Role of Mast Cells in Antigen-Induced Airway Inflammation and Bronchial Hyperresponsiveness in Rats

Naoki Kawada, Hiroyuki Tanaka, Toshiaki Takizawa, Takatoshi Yamada, Yoshimasa Takahashi, Taisei Masuda, Naoki Inagaki and Hiroichi Nagai*

Department of Pharmacology, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan

ABSTRACT—The participation of mast cells in the induction of antigen-induced airway inflammation and bronchial hyperresponsiveness (BHR) to acetylcholine (ACh) was investigated using pharmacological agents and mast cell-deficient rats (Ws/Ws). A significant increase in the number of leukocytes in bronchoalveolar lavage fluid (BALF) and bronchial responsiveness to ACh were observed 24 h after antigen (ovalbumin) challenge in sensitized Brown-Norway (BN) rats. Disodium cromoglycate and terfenadine did not inhibit antigen-induced airway inflammation and BHR in sensitized BN rats. In contrast, cyclosporin A (CyA), FK-506 and prednisolone significantly inhibited antigen-induced airway inflammation and BHR in sensitized BN rats. In addition, disodium cromoglycate, terfenadine and prednisolone, but not CyA and FK-506, inhibited homologous passive cutaneous anaphylaxis in rats. Furthermore, a significant increase in the number of leukocytes in BALF and BHR was also observed in Ws/Ws rats 24 h after inhalation of antigen; however, the magnitude of BHR in Ws/Ws rats was lower than that in the congenic rats. These findings suggest that mast cells play a partial role in the development of antigen-induced BHR in rats and that the induction of BHR is barely suppressed by mast cell stabilizing agents.

Keywords: Airway inflammation, Bronchial hyperresponsiveness, Brown-Norway rat, Mast cell, Ws/Ws rat

Bronchial hyperresponsiveness (BHR) is a characteristic feature of most asthmatics, which correlates with the severity of the disease (1), although its precise mechanism remains unclear. In allergic bronchial asthma, the stimulation of allergen-specific IgE production is believed to contribute to the development of BHR (2). Mast cells and derivative mediators are believed to be crucial in the development of allergic bronchial asthma. IgE, bound to its receptor on mast cells and other cells, triggers the release of pre-formed and newly-generated mediators (3). After antigen cross-linking, mast cells produce mediators, including tryptase, leukotrienes and platelet activating factor (4 – 6), which can potentially induce BHR (7 – 9). Furthermore, mast cells contain and produce several pro-inflammatory cytokines, including granulocyte-macrophage colony-stimulating factor, tumor necrosis factor, interleukin (IL)-3, IL-4 and IL-5, which may contribute to eosinophil recruitment to the airway and BHR (10, 11).

Recently, Martin et al. (12) and we (13) demonstrated that mast cells are required for the development of antigen-induced BHR using mast cell-deficient mice. In contrast, Elwood et al. reported that 5-hydroxytryptamine (5-HT), lipoxygenase metabolites and cyclooxygenase metabolites are not involved in antigen-induced BHR in Brown-Norway (BN) rats (14). However, there are no reports that evaluate the role of mast cells in the development of antigen-induced BHR in rats.

Therefore, in the present study, we examined the role of mast cells in the development of antigen-induced BHR by testing the effect of four drugs showing mast cell stabilizing activities in rats. The four drugs were disodium cromoglycate (DSCG), terfenadine, cyclosporin A (CyA) and FK-506. DSCG and terfenadine are known to be mast cell stabilizers and recent studies indicated that CyA and FK-506 have also shown mast cell stabilizing activities in addition to the immunosuppressive activities to T cells (15, 16). In addition, we have also investigated the role of mast cells in the development of antigen-induced BHR using mast cell-deficient rats (Ws/Ws) (17) and their congenic rats (+/+).
MATERIALS AND METHODS

Animals

Female BN rats (6- to 11-week-old; body weight, 75 – 100 g) obtained from Japan Charles River, Yokohama and mast cell-deficient (Ws/Ws) and congenic wild-type (+/+) rats (6-week-old; body weight, 80 – 100 g) obtained from Japan SLC, Inc., Shizuoka, were housed in stainless steel cages in an air-conditioned room at 22 ± 1°C with a relative humidity of 60 ± 1%, fed a standard laboratory diet and given water ad libitum. In the Ws/Ws rats, no mast cells were detected in the spleen, bone marrow, liver, stomach, small and large intestines, lung and brain (17). Female Wistar rats (7-week-old; body weight, 120 – 150 g) were also purchased from Japan SLC, Inc. Experiments were performed following the Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animals Science in 1987.

Agents

Ovalbumin (OA) (Seikagaku Kogyo), acetylcholine chloride (ACh) (Nacalai Tesque, Kyoto), bovine serum albumin (BSA) (Seikagaku Kogyo), Turk solution (Wako Pure Chemical Industries, Ltd., Osaka), succinylcholine chloride (Sigma, St. Louis, MO, USA), urethane (Sigma), disodium ethylenediaminetetraacetic acid (EDTA-2Na) (Nacalai Tesque), Diff-Quick solution (International Reagent Corp., Kobe) and prednisolone acetate (Shionogi & Co., Ltd., Osaka) were purchased commercially.

Disodium cromoglycate (DSCG) and FK-506 were donated by Sandoz (Novartis) Japan Co., Ltd. CyA was donated by Sankyo Pharmaceutical Co., Ltd. (Tokyo). Terfenadine (Tanabe Pharmaceutical Co., Osaka) were purchased commercially.

Anti-dinitrophenol (DNP) monoclonal IgE antibody was prepared as reported previously (18). The IgE titer of the preparation used to estimate passive cutaneous anaphylaxis (PCA) was 1:1,000 or greater.

Sensitization and challenge

Rats were actively sensitized by intraperitoneal injections of 1 mg OA with 100 mg aluminium hydroxide [Al(OH)3] on days 0 and 2. On day 21, they were exposed to aerosolized OA (1% w/v diluted in sterile saline) for 30 min according to the modified methods previously reported (14). Negative control animals were injected with sterile saline and exposed to aerosolized saline in a similar manner. The aerosol (particle size of 2.0 – 6.0 μm) was generated by a nebulizer (Ultrasonic nebulizer TUR-3200; Nihon Kohden, Tokyo) driven by filling a perspex cylinder chamber (diameter, 5.5 cm; height, 12 cm) with a vaporized solution.

Bronchoalveolar lavage study

To assess antigen-induced airway inflammation, we studied the accumulation of inflammatory cells in bronchoalveolar lavage fluid (BALF) according to modified methods previously reported (19). Briefly, at various times after antigen challenge, animals were sacrificed with an intraperitoneal injection of urethane (2 g/kg). The trachea was cannulated and the air lumen was washed 4 times with 2.5 ml calcium- and magnesium-free phosphate-buffered saline (PBS) containing 0.1% BSA and 0.05 mM EDTA-2 Na, and this procedure was repeated twice (total volume of 5 ml, recovery >80%). BALF from each animal was collected in a plastic tube, cooled on ice and centrifuged (150 × g) at 4°C for 10 min. Cell pellets were resuspended in the same buffer (2 ml). BALF was stained with Turk solution and the number of nucleated cells was counted in a Bürker chamber. A differential count was made on a smear prepared with a cyt centrifuge (Cytospin II; Shandon Scientific Ltd., Cheshire, England) and stained with Diff-Quick solution, (based on standard morphologic criteria) on at least 300 cells (magnification ×500).

Measurement of airway function

Measurement of bronchial responsiveness to ACh at various times after antigen inhalation was carried out according to modified methods previously described (14). Briefly, to measure bronchial responsiveness to ACh, rats were anesthetized with urethane (1 g/kg, i.p.) and the jugular vein was cannulated for intravenous injection of ACh. Rats were injected with succinylcholine chloride (1.2 mg/kg, i.v.), to suppress spontaneous respiration, and were ventilated with a rodent ventilator (New England Medical Instruments Inc., Medway, MA, USA) with oxygen supplemented air at 60 strokes/min, at a stroke volume of 1 ml/100 g body weight. Bronchostenosption was measured according to the overflow method, using a bronchospsasm transducer (Ugo Basil 7020, Milan, Italy) connected to the tracheal cannula. To measure bronchial responsiveness to ACh, changes in respiratory overflow volume were measured using increasing doses of ACh. The increase in respiratory overflow volume induced by ACh was represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. The area under the curve (AUC) calculated from dose-response curves for ACh are expressed as a magnitude of BHR. Briefly, each dose was converted logarithmically, and then AUC was calculated and represented as arbitrary units.

Passive cutaneous anaphylaxis (PCA)

PCA was carried out according to the previously described method (18). The dorsal skin of Wistar rats was shaved, and 0.1 ml diluted (20×) monoclonal anti-DNP IgE was injected intradermally for passive sensitization.
Concomitantly, 0.1 ml saline was injected into another site as the control for PCA. Forty-eight hours after the sensitization, 1 mg of DNP-BSA and 5 mg of Evans Blue dye dissolved in saline was injected intravenously. After 30 min, the rats were sacrificed, and the reaction sites were excised for the determination of the extravasated dye. Extravasated dye at each reaction site was extracted and determined colorimetrically according to the previously described method (20).

Administration of drugs

DSCG was dissolved in sterile saline and given intravenously immediately before antigen challenge. Terfenadine was dissolved in sterile saline and given orally 1 h before antigen challenge. CyA, dissolved in olive oil, FK-506, dissolved in sterile saline, or prednisolone acetate, suspended in 0.5% sodium carboxymethylcellulose, was given orally for 3 consecutive days before antigen challenge (days 19, 20 and 21).

Statistical analyses

Values are presented as the mean ± S.E.M. of 5–6 animals. Statistical significance between saline-inhaled and OA-inhaled animals or between wild-type and mast cell-deficient animals was estimated using the unpaired two-tailed Student’s t-test or Mann-Whitney U-test. To define significant differences among the groups for the inhibitory effects, data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett’s multiple range test. P values less than 0.05 were considered significant.

RESULTS

Antigen-induced airway inflammation and BHR to ACh in sensitized BN rats

Time course studies for antigen-induced airway inflammation and BHR in response to ACh in BN rats are shown in Fig. 1.

In the preliminary experiments, BHR after antigen exposure was observed only in the sensitized antigen-challenged group, but not in the sensitized saline-challenged or nonsensitized antigen challenged groups, suggesting BHR after challenge is allergic reactions-dependent.

The number of inflammatory cells including macrophages, eosinophils and lymphocytes in BALF increased significantly between 8 and 48 h after challenge (except for macrophages at 8 and 24 h). Neutrophils showed a slight increase at 24 h after the antigen challenge (data not shown). As for the airway responsiveness to ACh, AUC clearly increased between 8 and 48 h after the antigen challenge. A significant correlation between the number of eosinophils, lymphocytes and BHR was observed in sensitized BN rats (r=0.95 and r=0.64, respectively, at 24 h, r=0.73 and r=0.86, respectively, at 48 h).

From these findings, the following experiments were carried out 24 h after the antigen challenge.

Effect of DSCG, terfenadine and prednisolone on antigen-induced BHR in BN rats

To study the effect of mast cell stabilizer on antigen-induced BHR in BN rats, the effects of DSCG and terfenadine were examined. As shown in Fig. 2, BHR to ACh was observed 24 h after antigen challenge in sensitized BN rats. DSCG at doses between 1 and 10 mg/kg and terfenadine at doses between 10 and 100 mg/kg did not inhibit BHR. In addition, the increase in the number of leukocytes, including eosinophils and lymphocytes, was not affected by administration of DSCG and terfenadine (Fig. 3). Prednisolone (used as a reference drug) at a dose of 10 mg/kg clearly inhibited antigen-induced BHR and airway inflammation.

Effect of CyA, FK-506 and prednisolone on antigen-induced airway inflammation and BHR in BN rats

To investigate the effect of T cell immunosuppressors with mast cell stabilizing activity on the development of BHR, the effects of CyA and FK-506 were examined. As shown in Fig. 4, 30 or 100 mg/kg of CyA, 0.3 or 3 mg/kg of FK-506 and 10 mg/kg of prednisolone clearly inhibited antigen-induced BHR. The antigen-induced increases in number of total leukocytes, eosinophils and lymphocytes 24 h after antigen inhalation were significantly decreased by the treatment with CyA, FK-506 and prednisolone (Fig. 5).

Effect of DSCG, terfenadine, CyA, FK-506 and prednisolone on PCA

To study the effect of examined drugs on mast cell dependent allergic reactions, the effect of drugs on PCA was examined. As shown in Table 1, DSCG, terfenadine and prednisolone clearly inhibited the dye leakage by PCA, but CyA and FK-506 did not.

Effect of antigen challenge on airway inflammation and bronchial responsiveness to ACh in Ws/Ws rats

Antigen challenge in actively sensitized +/- and Ws/Ws rats induced significant increases in the number of eosinophils and lymphocytes in BALF compared with the saline-exposed group; however, there was no significant difference between the increase in eosinophilia in +/- and Ws/Ws rats (Table 2). To investigate the effect of mast cell deficiency on airway function, bronchial responsiveness to ACh after antigen challenge was examined. Figure 6 shows the dose-response curve of bronchial responsiveness to ACh 24 h after challenge and the value of AUC. In the saline-exposed group, BHR in response to ACh was not observed. In contrast, antigen challenge in sensitized rats caused a signifi-
cant increase in bronchial responsiveness compared with the saline-treated group in both +/+ and Ws/Ws rats (Fig. 6), although the magnitude of BHR in Ws/Ws rats was clearly lower than that in +/+ rats.

**DISCUSSION**

In the present study, we demonstrated a partial role of mast cells in antigen-induced BHR, but not in airway in-
flammation. Whereas DSCG and terfenadine did not affect an antigen-induced BHR and airway inflammation, CyA and FK-506 inhibited the development of BHR and airway inflammation. Furthermore, the degree of BHR after antigen challenge in Ws/Ws rats was clearly lower than that in Gb1/Gb1 rats. However, allergen-induced airway inflammation was not influenced by the deficiency of mast cells.

First, we investigated the characteristics of asthmatic responses after allergen challenge in the present animal model. As expected, antigen provocation induced airway inflammation including airway eosinophilia in a time-dependent manner up to 48 h. We also found that BHR in response to cholinergic stimuli was induced after single allergen challenge. Furthermore, the correlation between the presence of inflammatory cells in the airways and BHR exists in this model 24–48 h after inhalation of allergen as reported in human subjects (21, 22). Therefore, we chose the time point of 24 h after the challenge for further examinations of the present study. In contrast, the degree of BHR at 8 h after the challenge was greater than those observed 24 or 48 h after the provocation, whereas the number of inflammatory cells in BALF was increased in a time-dependent manner. The reason for the dissociation is not clear; however, mediators and/or cytokines released by mast cells or inflammatory cells are proposed to be responsible for the induction of BHR in humans and several different animals (23). Thus, there is a possibility that these functional molecules may affect the bronchial responsiveness directly or synergistically with the inflammatory cells in the airways.

As for the effect of DSCG and terfenadine, there was little opportunity to clarify their efficacy in BHR. In the present study, both drugs did not affect BHR and airway inflammation. The dose of both drugs was sufficient to suppress the mast cell activation, because both drugs clearly inhibited PCA in rats. These findings may also indicate a minor role of mast cell-derived histamine in the development of BHR.

The inhibition of BHR and airway inflammation by CyA...
Airway Responsiveness and Mast Cells

and FK-506 may indicate the involvement of T cells in the induction of BHR in rats because they produced decreases in the number of lymphocytes in BALF and did not inhibit the PCA reaction. CyA and FK-506 are novel immunosuppressors that inhibit T cell proliferation, cytotoxicity and cytokine production (24, 25) through the inhibition of calcium/calmodulin-activated protein phosphatase, calcineurin (26). Calcineurin is required to activate the cytoplasmic component in nuclear transcription of activated T cell (NF-AT). Both agents inhibit NF-AT activation and transcription of many cytokine genes (27). In addition to T cells, CyA and FK-506 inhibited mast cell activation in vitro (15, 16), probably without affecting NF-AT activation.

In the present study, these two agents clearly inhibited antigen-induced BHR and airway inflammation. These findings are clearly in agreement with our previous murine study (28). In addition, Akutsu et al. (29) and Eum et al. (30) reported similar findings in guinea pigs and mice. However, Elwood et al. demonstrated that CyA did not affect an antigen-induced airway eosinophilia and BHR in BN-rats (31). These discrepancies in the effect of CyA may be due to the experimental protocols including doses and administration period of CyA. Sequential administration appears to be necessary to obtain efficacy of the agents.

Both agents were administered for three days before an antigen challenge in the present experiments. As shown by the findings, BHR and airway inflammation, but not PCA, were clearly inhibited by the three administrations of CyA and FK-506. Moreover, mast cell depletion (Ws/Ws rats) did not affect the development of airway inflammation. In addition, both agents do not affect the release of histamine or serotonin from mast cells in vivo since the PCA reaction was not affected by the three administrations of drugs. These findings suggest that the inhibition of airway inflammation would be mainly due to the inhibition of cytokine production by T cells.

Clinical and experimental studies demonstrated that antigen-specific CD4+ T helper type 2 cells and the cytokines, IL-4 and IL-5, play central roles in initiating and sustaining an asthmatic response by regulating the activation and recruitment of mast cells and eosinophils (32, 33). Gavett et al. (34) and Li et al. (35) have clearly demonstrated that requirement of CD4+ T lymphocytes in the development of BHR as well as airway eosinophilic inflammation. Moreover, the role of IL-4 and IL-5 in the induction of BHR have been reported (36, 37), although the controversial reports exist (38, 39). Regarding these observations, Drazen et al. recently proposed that BHR could be induced by two distinct mech-

![Fig. 3. Effect of DSCG and terfenadine on antigen-induced increases in leukocytes in BALF obtained from BN rats. DSCG was given intravenously immediately before antigen challenge. Terfenadine was given orally 1 h before antigen challenge. BALF was obtained at 24 h after challenge. Each value represents the mean ± S.E.M. of 5 animals. *P<0.05, **P<0.01 (vs OA-exposed group).](image-url)
mechanisms; one could be mediated by an IgE-mast cells pathway, whereas the other could be induced by an IL-5-eosinophils pathway (40). Thus, the relative contribution of these mechanisms to the induction of allergen-induced airway inflammation and BHR could be different in each patient and experimental method and influenced by the nature of allergen, sensitization period or frequency of allergen challenge. However, to clarify the inhibitory mechanisms of CyA and FK-506 on BHR and also the effect of these agents on lung mast cells activation in vivo, further experiments will be needed to examine the effect of CyA and FK-506 for the development of BHR in Ws/Ws rats.

Fig. 4. Effect of cyclosporin A (A, B) and FK-506 (C, D) on antigen-induced BHR in BN rats. Each drug was given orally for 3 consecutive days before antigen challenge. The bronchial responsiveness was measured at 24 h after challenge and was expressed as AUC. Each value represents the mean (A and C) or mean ± S.E.M. (B and D) of 5 – 6 animals. *P<0.05, **P<0.01 (vs OA-exposed group).

An antigen-induced BHR was clearly observed in Ws/Ws (mast cell deficient) rats, but the magnitude of BHR was weaker than that in +/+ rats. Airway inflammation in terms of increase in inflammatory cells in BALF was not affected by mast cell depletion. These observations are in agreement with our previous data (13) and others (41). As for the investigation of the role of mast cells in BHR by employing mast cell deficient animals, mast cell deficient mice (W/Wv or Sl/Slm mice) were often used. The term Ws is derived from “White spotting”, which refers to black-eyed whites; and Ws/Ws rats have a similar phenotype to the W/Wv mice, exhibiting anemia and mast cell deficiency (17) due to the lack of the tyrosine kinase of the c-kit gene (42). These findings suggest that Ws/Ws rats may be as equally useful as W/Wv mice. We previously reported that mast cells were involved in the antigen-induced BHR but not airway inflammation, especially eosinophilia in actively sensitized W/Wv mice (13). In contrast, a recent study has reported that allergen-induced airway eosinophilia and BHR were observed in W/Wv mice, as in their congenic mice (41). These discrepancies could result from different experimental conditions including sensitization, allergen provocation and others, as reported by Kobayashi et al. (43) and Williams and Galli (44). In the present study, rats were sensitized by a single injection of antigen and challenged by a single inhalation of aeroantigen. This is not a chronic condition; therefore, more chronic provocation will be necessary to clarify the role of mast cells in a chronic allergic inflammatory response such as bronchial asthma.

In conclusion, the present findings indicate that mast cells play a partial role in the development of antigen-induced BHR but not airway inflammation in rats. More-
Fig. 5. Effect of cyclosporin A and FK-506 on antigen-induced increases in leukocytes in BALF obtained from BN rats. Each drug was given orally for 3 consecutive days before antigen challenge. BALF was obtained at 24 h after challenge. Each value represents the mean ± S.E.M. of 5 animals. *P<0.05, **P<0.01, ***P<0.001 (vs OA-exposed group).

Table 1. Effect of DSCG, terfenadine, cyclosporin A, FK-506 and prednisolone on homologous PCA in rats

<table>
<thead>
<tr>
<th>Amount of dye (µg/site)</th>
<th>Saline Control</th>
<th>DSCG (10 mg/kg)</th>
<th>Terfenadine (100 mg/kg)</th>
<th>CyA (100 mg/kg)</th>
<th>FK-506 (3 mg/kg)</th>
<th>Prednisolone (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13.8 ± 3.78**</td>
<td>84.6 ± 13.12</td>
<td>15.0 ± 4.68**</td>
<td>19.4 ± 3.55**</td>
<td>87.2 ± 29.00</td>
<td>75.5 ± 13.70</td>
</tr>
</tbody>
</table>

DSCG was administered intravenously immediately before antigen challenge. Terfenadine was administered orally 1 h before antigen challenge. Other drugs were administered orally for 3 days before antigen challenge. Each value represents the mean ± S.E.M. of 5–6 animals. **P<0.01 (vs control).

Table 2. The number of leukocytes in bronchoalveolar lavage fluid after antigen challenge in Ws/Ws and congenic rats

<table>
<thead>
<tr>
<th>Bronchoalveolar lavage study</th>
<th>Genotype</th>
<th>Sensitization day 0 and 2</th>
<th>Antigen provocation day 21</th>
<th>Total leukocytes (x 10³)</th>
<th>Eosinophils (x 10³)</th>
<th>Lymphocytes (x 10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>SAL</td>
<td>SAL</td>
<td>4.60 ± 0.24</td>
<td>N.D.</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Wild-type</td>
<td>OA</td>
<td>OA</td>
<td>7.60 ± 0.68**</td>
<td>0.94 ± 0.10**</td>
<td>0.89 ± 0.24**</td>
</tr>
<tr>
<td></td>
<td>Ws/Ws</td>
<td>SAL</td>
<td>SAL</td>
<td>5.76 ± 0.56</td>
<td>N.D.</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Ws/Ws</td>
<td>OA</td>
<td>OA</td>
<td>8.08 ± 0.86**</td>
<td>0.77 ± 0.07</td>
<td>0.75 ± 0.12**</td>
</tr>
</tbody>
</table>

Bronchoalveolar lavage fluid was obtained at 24 h after challenge. Each value represents the mean ± S.E.M. of 5 animals. SAL: saline, OA: ovalbumin, N.D.: not detected. **P<0.01 (vs SAL).
over, mast cell stabilizing agents, DSCG and terfenadine, are not effective for suppression of BHR, whereas T cell immunosuppressors, CyA and FK-506, are effective for suppression of BHR and airway inflammation.

REFERENCES

21 De Monchy JG, Kauffman HF, Venge P, Koeter GH, Jansen...
Airway Responsiveness and Mast Cells

259


