (47, 48). Interestingly, mucosal mast cells in S. ratti-infected mice do not show signs of usual degranulation, but they disintegrate near the surface of the epithelium (49). Our results indicate that the local concentration of glycosaminoglycans has to be high to inhibit invasion in vivo. A detailed study on the degranulation processes of mucosal mast cells in nematode infections is necessary. Furthermore, it would be interesting to examine the mucosal invasion of S. venezuelensis in IL-9 transgenic mice, which show increased mast cell infiltration in the intestinal epithelium with no signs of massive degranulation (50).

Another possible mechanism for worm expulsion by mast cell glycosaminoglycans is an inhibitory effect of glycosaminoglycans on parasite enzymes. It is well known that heparin and related compounds bind to a wide range of proteins including proteases either specifically or electrostatically (34, 51, 52). Therefore, mast cell glycosaminoglycans would bind not only to the adhesion substances but also the proteolytic enzymes of S. venezuelensis that are required for the invasion of the intestinal epithelial layer. Although nothing has been known about the secreted proteases of adult Strongyloides nematodes, protease activities have been shown in secreted Ags in tissue-invading intestinal nematodes (53-55). Further investigation about the invasion processes of S. venezuelensis will help us to understand defense mechanisms against intestinal infections.

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References
