Evidence for a role of mast cells in the mucosal injury induced by Newcastle disease virus

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ABSTRACT We have previously demonstrated that mast cells were significantly increased during Newcastle disease virus (NDV) infection, but their precise role in the process is unknown. In this study, we investigated the role of mast cells in this process by using ketotifen, a mast cell membrane stabilizer. A total of 60 specific-pathogen-free chickens were randomly divided into 3 groups of 20 birds each (NDV-infected group, ketotifen-pretreated group, and the control group). The ketotifen-pretreated group was administered orally with ketotifen before NDV infection. On 12, 24, and 48 h postinfection, 5 chickens from each treatment were killed. Tissues of proventriculus were collected to quantify mast cells, the content of tryptase and histamine by cytochemistry, immunohistochemistry, and fluorescence analysis, respectively. The results showed that the population of mast cells and the content of tryptase and histamine were increased significantly in the proventriculus (P < 0.01) of infected birds compared with the control group. An acute mucosal injury was observed in the infected chickens. In contrast, among chickens pretreated with ketotifen, followed by NDV infection, the mast cells number and the content of tryptase and histamine were decreased significantly (P < 0.01). Likely as a result, the mucosal injury was remitted remarkably. The overall results of this experiment suggest that mast cells are implicated in NDV-induced mucosal injury. Inhibition of mast cell mediator release may represent a novel strategy to modulate this process.

Key words: mast cell, tryptase, histamine, Newcastle disease virus

INTRODUCTION

Newcastle disease is a worldwide disease of poultry caused by Newcastle disease virus (NDV), a member of the genus Rubulavirus, family Paramyxoviridae (Awan et al., 1994; Brown et al., 1999; Swayne and King, 2003). Newcastle disease virus strains are grouped into 5 pathotypes based on clinical signs induced in infected chickens (Beard and Hanson, 1984): (I) viscerotropic, (II) neurotropic, (III) mesogenic, (IV) lentogenic, and (V) asymptomatic enteritic. Both velogenic pathotypes cause severe clinical disease and infect multiple tissues. These lesions are often particularly prominent in the proventriculus, ceca, and small intestine, which are usually more extensive and severe with viscerotropic velogenic (VVNDV) than with neurotropic velogenic (NVNDV), characterized by hemorrhagic and necrotic lesions in the intestinal mucosa of dead birds (Heckert et al., 1996; Brown et al., 1999).

The mucosa has long been regarded as a physical barrier to invading pathogens, including NDV, but studies over the last decade have shown that the mucosa contributes to host protection more than initially thought. Evidence accumulated from several independent studies suggests that the mucosa may directly influence adaptive immune responses (Neutra et al., 1996; Mowat and Viney, 1997).

Mast cells, the key inflammatory cell, are resident in tissues throughout the body but are most common at sites that are exposed to the external environment, such as the skin, airways, and gastrointestinal tract (Galli et al., 1999). As long-lived cells, they can have an enormous effect on the tissue microenvironment through the selective release of a wide variety of preformed and newly derived mediators (Dawicki and Marshall, 2007). Mast cells play an important role in host defense against various pathogens, but their role in viral infection has not been clear (Marshall, 2004). It was reported that Toll-like receptor 3-induced activation of mast cells stimulates CD8+ T-cell recruitment.
after stimulation with NDV (Orinska et al., 2005). Also, it has been revealed that mast cells and tryptase were crucial to the inflammation of acute infectious bursal disease (Wang et al., 2008). Recently, we found that mast cell population was significantly increased in NDV-infected specific-pathogen-free chickens. More intriguing, the greatest number of mast cells were found in the proventriculus, which also showed the greatest level of NDV antigen (Sun et al., 2008). These findings indicated that mast cells may contribute to the inflammation response during the disease.

Ketotifen is a mast cell membrane stabilizer; the capacity of the histamine H1 receptor antagonist ketotifen to inhibit mast cell degranulation and the release of histamine and proinflammatory mediators from mast cells has been known for some time (Kubes et al., 1993). Its effects on reducing chicken mucosal injury during NDV infection have not been investigated previously. To determine the role of mast cells in the NDV-induced mucosal injury, in this study, ketotifen was used to inhibit the activation of mast cells.

**MATERIALS AND METHODS**

**Birds and Experimental Treatments**

Sixty 4-wk-old healthy specific-pathogen-free chickens (White Avian, Merial Co. Ltd., Beijing, China) were housed in separate isolators and provided with feed and water ad libitum. All chickens were divided into 3 groups of 20 birds each (NDV-infected group, ketotifen-pretreated group, and the control group). The NDV F48E8 strain was obtained from the China Institute of Veterinary Drug Control (Beijing). The virus was propagated in 9-d-old embryonated chicken eggs and glutaraldehyde-polyoxymethylene solution and kept at −80°C for later histamine analysis. Ketotifen is a mast cell membrane stabilizer; the capacity of the histamine H1 receptor antagonist ketotifen to inhibit mast cell degranulation and the release of histamine and proinflammatory mediators from mast cells has been known for some time (Kubes et al., 1993). Its effects on reducing chicken mucosal injury during NDV infection have not been investigated previously. To determine the role of mast cells in the NDV-induced mucosal injury, in this study, ketotifen was used to inhibit the activation of mast cells.

**Sample Collection**

On 12, 24, and 48 h postinfection (hpi), 5 birds in each group were selected randomly, and 10 samples of each proventriculus tissue, about 2 cm in length, were fixed immediately by immersion in 2.5% (vol/vol) glutaraldehyde-polyoxymethylene solution and kept at room temperature for 72 h until used for cytochemistry and immunohistochemistry staining. Another sample of each proventriculus tissue, about 10 cm in length, was immediately immersed in liquid nitrogen and preserved at −80°C for later histamine analysis.

**Histological Studies**

For histological studies, the fixed proventriculus tissues were dehydrated by increasing concentrations of ethanol, and the tissues were embedded in paraffin. Thereafter, sections of tissue were cut at 5 μm, mounted on clean glass slides, and dried overnight at 37°C. Sections were cleared, hydrated, and stained with hematoxylin and eosin for histological damage evaluation, according to standard protocol, and the slides were coded to prevent observer bias during evaluation. All tissue sections were examined with an Olympus BH-2 microscope (Olympus Optical Co. Ltd., Beijing, China). Motic Images 2000 (Motic China Group Co. Ltd, Guangzhou, China) was used for characterization of histopathological changes.

**Improved Toluidine Blue Staining Procedure for Mast Cells**

Mast cells were examined by an improved toluidine blue staining method based on previous reports (Franzogiannis et al., 1999; Xu et al., 2001). Briefly, tissue sections were dewaxed, rehydrated, and immersed in 0.1% toluidine blue for 10 min. Slides were then washed with distilled water for 30 s and were immediately put into 95% alcohol until the cells appeared deep reddish purple under the microscope, then were immersed in 100% alcohol, alcohol-xyol (1:1, vol/vol), and xyol for 3 min, respectively, and then mounted with neutral gums.

Quantitative analysis of mast cell density in tissues was performed by counting the number of mast cells in 10 high-power fields (40× magnification) and the mean was calculated. The whole section was scanned for general qualitative observations, but detailed examination focused on mast cells. Mast cell density was expressed as cells per square millimeter.

**Measurement of Tryptase with Immunohistochemical Staining**

Tryptase is stored almost exclusively in mast cells. It is secreted along with other mast cell granule products on mast cell degranulation (Schwartz et al., 1981; Walls et al., 1990). To detect the distribution and contents of tryptase in tissues, an immunohistochemical staining was performed. Briefly, sections were deparaffinized in xylene and descending grades of ethanol to PBS. Endogenous peroxidase activity was blocked by incubation in 3% H2O2 in absolute methanol for 15 min, followed by a 30-min incubation in 20% normal goat...
serum in 80% PBS. Slides were then incubated with the monoclonal mouse anti-human mast cell tryptase (Sigma) primary antibody at a dilution of 1:2,000 for 1 h at room temperature, followed by incubation with goat anti-mouse IgG poly-horseradish peroxidase (Sigma) secondary antibody at a dilution of 1:20 for 30 min. The reaction was developed at 37°C for 8 min with 3,3-diaminobenzidine (Zymed Laboratories Inc., San Diego, CA). Slides were then counterstained with hematoxylin, dehydrated, and mounted. For each tissue specimen, negative controls were prepared by omitting the incubation with the primary antibody.

Tryptase-positive cells in proventriculus were counted in 10 high-power fields at 40× magnification. The results were expressed as area density (area of the positives/area of the whole field).

**Fluorescent Assay of Histamine**

The content of histamine in tissues can be determined by their fluorescence intensity. A fluorometric method used for the determination of histamine was described previously (Håkanson et al., 1972). Tissues were homogenized with 2 mL of 25% (wt/vol) trichloroacetic acid. After tissue homogenization and extraction, 1.0 mL of HCl phase was diluted by double-distilled water into 2.0 mL, then 0.5 mL, 0.4 N NaOH and 0.1% o-phthalaldehyde methanol was added. The mixtures reacted at 22°C for 10 min, and then were stopped by adding 0.5 mL of 0.5 N HCl. As the blank, another 2.0 mL HCl phase was added orderly by 0.5 mL 0.5 N HCl, 0.1% o-phthalaldehyde methanol, and 0.5 mL of 0.4 N NaOH. Then the fluorescence intensity of the sample was determined by a fluorescence spectrometer (Shantou Keyi Instrument & Equipment Co. Ltd., Shantou, China). The activation wavelength was 360 nm and the fluorescence wavelength was 450 nm.

**Statistical Analysis**

Experimental data were analyzed by 1-way ANOVA of the SAS statistical program (SAS Institute Inc., Cary, NC). The results were expressed as means and SE. Differences were considered statistically significant at $P < 0.05$ or $P < 0.01$.

**RESULTS**

**Histopathological Changes in Proventriculus**

Histopathological lesions were mainly observed in the mucous membrane of the proventriculus in NDV-infected birds. In the control birds, the mucosal epithelium was complete and inflammatory cells were scarcely present in the lamina propria of the mucosa (Figure 1A). In the infected birds, extensive necrotic lesions of overlying mucosal epithelium were observed. Microscopically, the villi were swollen and had various degrees of degeneration and necrosis. In addition, plenty of inflammatory cells had infiltrated the proper layer of the villi (Figure 1B). In chickens pretreated with ketotifen, the damage to villi was remitted. The villi were relatively complete compared with the infected birds. Few inflammatory cells were observed (Figure 1C).

**The Distribution and Numbers of Mast Cells**

Toluidine blue staining was used to identify mast cells in tissues. Mast cells were recognized as round or elongated cells with metachromatic granular staining. Mast cells in the glandular stomach of birds were mostly distributed in the lamina propria of the mucosa (Figure 2). There was no obvious difference in the distribution between the 3 groups. Meanwhile, the morphology of mast cells changed over the course of the infection. Mast cells in the control group were all nearly

![Figure 1. Histopathological lesions in the mucosa of chicken proventriculus at 48 h after Newcastle disease virus infection. (A) In control chickens, the epithelial cells are polarized and the nuclei are localized at their base. (B) In infected chickens, the villi are extremely swollen and many nuclei became karyolitic. The epithelial cells had various degrees of degeneration and necrosis (hollow arrow), with a large number of inflammatory cell infiltration (arrow). (C) In the ketotifen-pretreated chickens, the structure of the villus was relatively complete (hollow arrow) and few inflammatory cells were observed (arrow). Hematoxylin and eosin stain. Original magnification ×200.](https://academic.oup.com/ps/article-abstract/88/3/554/1511835)
intact (Figure 2A, B, and C). However, in the infected group, mast cells exhibited long tail-like cytoplasmic projections filled with granular content. Some extruded granules were dispersed around the cell body (Figure 2D, E, and F). Compared with the birds in the infected group, mast cells in the ketotifen-pretreated birds were relatively intact (Figure 2G, H, and I).

There was a significant ketotifen effect on the number of mast cells in the proventriculus at 12, 24, and 48 hpi (Figure 3), with the number of mast cells greater \( (P < 0.01) \) in the NDV-infected group compared with the control (13.91 vs. 2.56, 42.75 vs. 3.07, 23.83 vs. 2.73), whereas the number of mast cells was statistically decreased \( (P < 0.01) \) in the inhibited group compared with the infected group (6.36 vs. 13.91, 9.87 vs. 42.75, 8.72 vs. 23.83).

**The Expression of Tryptase in Glandular Stomach**

Tryptase is an important protease released by mast cells, and we also examined expression of tryptase using an established histochemical technique (Slater et al., 1996; He et al., 1998). In the control group, tryptase-positive cells were hardly observed (Figure 4A, B, and C), whereas they were expressed extensively in the NDV-infected group (Figure 4D, E, and F) and ketotifen-pretreated group (Figure 4G, H, and I). Tryptase-

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**Figure 2.** The distribution of mast cells in proventriculus. Mast cells in the proventriculus of the birds are distributed throughout lamina propria of mucosa from 12 to 48 h postinfection (hpi). C = control group (A, B, and C); N = Newcastle disease virus-infected group (D, E, and F); K = ketotifen-pretreated group (G, H, and I). Improved toluidine blue stain. Original magnification ×400.

**Figure 3.** The change of mast cell number in proventriculus. Toluidine blue staining for mast cells. Results are expressed as mean mast cells per square millimeter ± SEM. Double asterisks = significantly different from control at \( P < 0.01 \); double stars = significantly different from the infected group at \( P < 0.01 \). C = control group; N = Newcastle disease virus-infected group; K = ketotifen-pretreated group.
positive cells were mostly located in the lamina propria of the mucosa. The production of tryptase-positive granules was significantly increased from 12 to 48 hpi in infected birds compared with the control ($P < 0.01$; Figure 5). In addition, the content was reduced from 12 to 48 hpi in ketotifen-pretreated birds compared with the NDV-infected group ($P < 0.01$). Tryptase expression had a positive correlation with mast cell distribution in the 3 groups.

**Histamine Content in Proventriculus**

The histamine content was significantly increased from 12 to 48 hpi in the proventriculus of all NDV-infected birds compared with the control birds ($P < 0.01$), in which the concentration reaches a peak at 24 hpi. The ketotifen-pretreated group significantly reduced the histamine content in the proventriculus ($P < 0.01$; Figure 6).

**Figure 4.** The distribution of tryptase positives in proventriculus. In the control group (A, B, and C), tryptase-positive cells were hardly observed in the lamina propria of mucosa. In the infected (D, E, and F) and ketotifen-pretreated birds (G, H, and I), tryptase-positive cells (brown) were observed extensively in the lamina propria of mucosa. (J) No tryptase-positive cells were present in the negative control. C = control group; N = Newcastle disease virus-infected group; K = ketotifen-pretreated group. Immunohistochemistry, Mayer’s hematoxylin counterstain. Original magnification $\times 400$. hpi = hours postinfection.
DISCUSSION

In this study, we evaluated the presence of mast cells and mucosal injury of the proventriculus induced by NDV infection. We found that the population of mast cells was increased significantly in NDV-infected birds. Acute mucosal injury was observed in infected birds. However, pretreatment with ketotifen before NDV infection decreased the number of mast cells significantly compared with the infected group. Furthermore, mucosal injury in the pretreated group was remitted compared with the infected group. These data indicated that mast cells were implicated in mucosal injury during NDV infection.

Mast cells are present in the skin, the entire gastrointestinal tract, and the airways, where they are in close contact with the outside environment. Mast cells within all layers of the gut wall play a key role in the gastrointestinal tract inflammatory processes and the immune response (De Jonge et al., 2002; Schneider et al., 2002). Studies in mast cell-deficient and reconstituted mice have demonstrated that mast cells in specific, albeit limited, models of inflammation amplify many features of immunologically nonspecific acute inflammatory responses (Qureshi and Jakschik, 1988; Wershil et al., 1988). In addition, mast cells are known to release several mediators that have been suggested to induce gastric injury, resulting in severe hemorrhage and tissue necrosis in rats (Ogle and Lau, 1980; Takeuchi et al., 1986). The role of these cells in the pathogenesis of acute mucosal injury during NDV infection remains unclear.

An important finding of the current study is that the number of mast cells was increased in the infected tissues, with infiltration of inflammatory cells. However, inflammatory cells were rarely observed in the ketotifen-pretreated group. This may be explained if mast cells serve as a primary detector of tissue infection or invasion and release proinflammatory mediators that recruit leukocytes to the appropriate site at risk (Gaboury et al., 1995).

Although there have been few studies in this area, mast cells might also have a crucial role in avian diseases. Previous studies have shown that mast cells are crucial in acute phase responses in the intestines of chickens experimentally challenged with *Eimeria* (Morris et al., 2004). In addition, an increase in mast cell numbers may result in an increase in the production and release of active mediators that may contribute to the inflammation of acute infectious bursal disease induced by very virulent infectious bursal disease virus infection (Wang et al., 2008). More recently, we found that mast cell populations were increased markedly in the proventriculus after NDV infection, which also showed the greatest level of NDV antigen and severe mucosal injury (Sun et al., 2008).

Mast cells release large amounts of active mediators including tryptase, histamine, and other cytokines (Marshall, 2004; Galli et al., 2005). Tryptase is stored almost exclusively in mast cells. It is secreted along with other mast cell granule products during mast cell degranulation (Schwartz et al., 1981; Walls et al., 1990). Because mast cell degranulation predominantly occurs during inflammatory conditions, tryptase can be expected to have a role in the regulation of inflammatory responses (Hallgren and Pejler, 2006). It has been reported that elevated tryptase can induce an inflammatory reaction characterized by granulocyte infiltration, increased wall thickness, and tissue damage in inflammatory bowel diseases (Cenac et al., 2002).

**Figure 5.** The change of tryptase content in proventriculus. Changes in content of tryptase in proventriculus from 12 to 48 h postinfection. Double asterisks = significantly different from control at $P < 0.01$; double stars = significantly different from the infected group at $P < 0.01$. C = control group; N = Newcastle disease virus-infected group; K = ketotifen-pretreated group.

**Figure 6.** The change of histamine content in proventriculus. Changes in content of histamine in proventriculus from 12 to 48 h postinfection. Double asterisks = significantly different from control at $P < 0.01$; double stars = significantly different from the infected group at $P < 0.01$. C = control group; N = Newcastle disease virus-infected group; K = ketotifen-pretreated group.
In this study, we measured the production of tryptase using the immunocytochemical method. Interestingly, we found that tryptase expression had a positive correlation with mast cell distribution in the 3 groups. This finding effectively confirmed the same finding of mast cell populations. In addition, the tryptase content in the proventriculus was corrected with the number of inflammatory cells in these tissues. The fact that mast cells can induce cellular recruitment through tryptase (Compton et al., 1998, 2000; Huang et al., 1998) could partially explain the increased inflammatory cells in infected birds.

Histamine is a multifunctional biogenic amine mainly produced by mucosal mast cells in the gastric mucosa. Mast cells are involved in gastric mucosal injury-repair mechanisms, mediated by histamine release (Becho et al., 1995). Histamine-containing mast cells are numerous in chickens and are regarded as mercurial messengers in the gut (Rangachari, 1992). Histamine can contribute to the progression of inflammatory responses by enhancement of the secretion of proinflammatory cytokines, such as IL-1α, IL-1β, and IL-6 both in several cell types and in local tissues (Jeanmin et al., 1994; Bayram et al., 1999).

The results of the current study showed that the histamine contents in the proventriculus of infected chickens were higher than that in the control birds. In addition, the histamine content was correlated with the number of mast cells in these tissues. In contrast, the number of mast cells and the content of histamine were decreased in the ketotifen-pretreated birds, and mucosal injury was remitted. It is possible that histamine release from mast cells is involved in the damage. The current study provides further evidence associating mast cells with histamine and tryptase in chickens. However, a contributing role of other mast cell mediators in the mechanism of NDV-induced damage cannot be ruled out without further investigation.

Another important finding of the current study is that the mast cell numbers, tryptase release, and histamine contents were the highest levels at 24 hpi, and then they were decreased at 48 hpi in the proventriculus in the NDV-infected group and ketotifen-pretreated group. It may be explained that the early phase of NDV replication stimulated mast cells in a direct or indirect way. Activated mast cells enhance the recruitment of lymphocytes and macrophages through the release of inflammatory mediators and cytokines. And in the late phase of response, mast cells may not contribute so much to the pathophysiology of this disease. The recruitment of effector cells plays a more important role than mast cells in the process of phagocytosis and clearance. However, the mechanisms of mast cell activation during NDV invasion are yet to be fully characterized.

In conclusion, the results of the current study show that increased mast cell and subsequent tryptase and histamine release are major contributing factors in the proventriculus mucosal injury induced by NDV infection. It appears that by preventing mast cell degranulation, other defense mechanisms may be employed, such as a reduction in the infiltration of inflammatory cells. Despite this wealth of correlative data, further studies are needed to determine the role of mast cells in the pathogenesis of Newcastle disease.

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