Establishment of an asthma model by sensitization with mite antigen alone in C57BL/6J mice

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ABSTRACT Bronchial asthma is characterized by the bronchial hyperresponsiveness and airway obstruction related to airway smooth muscle contraction. Eosinophilic airway inflammation is involved in its pathogenesis. To reproduce the condition, various animal models have been prepared. However, there are many models that do not reflect the spontaneous history of bronchial asthma onset in humans due to the mouse strain, sensitizing antigen, or administration method. In this study, we prepared a mouse model of which the mechanism is similar to that of human bronchial asthma.

Mite Extract-*Dermatophagoides farinae* (Derf) antigen was transnasally administered to wild-type C57BL/6J mice (WT) 13 times. Subsequently, an airway hypersensitivity test (Mch PC_{200}), specific antigen exposure test (ΔSRaw), bronchoalveolar lavage (BAL), and blood collection were performed to examine the presence or absence of asthma acquisition and differences in the local pulmonary levels of cytokines/chemokines in comparison with the physiological saline-treated group.

In the mite antigen-treated mice (WT/-Derf), bronchial hyperresponsiveness was enhanced, antigen-specific was increased airway resistance in comparison with physiological saline-treated mice (WT/-Saline). In addition, the number of eosinophils in BAL fluid (BALF) was greater. Furthermore, there was a correlation among leukotrienes, eotaxin, and tissue inhibitors of metalloproteinase 1 in BALF, suggesting that the mechanism concerning eosinophilic airway inflammation involving in human bronchial asthma was reproduced.

In this study, we successfully established a mouse bronchial asthma model in which the pathogenesis resembles that in humans in comparison with conventional models, using Derf antigen alone and C57BL/6J mice.

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Key words：**Asthma, Mite Extract-*Dermatophagoides farinae*, Cytokine, Chemokine Asthma model mice, C57BL/6J**

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INTRODUCTION

Bronchial asthma is characterized by the enhancement of airway hypersensitivity and airway obstruction related to airway smooth muscle contraction. Eosinophilic airway inflammation is involved in its pathogenesis[1]. Various animal models have been prepared to clarify the pathogenesis of bronchial asthma and develop drugs[2]. In particular, an ovalbumin (OVA)-sensitized model, which was prepared using BALB/c mice, is known. However, Walsh *et al*. reported that, among similar OVA-sensitized models, airway hypersensitivity was acquired even in an eosinophil defect model consisting of Th2 lymphocyte-predominant BALB/c mice, but not in another eosinophil defect model consisting of C57BL/6J mice, in which Th1 lymphocytes were predominant, as demonstrated in humans[3]. In the pathogenesis of bronchial asthma in humans, tissue disorder related to granular protein released by activated eosinophils may induce airway hypersensitivity[4]. However, Fattouh *et al*. indicated that airway hypersensitivity was enhanced in mite antigen-sensitized BALB/c mice with an eosinophil defect, as observed in wild-type mice[5]. Thus, Th2-predominant BALB/c mice may be useful for acquiring airway hypersensitivity, but the use of OVA, which is not a primary antigen for human bronchial asthma, and an intraperitoneal route for sensitization, which is not probable in the spontaneous course of human bronchial asthma onset, in many models raises issues. If the inhalation of a foreign antigen induces airway inflammation with eosinophil infiltration and airway hypersensitivity to acetylcholine/methacholine, many characteristics of the condition may be common with those of human bronchial asthma. In C57BL/6 mice, transgenic and knock-out mice involving many protein genes have been established. The preparation of a basic model with C57BL/6 background may be useful for clarifying the pathogenesis of allergic asthma and drug responsiveness. The grade of inflammation depends on the method of airway sensitization, the frequency of antigen administration, dose, period, and interval from the final dosing until analysis; the results must be carefully interpreted[6].

In this study, we prepared a mouse model resembling human bronchial asthma by conducting trans-airway sensitization with a primary antigen for human bronchial asthma, Mite Extract-*Dermatophagoides farinae* (Derf) antigen, in C57BL/6J mice.

MATERIALS AND METHODS

Animal Model

Mite antigen was transnasally administered to 8-week-old, twenty-four female wild-type C57BL/6J mice (WT) as a mouse bronchial asthma model (sensitization group, WT/-Derf). Physiological saline was administered to control mice (control group, WT/-Saline). The results were compared between the two groups to confirm whether eosinophilic airway inflammation, the enhancement of airway hypersensitivity, and specific antigen exposure-induced airway obstruction, which are characteristics of bronchial asthma, are acquired.

Mite Extract-*Dermatophagoides farinae* (Derf) (LSL Co., Tokyo, Japan) was dissolved with distilled water and adjusted to 2 mg/ml with saline. Animals were anesthetized by the intraperitoneal administration of 50 mg/kg pentobarbital. After confirming sufficient anesthesia, 50 μl of 2-mg/ml Derf was administered into the nasal cavity. The antigen was administered 13 times at 4 times a week. Saline was similarly administered at the same volume and schedule. All experimental animals used in this study were under a protocol approved by the institutional animal care and use committee of Kawasaki Medical School (Differential Number 10-026/ 12-012).

Airway hypersensitivity test

Using a nebulizer, mice were allowed to inhale methacholine at concentrations of 6.25, 12.5, 25, 50, and 100 mg/ml. We compared concentrations (Mch PC_{200}) at which the airway resistance increased to 2-fold the value on the inhalation of physiological saline as a control value. Inhalation was performed for 3 minutes. After a 1-minute rest, the airway resistance was measured for 2 minutes (Pulmos, MIPS, Osaka, Japan). When Mch PC_{200} was significantly lower than in the control group (physiological saline group), mice were regarded as positive.

Specific antigen exposure test

The airway resistance was measured before and after transnasal Derf administration to evaluate the rate of increase (ΔsRaw) in comparison with the pretreatment airway resistance. When there was a significant increase in comparison with the control group, mice were regarded as positive.

Collection of blood/BAL fluid/lung tissue

250 mg/kg pentobarbital was intraperitoneally administered. After respiratory arrest was confirmed, blood and BAL fluid (BALF) were collected. In some mice, BALF was not conducted, and May-Giemsa staining was performed in lung tissue specimens for pathological investigation.

ELISA

IL-5, IL-13, IL-17, eotaxin, TNF-α, IFN-γ, matrix metalloproteinase 9 (MMP9), issue inhibitors of metalloproteinase 1 (TIMP1) (R&D Systems, Minneapolis, MN), and cysteinyl leukotrienes (C4, D4, E4) (Cyman Chemical Company, Ann Arbor, MI) in BALF were measured using ELISA. The detection limits were 15.6, 7.8, 10.9, 15.6, 10.9, 9.4, 78.0, 37.5 and 7.8 pg/ml for IL-5, IL-13, IL-17, eotaxin, TNF-α, IFN-γ, MMP9, TIMP1 1 and cysteinyl leukotrienes, respectively. The Derf-specific serum IgE and IgG1 were measured by ELISA as previously described[7].

Statistical analysis

For statistical analysis, Stat View 5.0® software (SAS Institute, Inc., Cary, NC, U.S.A.) was used. The results were compared between the two groups using the Mann-Whitney U test. To examine the correlation among factors, Spearman's rank correlation coefficient (non-parametric method) was used. Independent factors were identified using variable selection-multiple regression analysis. $P<0.05$ was regarded as significant.

RESULTS

The serum levels of Derf-specific IgG_1 and IgE significantly increased after Derf administration (Derf-specific IgG_1: 1.44 ± 0.43 vs. 0.001 ± 0.003 O.D., respectively, $p<0.01$; Derf-specific IgE: 0.24 ± 0.02 vs. 0.21 ± 0.01 O.D., respectively, $p<0.01$; Table 1).

Concerning airway hypersensitivity, a methacholine inhalation test showed that the Mch PC_{200} value in the WT/-Derf was significantly lower than in the WT/-Saline (14.9 ± 10.6 vs. 24.7 ± 12.6 mg/mL, respectively, $p<0.05$;Table 1).

We investigated the airway resistance after exposure to a specific antigen. In the WT/-Derf, the airway resistance (ΔsRaw) 20 minutes after exposure was significantly higher than in the WT/-Saline (25.8 ± 24.9 vs. 3.99 ± 1.79, respectively, $p<0.01$; Table 1).

The total number of leukocytes in the BALF in the WT/-Derf was significantly higher than that in the WT/-Saline (91.7 ± 43.2 vs. 15.8 ± 10.2 x $10^4/\mu$L, respectively, $p<0.05$; Table 1). With respect to leukocyte fractions, the concentrations of eosinophils (35.3 ± 31.7 vs. 0 ± 0 x $10^4/\mu$L, respectively, $p<0.01$), neutrophils (28.1 ± 19.8 vs. 0.05 ± 0.09 x $10^4/\mu$L, respectively, $p<0.01$), and lymphocytes (14.2 ± 12.7 vs. 1.24 ± 3.72 x $10^4/$

μL, respectively, p<0.01) in the WT/-Derf were significantly higher than in the WT/-Saline, as demonstrated for the cell concentration.

The comparison of the BALF levels of cytokines and chemokines is shown in Table 1. The IL-13, IL-17, eotaxin and cysteinyl leukotriene levels in the WT/-Derf were significantly higher than in the WT/-Saline (p<0.05). Furthermore, the TIMP1 level in the WT/-Derf was also significantly higher than in the WT/-Saline (p<0.01). However, there were no significant differences in the MMP9 level or MMP9/TIMP1 ratio. On single correlation analysis involving Derf mice, there were strong correlations between eotaxin and leukotriene (r=0.87, p=0.02), between leukotriene and TIMP1 (r=0.76, p=0.04), and between eotaxin and TIMP1 (r=0.81, p=0.03; Table 2). Furthermore, there was a tendency of correlation between IL-13 and TIMP1 (r=0.68, p=0.07). Concerning factors correlated with airway hypersensitivity, there were no significant independent variables on multiple regression analysis.

We compared lung tissue specimens after May-Giemsa staining (Fig.1). In the WT/-Derf, the infiltration of eosinophils was observed around the bronchus and blood vessels. However, there was no infiltration in the WT/-Saline. Neither the WT/-Derf nor WT/-Saline showed fibrin deposition to the airway wall. Pathological remodeling could not be confirmed.

Table 1. Characteristics of Derf induced mouse asthma model.

	WT/-Derf (n=9)	WT/-Saline (n=10)	p
PC200, mg/ml	14.9 [8.1, 21.6]	24.7 [16.7, 32.7]	0.02
Delta SRaw	25.8 [0.90, 50.8]	3.99 [2.20, 5.77]	< 0.01
Serum Df IgG1, o.d.	1.44 [1.1, 1.8]	0.001 [-0.001, 0.004]	< 0.01
Serum Df IgE, o.d.	0.24 [0.23, 0.26]	0.21 [0.19, 0.22]	< 0.01
BALF total number of leukocytes, x 10^4/μL	91.7 [37.6, 145.1]	15.8 [5.6, 32.8]	< 0.01
BALF Neutro, %	32.0 [10.9, 66.8]	0.55 [0.0, 2.32]	< 0.01
BALF Eosino, %	37.4 [19.5, 55.4]	N.D.	< 0.01
BALF Eotaxin, pg/ml	8.6 [-0.92, 18.1]	N.D.	0.02
BALF IL-5, pg/ml	N.D.	N.D.	
BALF IFN-γ, pg/ml	N.D.	N.D.	
BALF IL-13, pg/ml	17.1 [6.6, 27.7]	N.D.	< 0.01
BALF IL-17, pg/ml	23.5 [2.1, 44.9]	N.D.	< 0.01
BALF Leukotriene C4+D4+E4, pg/ml	6,105 [5,047, 7,163]	722 [128, 1,317]	< 0.01
BALF MMP-9, pg/ml	778 [-21, 1576]	14 [5, 23]	0.83
BALF TIMP-1, pg/ml	488 [208, 769]	43 [37, 49]	< 0.01
BALF MMP-9/TIMP-1 ratio	3.5 [0.35, 6.7]	0.33 [0.12, 0.53]	0.58

mean [95%CI], Mann-Whitney U test was used for comparing WT/-Derf and WT/-Saline mice.
Abbreviation is a in the text.
N.D.: not detected, under the detection limit

Table 2. The significant relationships among factors in serum and bronchial lavage fluid in Derf-sensitized C57BL mice

	r	p-values
Serum IgE vs. BALF MMP-9/TIMP1 ratio	0.82	0.04
BALF eotaxin vs. BALF TIMP-1	0.81	0.03
BALF eotaxin vs. BALF leukotriene	0.87	0.02
BALF leukotriene vs. BALF TIMP-1	0.76	0.04
BALF IL-13 vs. BALF TIMP-1	0.68	0.07

To examine the correlation among factors, Spearman's rank correlation coefficient (non-parametric method) was used.
Abbreviation is a in the text.

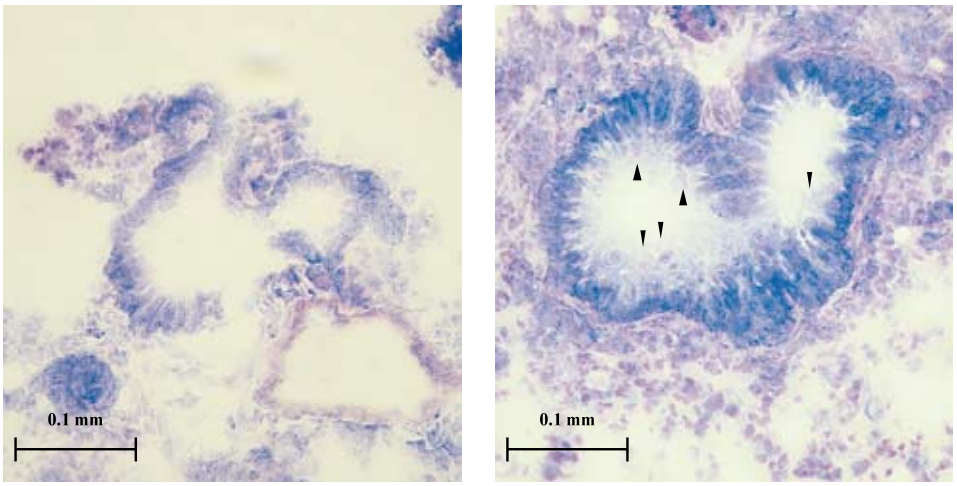

Fig. 1. The tissue-based microscopic findings of bronchi of WT/-Saline (left) and WT/-Derf (right) Eosinophils (allow head) is infiltrated in airway epithelium.

DISCUSSION

In previous studies involving the preparation of a mouse model of bronchial asthma, sensitization/intra-airway antigen administration methods varied, and the grade of inflammation depended on the frequency of antigen administration, dose, interval, and interval from the final dosing until analysis; the results must be carefully interpreted. As described by Walsh *et al*. and Fattouh *et al*., requirements to acquire airway hypersensitivity depend on the mouse strain[3,5]. Therefore, a mouse model reflecting the mode of the development of asthma in humans was necessary to clarify the pathogenesis of bronchial asthma and establish remedies. We prepared asthma model in C57BL/6 mouse, assuming that these may be overcome by increasing the period/frequency of sensitization at a 2-fold volume of mites, as considered the previous studies[2]. This is the first report on stable eosinophilic airway inflammation achieved by 13 sessions of airway sensitization at 50 μl of 2-mg/ml Derf in C57BL/6J background mice. In this model, after transnasal Derf administration, the WT/-Derf met the following conditions: positive reactions on a specific antigen exposure test (ΔsRaw), positive reactions on a methacholine inhalation test (Mch PC_{200}), increases in the serum levels of Derf-specific IgE and IgG1, and an increase in the number of eosinophils in the BALF, confirming that the characteristics of bronchial asthma could be reproduced. This mouse model may reflect the pathogenesis of human bronchial asthma, because the type of antigen, mouse strain, and route of sensitization differ from those in conventional models such as an OVA-sensitized model and that using BALB/c mice.

The correlations between various chemokines and cytokines in BALF were evaluated. Eotaxin is a cytokine that induces the chemotaxis of eosinophils to local areas[8]. Eosinophils are one of the main producer of leukotrienes. In addition, eosinophils are stimulated by the leukotrienes that they produce, inducing airway contraction, mucous secretion, and the enhancement of vascular permeability[9]. The correlation between leukotrienes and eotaxin in BALF may reflect such a mechanism. Mast cells were not found in sites of lung inflammation and in the BALF of WT/Derf, although they were also one of the main leukotrienes producing cells. Mast cells may not play an important role in the development of airway hypersensitivity and airway inflammation in this model. Further examination is required to clarify the main leukotriens producing cells.

Bronchial asthma is a chronic inflammatory disease of the airway, as described above. Under such an environment, an imbalance between extracellular matrix-decomposing enzymes, MMPs, and TIMPs may induce remodeling[10,11]. The correlations between BALF leukotriene/Eotaxin/IL-13, which are involved in local eosinophilic inflammation, and TIMP1, which is involved in the inhibition of remodeling, may reflect the development and reduction of eosinophilic airway inflammation[12]. However, MMP9 or MMP9/TIMP-1, which are index of the airway remodeling were not increased in WT/-Derf. Pathological examination did not confirm remodeling of the WT/-Derf mice airway. To reproduce these characteristics, repeated antigen exposure stimulation may be needed. IFN-γ and IL-5, which could not be measured in this study (below the detection limit), may be detected by increasing exposure stimulation and/or by improvement of the measurement sensitivity such as concentration techniques. In the future, further examination is require.

As the conclusions, this model may reproduce not only the characteristics of human bronchial asthma, such as eosinophilic inflammation of the airway, the enhancement of airway hypersensitivity, and specific antigen exposure-related obstruction of the airway, but also the chemotaxis of eosinophils to local areas and leukotriene-related inflammation, which are considered to be etiological factors for these characteristics, as well as a TIMP1-mediated inhibitory mechanism. In this study, we successfully established a mouse bronchial asthma model reflecting the mode of onset in humans in comparison with conventional models, using Derf antigen and C57BL/6J mice. The results of analysis with this model may be useful for clarifying the pathogenesis of bronchial asthma and developing drugs in the future.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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