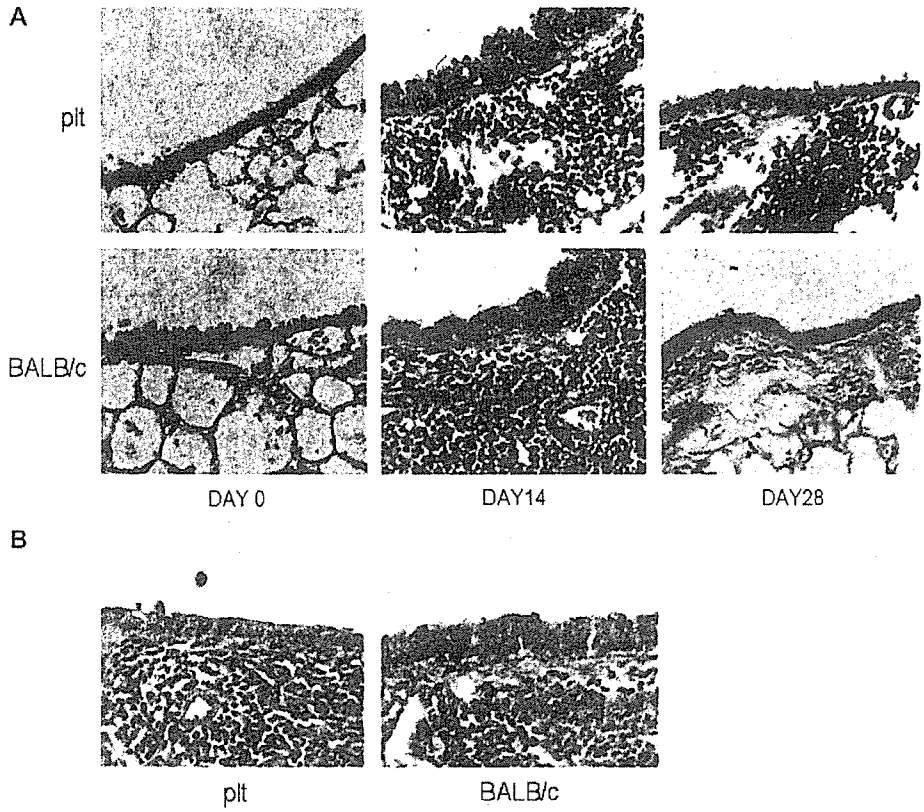

FIG 4. Histologic analysis was performed by hematoxylin-eosin staining **(A)** and PAS staining **(B)** in BALB/c and plt mice at the indicated time. Figures are representative data of histologic examination of 5 mice. Original magnification ×200.

Analysis of CCR7 and CD25 expression

To clarify the mechanisms of delay in resolution of airway inflammation, we performed flow-cytometric analysis to assess CCR7, receptor for CCL19 and CCL21, on T cells, because it has been reported that CCR7 expression is critical for T-cell exit from peripheral tissues.[24,25] $CCR7^+$ cells were present in the $CD4^+$ cells of lung after ovalbumin inhalation both in BALB/c (Fig 5, *A*) and plt mice (Fig 5, *B*). When we examined after cessation of ovalbumin inhalation in plt mice, there were $CD4^+CCR7^+$ cells, but the majority were $CD4^+CCR7^-$ cells (Fig 5, *B*).

Regulatory T cells, which are known to suppress airway inflammation, express CCR7 and are activated by pulmonary DCs at the secondary lymph nodes.[26,27] Therefore, we next examined the expression of CD25 on $CD4^+$ cells. $CD4^+CD25^+$ cells appeared in the BALF after ovalbumin inhalation. Seven days after cessation of ovalbumin inhalation, the number of $CD4^+CD25^+$ cells in the BALF was significantly smaller in plt mice than that in BALB/c mice (Fig 5, *C*). $CD4^+CD25^+$ cells marginally existed in mediastinal lymph node of plt mice (Fig 5, *C*). There were no differences in the number of $CD4^+CD25^+$ cells in the blood and spleen between plt and BALB/c mice (Fig 5, *C*). Although the number of $CD4^+CD25^+$ cells in the BALF was decreased, a significant number of $CD4^+CD25^+$ cells was found in the BALF of plt mice.

Role of CCL19 in resolution of airway inflammation

To investigate the mechanisms of delayed resolution of allergic inflammation in plt mice, we added CCL19, another ligand of CCR7, for which recombinant protein is available. After 14 days of ovalbumin exposure, we added CCL19 (5 μg/mouse by osmotic pump) continuously. For BALB/c mice, saline was added instead of CCL19. After 1 week of administration, lung lymphocytes were separated and were cultured for 2 days, after which the supernatants were harvested. We measured IL-10 because this protein contributes to the resolution of allergic inflammation. As shown in Fig 6, *A* (in the BALF), and Fig 6, *B* (in the cultured supernatants), IL-10 production in BALB/c mice was significantly greater than in plt mice. The treatment with CCL19 significantly enhanced IL-10 production in plt mice (Fig 6, *B*), suggesting that the defect in CCL19 and CCL21 impairs IL-10 production in lung cells. To assess the effect of CCL19 administration, we also analyzed BALF cells and airway response after 1 week of CCL19 administration. Although lymphocytes in CCL19-treated plt mice did not reach the same level in BALB/c mice, CCL19-treated plt mice exhibited a significantly lower number of lymphocytes in the BALF and Penh level after 100 mg acetylcholine inhalation compared with nontreated plt mice (Fig 6, *C and D*).

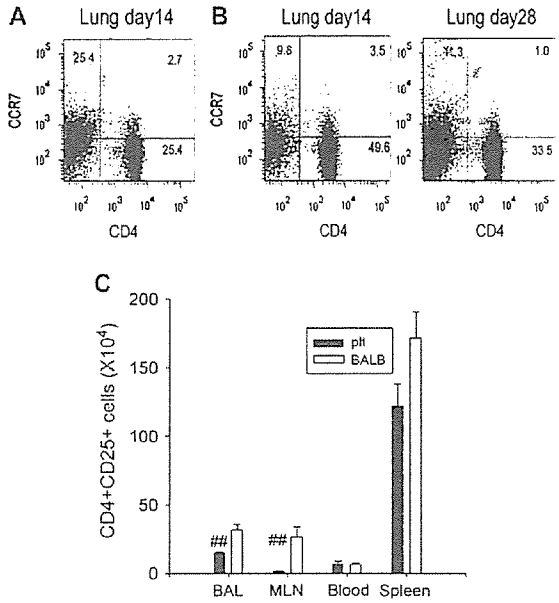

FIG 5. Flow-cytometric analysis of BALF cells, lung, secondary lymph node, blood, and spleen. **A,** CCR7 expression in BALB/c mice. **B,** CCR7 expression in plt mice. **C,** Number of $CD4^+CD25^+$ T cells on day 21 (1 week after cessation of ovalbumin inhalation). *BAL,* Bronchoalveolar lavage; *MLN,* mediastinal lymph node.

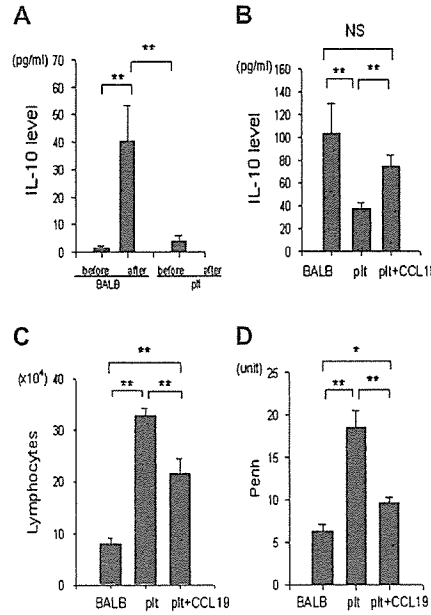

FIG 6. IL-10 production after cessation of allergen inhalation and effect of CCL19 administration. **A,** IL-10 level in the BALF before and after ovalbumin inhalation. **B,** IL-10 level in the cultured cells. Data shown are means ± SEMs of triplicate examination from 4 mice per group. The effect of CCL19 administration on airway inflammation **(C)** and on airway response **(D).** $*P < .05$; $**P < .01$. *NS,* Not significant.

DISCUSSION

In the current study, we showed that CCL19 and CCL21 are important but not critical for sensitization and induction of allergic inflammation. In plt mice, which are genetically defective with regard to CCL19 and CCL21,[17] the ovalbumin-specific IgE response was delayed, although the same level of response was noted as in BALB/c mice after the final immunization. Although airway inflammation and airway response to acetylcholine were significantly reduced in plt mice compared with BALB/c mice, significant eosinophilic inflammation and hyperresponsiveness were also observed in plt mice after 2 weeks of inhalation. However, 4 weeks after cessation of inhalation, airway inflammation and airway hyperresponsiveness in plt mice were greater than in BALB/c. At the time of resolution of airway inflammation, IL-10 production was enhanced in BALB/c but not in plt mice.

Enhanced T-cell immune response was observed in plt mice when examined by contact sensitization.[23] CCL21 is present in the luminal surface of venules in peripheral lymph nodes of wild-type mice but not plt/plt.[25] High endothelial venules in plt mice did not express CCL21 and did not support T-cell sticking, resulting in a marked reduction of T-cell homing to peripheral lymph nodes.[23] After immunization of plt mice, T cells and DCs located in the superficial cortex of lymph nodes and in splenic bridging channels.[23] It has been reported that airway inflammation can be induced without secondary lymph nodes.[28] In that study, lymphotoxin α/β knockout mice were used for impairment of secondary lymph nodes. Consistent with previous reports, our data showed that significant airway inflammation in a lesser magnitude was induced without secondary lymph nodes.

Blockade of CCL21 by neutralizing antibody exacerbates lung T_H1-dominant inflammation induced by propionibacterium acnes, which is thought to possess linkage to sarcoidosis.[16] Immune response in CCR7 receptor knockout mice was also reported.[12] CCR7 is the receptor for CCL19 and CCL21, and its knockout mice possess impairment of secondary lymph nodes similar to plt mice. However, the impairment of contact sensitivity is severely suppressed compared with plt mice. Antibody response to subcutaneous immunization with complete Freund adjuvant is also depressed in the first 10 days and reaches a level similar to that in wild-type mice after 20 days. Although the immunization protocol and antigens are different, our data with plt mice exhibited similar kinetics.

Plt mice exhibited impaired resolution of airway inflammation. Recently, it has been reported that CCR7 plays a critical role in the lymphocyte exit from peripheral tissue and entry into afferent lymphatics.[24,25] As shown in Fig 5, both $CCR7^+$ and $CCR7^-$ CD4 T cells existed in the lung of both plt and BALB/c mice. Even at the resolution phase, both $CCR7^+$ and $CCR7^-$ cells exist in the lung of plt mice, suggesting that we could not ascribe delayed resolution of inflammation only to impairment of T-cell exit in plt mice. Because IL-10 regulates airway inflammation,[29-31] our data on impairment of IL-10 production in plt mice after cessation of ovalbumin exposure might be

critical in elucidating factors for resolution of airway inflammation. Furthermore, administration of CCL19 resulted in recovery of impaired IL-10 production, suggesting that the defect in IL-10 production was directly related to genetic defects of plt mice. Natural recovery from autoimmune encephalomyelitis has been ascribed to IL-10 producing $CD4^+CD25^+$ regulatory T cells,[32] which are known to suppress airway inflammation and produce IL-10 or TGF-β.[6,7,26,30,33,34] Plt mice also demonstrate defective DC localization,[19] which is a regulator of adaptive immune response as well as a critical inducer.[35] Pulmonary DCs also activate regulatory T cells at the secondary lymph nodes.[27] Although we could not show phenotypical defects in $CD4^+CD25^+$ cells in plt mice, we identify functional impairment of induction of IL-10 production in plt mice, which might be ascribed to functional impairment of regulatory T cells or DCs. Another possible mechanism of delayed resolution is a defect in apoptosis in plt mice, which had been reported previously in T_H1-dominant inflammation.[36]

REFERENCES

1. Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP, et al. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. J Exp Med 1996;183:109-17.
2. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J Exp Med 1996;183:195-201.
3. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998;282:2261-3.
4. Vogel G. Interleukin-13's key role in asthma shown. Science 1998;282: 2168.
5. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. Science 1998; 282:2258-61.
6. Chinen J, Shearer WT. Basic and clinical immunology. J Allergy Clin Immunol 2005;116:411-8.
7. Chinen J, Shearer WT. Advances in asthma, allergy and immunology series 2004: basic and clinical immunology. J Allergy Clin Immunol 2004;114:398-405.
8. Lambrecht BN, Peleman RA, Bullock GR, Pauwels RA. Sensitization to inhaled antigen by intratracheal instillation of dendritic cells. Clin Exp Allergy 2000;30:214-24.
9. Lambrecht BN. Allergen uptake and presentation by dendritic cells. Curr Opin Allergy Clin Immunol 2001;1:51-9.
10. Vermaelen KY, Pauwels RA. Accelerated airway dendritic cell maturation, trafficking and elimination in a mouse model of asthma. Am J Respir Cell Mol Biol 2003;29:405-9.
11. Palucka K, Banchereau J. How dendritic cells and microbes interact to elicit or subvert protective immune responses. Curr Opin Immunol 2002;14:420-31.
12. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell 1999; 99:23-33.
13. Nakano H, Yanagita M, Gunn MD. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. J Exp Med 2001;194:1171-8.
14. Cyster JG. Chemokines and cell migration in secondary lymphoid organs. Science 1999;286:2098-102.
15. Sallusto F, Lanzavecchia A. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. Immunol Rev 2000;177:134-40.
16. Itakura M, Tokuda A, Kimura H, Nagai S, Yoneyama H, Onai N, et al. Blockade of secondary lymphoid tissue chemokine exacerbates propionibacterium acnes-induced acute lung inflammation. J Immunol 2001;166:2071-9.
17. Nakano H, Mori S, Yonekawa H, Nariuchi H, Matsuzawa A, Kakiuchi T. A novel mutant gene involved in T-lymphocyte-specific homing into peripheral lymphoid organs on mouse chromosome 4. Blood 1998;91: 2886-95.
18. Nakano H, Gunn MD. Gene duplications at the chemokine locus on mouse chromosome 4: multiple strain-specific haplotypes and the deletion of secondary lymphoid-organ chemokine and EBI-1 ligand chemokine genes in the plt mutation. J Immunol 2001;166:361-9.
19. Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT, et al. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. J Exp Med 1999;189:451-60.
20. Yamashita N, Tashimo H, Ishida H, Kaneko F, Nakano J, Kato H, et al. Attenuation of airway hyperresponsiveness in a murine asthma model by neutralization of granulocyte-macrophage colony-stimulating factor (GM-CSF). Cell Immunol 2002;219:92-7.
21. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am J Respir Crit Care Med 1997;156:766-75.
22. Yamashita N, Sekine K, Miyasaka T, Kawashima R, Nakajima Y Nakano J, et al. Platelet-derived growth factor is involved in the augmentation of airway responsiveness through remodeling of airways in diesel exhaust particulate-treated mice. J Allergy Clin Immunol 2001;107:135-42.
23. Mori S, Nakano H, Aritomi K, Wang CR, Gunn MD, Kakiuchi T. Mice lacking expression of the chemokines CCL21-ser and CCL19 (plt mice) demonstrate delayed but enhanced T cell immune responses. J Exp Med 2001;193:207-18.
24. Bromley SK, Thomas SY, Luster AD. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. Nat Immunol 2005;6:895-901.
25. Debes GF, Arnold CN, Young AJ, Krautwald S, Lipp M, Hay JB, et al. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. Nat Immunol 2005;6:889-94.
26. Huehn J, Siegmund K, Lehmann JC, Siewert C, Haubold U, Feuerer M, et al. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. J Exp Med 2004; 199:303-13.
27. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat Immunol 2001;2:725-31.
28. Gajewska BU, Alvarez D, Vidric M, Goncharova S, Stampfli MR, Coyle AJ, et al. Generation of experimental allergic airways inflammation in the absence of draining lymph nodes. J Clin Invest 2001;108:577-83.
29. Stampfli MR, Cwiartka M, Gajewska BU, Alvarez D, Ritz SA, Inman MD, et al. Interleukin-10 gene transfer to the airway regulates allergic mucosal sensitization in mice. Am J Respir Cell Mol Biol 1999;21:586-96.
30. Umetsu DT, Akbari O, Dekruyff RH. Regulatory T cells control the development of allergic disease and asthma. J Allergy Clin Immunol 2003;112:480-7; quiz 8.
31. Zemann B, Schwaerzler C, Griot-Wenk M, Nefzger M, Mayer P, Schneider H, et al. Oral administration of specific antigens to allergy-prone infant dogs induces IL-10 and TGF-beta expression and prevents allergy in adult life. J Allergy Clin Immunol 2003;111:1069-75.
32. McGeachy MJ, Stephens LA, Anderton SM. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+ CD25+ regulatory cells within the central nervous system. J Immunol 2005;175:3025-32.
33. McHugh RS, Shevach EM. The role of suppressor T cells in regulation of immune responses. J Allergy Clin Immunol 2002;110:693-702.
34. Johnston B, Kim CH, Soler D, Emoto M, Butcher EC. Differential chemokine responses and homing patterns of murine TCR alpha beta NKT cell subsets. J Immunol 2003;171:2960-9.
35. Morel PA, Feili-Hariri M, Coates PT, Thomson AW. Dendritic cells, T cell tolerance and therapy of adverse immune reactions. Clin Exp Immunol 2003;133:1-10.
36. Sanchez-Sanchez N, Riol-Blanco L, de la Rosa G, Puig-Kroger A, Garcia-Bordas J, Martin D, et al. Chemokine receptor CCR7 induces intracellular signaling that inhibits apoptosis of mature dendritic cells. Blood 2004;104:619-25.

Eur Respir J 2006; 27: 1–9
DOI: 10.1183/09031936.06.00107004

Role of macrophage migration inhibitory factor in ovalbumin-induced asthma in rats

M. Kobayashi*, Y. Nasuhara*, A. Kamachi#, Y. Tanino*, T. Betsuyaku*, E. Yamaguchi¶, J. Nishihira+ and M. Nishimura*

ABSTRACT: **Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that reportedly counteracts the anti-inflammatory effect of endogenous glucocorticoids. There have only been a few reports that demonstrate a potential link between MIF and bronchial asthma. In an attempt to further clarify the precise role of MIF in asthma, the present authors examined the effect of anti-MIF antibody (Ab) on airway inflammation and airway hyperresponsiveness in an ovalbumin-immunised rat asthma model.**

Actively immunised brown Norway rats received ovalbumin inhalation with or without treatment of anti-MIF Ab. The levels of MIF in bronchoalveolar lavage fluid were significantly elevated after the ovalbumin challenge.

An immunohistochemical study revealed positive immunostaining for MIF in bronchial epithelium, even in nonsensitised rats, and the MIF staining in bronchial epithelium was enhanced after the ovalbumin challenge. Anti-MIF Ab significantly decreased the number of total cells, neutrophils and eosinophils in the bronchoalveolar lavage fluid of the ovalbumin-challenged rats, and also attenuated the ovalbumin-induced airway hyperresponsiveness to ovalbumin and methacholine. However, anti-MIF Ab did not affect the level of serum ovalbumin-specific IgE, suggesting that anti-MIF Ab did not suppress immunisation itself.

The results indicate that macrophage migration inhibitory factor plays a crucial role in airway inflammation and airway hyperresponsiveness in asthma.

KEYWORDS: **Airway hyperresponsiveness, airway inflammation, asthma, eosinophil, macrophage migration inhibitory factor, ovalbumin**

Macrophage migration inhibitory factor (MIF) was first described as one of the earliest cytokines to be derived from activated T-cells and to prevent the random migration of macrophages [1, 2]. Cloning of human MIF cDNA has led to extensive studies using purified recombinant MIF [3]; this protein has been postulated to function as a pro-inflammatory cytokine [4, 5]. DONNELLY *et al.* [6] reported that the levels of MIF in bronchoalveolar lavage fluid (BALF) were increased in patients with acute respiratory distress syndrome. The present authors subsequently demonstrated that anti-MIF antibody (Ab) attenuated both lipopolysaccharide-induced neutrophil accumulation in rat lungs [7] and bleomycin-induced acute lung inflammation and mortality in mice [8]. These data support the idea that MIF is a pro-inflammatory cytokine involved in lung injury.

MIF is now known to be constitutively expressed in a variety of cells, including macrophages, T-cells and bronchial epithelial cells in the lungs [4, 7, 9]. It has the unique feature of overriding the anti-inflammatory and immunosuppressive effects of glucocorticoids [5, 10]. MIF also plays an important regulatory role in the activation of T-cells induced by mitogenic or antigenic stimuli [11]. The strong induction of MIF mRNA and protein has been observed from T-helper cell (Th) type 2 but not Th1 clones [11]. Accordingly, MIF is considered to be a pleiotropic peptide, functioning as a cytokine and/or hormone.

Only a few reports have examined the potential role of MIF in asthma [12–14]. ROSSI *et al.* [12] first reported that MIF levels were increased in BALF from asthmatic patients and that circulating eosinophils could produce MIF upon stimulation *in vitro*. However, one subsequent animal study could not support this argument of the role of MIF in asthma because anti-MIF serum did not affect allergic airway inflammation in mice [14]. The aim of the present study is to further clarify the role of MIF in asthma using rats. The study will demonstrate that anti-MIF Ab inhibits ovalbumin (OA)-induced airway inflammation as well as airway hyperresponsiveness in brown

AFFILIATIONS
*First Dept of Medicine and
+Central Research Institute, Hokkaido University School of Medicine, Sapporo, and
#Dept of Respiratory Medicine, Oji Municipal General Hospital, Tomakomai, and
¶Division of Respiratory Medical and Allergy Dept of Internal Medicine, Aichi Medical University School of Medicine, Aichi, Japan.

CORRESPONDENCE
Y. Nasuhara
First Dept of Medicine
Hokkaido University School of Medicine
North 15 West 7
Kita-ku
Sapporo 060-8638
Japan
Fax: 81 117067899
E-mail:
nasuhara@med.hokudai.ac.jp

Received:
September 14 2004
Accepted after revision:
January 05 2006

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

Norway rats, which have been used as a model of atopic asthma [15–17].

MATERIALS AND METHODS

Animals and immunisation

The research adhered to the Declaration of Helsinki and was approved by the Ethical Committee on Animal Research (Hokkaido University, Sapporo, Japan). Specific pathogen-free 6-week-old male brown Norway rats (weight range, 160–200 g) were purchased from Japan Charles River Co. (Yokohama, Japan). They were actively immunised to OA by subcutaneous injection with 1 mg OA containing 200 mg aluminum hydroxide. An adjuvant consisting of 1×10^9 heat-killed *Bordetella pertussis* organisms was intraperitoneally injected at the same time.

Preparation of rabbit polyclonal Ab against MIF

Polyclonal anti-rat MIF serum was generated by immunising New Zealand white rabbits with purified recombinant rat MIF. Rat MIF was expressed in *Escherichia coli* and purified to homogeneity, as described in a previous publication of the authors' [18]. In brief, the rabbits were inoculated intradermally with 100 mg of MIF emulsified in complete Freunds' adjuvant (Wako Pure Chemical Industries, Osaka, Japan) at weeks 1 and 2, and with 50 mg of MIF diluted in incomplete Freunds' adjuvant (Wako Pure Chemical Industries) at week 4. The immunoglobulin (Ig)G fraction was prepared using Protein A Sepharose (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according to the manufacturer's protocol.

Experimental protocol

The rats were divided into three groups: Naive group, OA group, and OA+anti-MIF Ab group. The Naive group did not receive immunisation and did not have any treatments. The OA and OA+anti-MIF Ab groups were actively immunised on day 0 and intraperitoneally injected with 2 mg of the non-immunized rabbit IgG or the anti-MIF polyclonal Ab every 2 days from day 0 to day 16. In the preliminary study, the present authors had confirmed that non-immunised rabbit IgG caused no changes in inflammatory cells of the OA-immunized lungs. Neither total cell nor eosinophil counts in BALF were significantly different between the OA immunised+untreated group and the OA immunized+non-immunized IgG group ($8.99 \pm 1.70 \times 10^6$ *versus* $7.05 \pm 0.89 \times 10^6$ n=3, and $5.79 \pm 0.76 \times 10^6$ *versus* $4.67 \pm 0.80 \times 10^6$ n=3, 3, respectively; unpublished data). The OA immunized+non-immunized IgG group were thus used as control in this experiment. The authors felt that administration of non-immunized rabbit IgG would be desirable to more specifically examine the effect of anti-MIF Ab. On day 14, the rats inhaled 2 % weight/volume OA for 15 minutes in an exposure chamber. Three days after OA inhalation, broncoalveolar lavage was performed, blood samples and lung tissues were taken, and the airway response to OA or methacholine (Mch) was measured.

Bronchoalveolar lavage and cell counting

The lungs were washed three times with 15 mL of sterile saline. After the lavage, the lungs were fixed with an intrabronchial infusion of 10% neutral formalin at a constant pressure of 25 cmH_2O for 48-h period. The lavage fluid was centrifuged and the cells were counted and processed for differential cell analysis. The supernatant was used for the measurement of MIF, eotaxin, or interleukin (IL)-13 concentrations.

Measurement of bronchial responsiveness to methacholine and ovalbumin

Three days after OA challenge, another set of three groups were anesthetised with an intraperitoneal injection of pentobarbital sodium (50 $mg \cdot kg^{-1}$). Intratrachial intubation was then performed with a metallic tube. The rats were mechanically ventilated (Rodent Ventilator Model 683; Harvard Apparatus, Holliston, MA, USA). A pressure transducer (TP-602T; Nihon Kohden Co., Tokyo, Japan) was connected to a side port of the metallic tube, and airway opening pressure (P_{ao}) was continuously measured. An aerosol of Mch or OA was administered through a reservoir box connected to the ventilator system. After measurement of baseline P_{ao}, an aerosol of saline followed by Mch or OA was administered.

Immunohistochemical study

Immunohistochemistry was performed according to the manufacturer's protocol on paraffin embedded tissue using a Catalized Signal Amplification kit (DAKO Japan, Kyoto, Japan). The primary Ab was anti-MIF diluted at 1:200 with PBS. The tissue sections were counterstained with methyl green and mounted. The anti-MIF Ab used for immunohistochemical study was the same as the Ab administered for treatment of rats.

Measurement of MIF levels by ELISA

The levels of MIF in the BALF were quantitated using the ELISA method, as described in a previous publication [19]. The anti-rat MIF Ab administered for treatment of rats was used in ELISA. Briefly, the anti-rat MIF Ab was added to each well of a 96-well microtitre plate. Wells were incubated with biotin-conjugated anti-MIF Ab for 1 h at room temperature. Avidin-conjugated horseradish peroxidase was added after washing. Substrate solution was then added to each well. The reaction was terminated with 2 M sulphuric acid. The absorbance was measured at 492 nm on an automated ELISA plate reader. The detection limit of this system was 1.5 $ng \cdot mL^{-1}$.

OA-specific IgE Ab assay

The levels of OA-specific IgE in serum were quantitated using an ELISA method, as previously described [20]. Briefly, the 96-well microtitre plates were coated with anti-rat IgE monoclonal Ab (Zymed, South San Francisco, CA, USA) at 4°C for 24 h. The plate was washed and incubated with standard serum or sample serum for 1 h at room temperature. After washing, horseradish peroxidase-streptavidin was plated into each well. After final washing, o-phenylenediamine solution containing 0.035% hydrogen peroxide was added to each well. The enzyme reaction was stopped by the addition of 2 M sulphuric acid and the absorbance was measured at 490 nm on a plate reader. The absorbance of standard serum diluted 1:100 was arbitrarily defined as $U \cdot mL^{-1}$.

Measurement of eotaxin and IL-13 concentrations by ELISA

Due to the high degree of similarity maintained in chemokines across species, a mouse ELISA kit (R&D Systems Inc.,

Minneapolis, MN, USA) containing a polyclonal Ab that recognizes mouse eotaxin was used to detect the rat cognate. Eotaxin levels in BALF were determined using this kit according to the manufacturer's instructions. IL-13 levels in BALF were determined using a rat-specific solid phase sandwich ELISA kit (Biosource International, Camarillo, CA, USA). The minimum detectable concentration of eotaxin was 3 $pg \cdot mL^{-1}$; IL-13 was 1.5 $pg \cdot mL^{-1}$.

Statistical analysis

Data are expressed as mean±SEM. Statistical analyses were performed on the data using single-factor ANOVA on the three groups and with a Student's unpaired t-test for comparisons of two groups. A p-value of <0.05 was assumed to be significant.

RESULTS

Expression of MIF in OA-induced airway inflammation

To investigate whether the expression of MIF in airways was enhanced in this model, the levels of MIF in BALF were measured 3 days after the OA challenge. They were significantly elevated in the OA group compared with those in the Naive group (14.7 ± 1.4 $ng \cdot mL^{-1}$ *versus* 1.3 ± 1.1 $ng \cdot mL^{-1}$, respectively, $p<0.05$; fig. 1).

Immunohistochemical localisation of MIF in lungs

Histological examination using the lung tissue confirmed that OA inhalation induced widespread peribronchiolar inflammation in OA-sensitised rats, which is characteristic of asthma. Positive immunostaining for MIF was observed within the bronchial epithelium, even in the Naive group (fig. 2a). There was a significant increase in immunostaining of the bronchial epithelial cells, epithelial submucosa and inflammatory cells in the alveoli of the OA group 3 days after the OA challenge (fig. 2b).

Effect of anti-MIF Ab on airway inflammation

Total and differential cell counts 3 days after the OA challenge are shown in figure 3. In the OA group, the numbers of total cells, macrophages, eosinophils and neutrophils were significantly elevated compared with those of the Naive group. Treatment with anti-MIF Ab significantly decreased the numbers of total cells, eosinophils and neutrophils compared with those of the OA group (total cells: $15.0 \pm 3.5 \times 10^6$ in the OA group *versus* $10.5 \pm 2.4 \times 10^6$ in the OA+anti-MIF Ab group, $p<0.01$; eosinophils: $10.5 \pm 2.7 \times 10^6$ in the OA group *versus* $6.2 \pm 2.7 \times 10^6$ in the OA+anti-MIF Ab group, $p<0.01$;

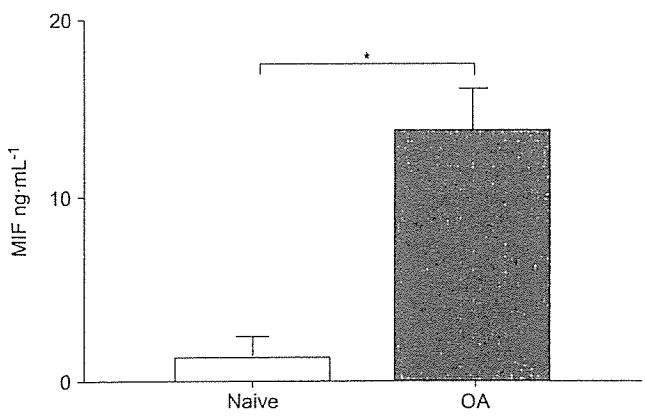

FIGURE 1. Levels of macrophage migration inhibitory factor (MIF) in bronchoalveolar lavage fluid (BALF). MIF in BALF significantly increased 3 days after the ovalbumin (OA) challenge in the OA group (n=6) compared with the Naive group (n=3). *: $p<0.05$.

FIGURE 2. Immunohistochemistry of macrophage migration inhibitory factor (MIF) in the lung. a) MIF was weakly detected in airway epithelium in the Naive group. b) MIF was prominent in airway epithelium in the ovalbumin (OA) group 3 days after the OA challenge. Scale bars=100 μm.

neutrophils: $1.4 \pm 1.2 \times 10^6$ in the OA group *versus* $0.16 \pm 0.27 \times 10^6$ in the OA+anti-MIF Ab group, $p<0.01$) and thus significantly attenuated airway inflammation.

Effect of anti-MIF Ab on antigen-specific airway contraction and nonspecific airway hyperresponsiveness

To investigate whether anti-MIF Ab suppressed airway hyperresponsiveness, OA-specific and Mch-induced airway contractions were measured. After measurement of the baseline pressure, an aerosol of OA was administered. The airway pressure was significantly increased in the OA group (fig. 4a) but not in the OA+anti-MIF Ab group (fig. 4b).

Similarly, after measurement of the baseline pressure, an aerosol of Mch was administered for 1 min in progressively doubled concentrations from 0.0625 $mg \cdot mL^{-1}$. In the OA group, the airway pressure was significantly increased. In contrast, the OA+anti-MIF Ab group did not respond to Mch (up to 16.0 $mg \cdot ml^{-1}$). The Naive group did not respond to either 5% OA or Mch (up to 16.0 $mg \cdot ml^{-1}$; data not shown).

Effect of anti-MIF Ab on the development of humoral immune responses

Elevated levels of IgE are known to be important in the development of an allergen-induced airway response [21]. The results described above may be a consequence of suppression of OA immunisation by treatment of anti-MIF Ab; the authors therefore examined the possibility that anti-MIF Ab might have influenced OA-specific IgE levels in serum. As shown in figure 5, as expected [16], the levels of OA-specific IgE in serum were significantly elevated in the OA group compared with those in Naive group (124.0 ± 41.3 $U \cdot mL^{-1}$ in the OA group *versus* 18.6 ± 5.7 $U \cdot mL^{-1}$ in the Naive group, $p<0.05$). Treatment with anti-MIF Ab similarly caused the elevation of OA-specific IgE in serum (153.3 ± 39.6 $U \cdot mL^{-1}$).

Effect of a single administration of anti-MIF Ab before airway challenge

The authors next considered whether the single administration of anti-MIF Ab before OA challenge might explain the results described above. A 2-mg aliquot of anti-MIF Ab or nonimmunised rabbit IgG was injected only once 2 h before OA challenge and bronchoalveolar lavage was performed 3 days after OA challenge. As shown in figure 6, a single administration of anti-MIF Ab did not change either the number of total cells or the differential cell counts in BALF.

Effect of anti-MIF Ab on eotaxin levels in BALF

To investigate the mechanism by which anti-MIF Ab attenuated eosinophil accumulation in the lungs, the levels of eotaxin, a potent chemokine of eosinophils, in BALF were

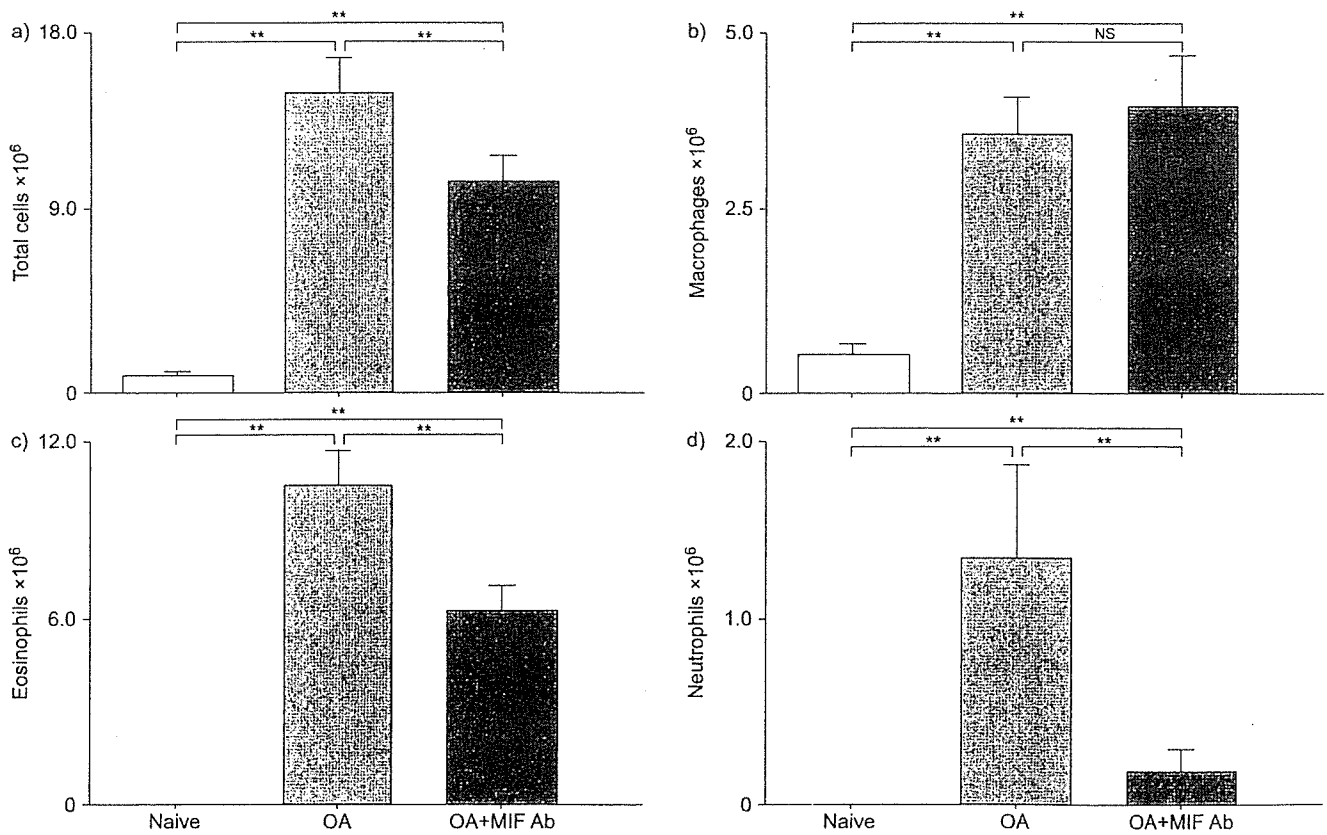

FIGURE 3. Total and differential cell counts in bronchoalveolar lavage fluid. Bronchoalveolar lavage was performed 3 days after ovalbumin (OA) challenge. The numbers of a) total cells, b) macrophages, c) eosinophils d) and neutrophils were significantly elevated in the OA group in comparison with the Naive group. Treatment with anti-MIF antibody (OA+MIF Ab) significantly decreased the numbers of total cells (a), eosinophils (c) and neutrophils (d) (n=6). **: $p<0.01$; NS: nonsignificant.

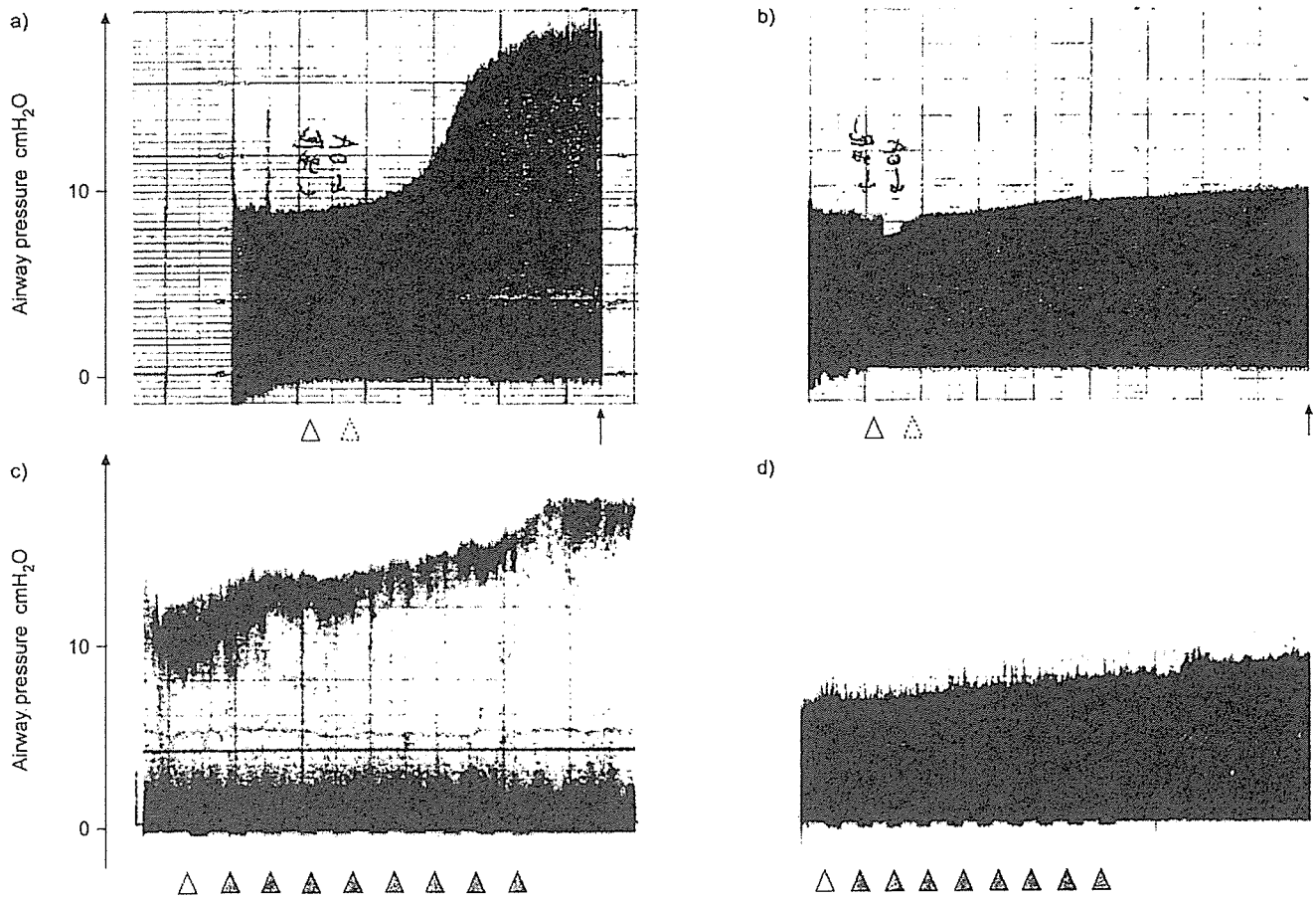

FIGURE 4. The effect of anti-macrophage migration inhibitory factor antibody (MIF Ab) on airway contraction. Both ovalbumin (OA)-specific and non-specific airway responsiveness were examined 3 days after OA challenge. After measurement of baseline pressure, an aerosol of 5% OA (indicated by the dotted arrowhead) was administered for 15 min (solid black arrow in a). a) In the OA group, the airway pressure was significantly increased. b) Converesly, the airway pressure was not increased in the OA+anti-MIF Ab group. An aerosol of methacholine (Mch) was administered for 1 min in progressively doubled concentrations from 0.0625 mg·mL^{-1} (grey arrowhead) after measurement of the baseline pressure. c) In the OA group, the airway pressure was significantly increased. d) In contrast, the OA+anti-MIF Ab group did not respond to Mch even at the maximum dose, 16 mg·mL^{-1}. Results are representative of three independent experiments. Solid black arrow in b): 30 min; open arrowhead: saline inhalation.

measured. In the study series up to 24 h after OA challenge, the levels of eotaxin in BALF began to increase at 4 h and reached peak levels at 8 h in the OA group; however, no appreciable increase was seen in the levels of the Naive group (data not shown). No significant difference was seen in eotaxin levels at 8 h after the OA challenge between the OA group and the OA+anti-MIF Ab group (8.24 ± 1.5 pg·mL^{-1} in the Naive group, 127.3 ± 38.0 pg·mL^{-1} in the OA group, and 160.0 ± 23.3 pg·mL^{-1} in the OA+anti-MIF Ab group; fig. 7a).

Effect of anti-MIF Ab on IL-13 levels in BALF

The levels of IL-13 in BALF were also measured. The levels were significantly elevated at 8 h after OA challenge in the OA group compared with the Naive group. However, no significant difference was seen in IL-13 levels between the OA group and the OA+anti-MIF Ab group (31.2 ± 5.2 pg·mL^{-1} in the Naive group, 63.0 ± 16.9 pg·mL^{-1} in the OA group, and 72.4 ± 8.1 pg·mL^{-1} in the OA+anti-MIF Ab group; fig. 7b).

DISCUSSION

This study first demonstrated that OA-sensitised rats had increased levels of MIF in BALF and enhanced expression of MIF in airway epithelium after OA challenge. These results are consistent with the previous observation in a human study in which BALF from patients with asthma contained significantly elevated levels of MIF as compared to normal volunteers [12]. In addition, it has been clearly demonstrated that treatment with anti-MIF Ab significantly suppressed airway inflammation and airway hyperresponsiveness, both of which are characteristic features in this rat model of atopic asthma. These results indicate that MIF plays a potent role in the pathogenesis of allergen-induced airway inflammation and that anti-MIF Ab may have a therapeutic potential for bronchial asthma.

The present study does not agree with a previous study in which anti-MIF serum did not affect the allergic inflammation of the airway in mice [14]. In that study, mice were exposed to

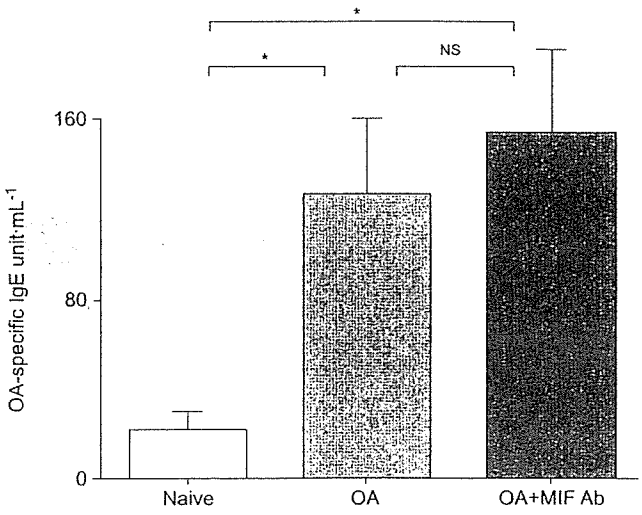

FIGURE 5. Effect of anti-macrophage migration inhibitory factor antibody (MIF Ab) on ovalbumin (OA)-specific immunoglobulin (Ig)E levels in serum. Serum was removed from the inferior vena cava 3 days after the OA challenge (n=6). The levels of OA-specific IgE in serum were significantly elevated in the OA group after OA challenge compared with those in the Naive group. There was no significant difference between the OA group and the OA+anti-MIF Ab group. *: $p<0.05$; NS: nonsignificant.

OA once daily for 7 days following active immunization by OA injection and were treated with anti-MIF serum every 3 days from the day before the first allergen challenge to the end of the experiment. Such treatment did not significantly reduce the number of eosinophils either in lung tissues or BALF. The discrepancy between the two studies with regard to the effect of anti-MIF on eosinophil recruitment into the airway requires some explanation. First, the eosinophilic inflammation induced in the other study was milder than that observed in the present study; the percentage of eosinophils in BALF was nearly 30% in the other study and 64.9±3.7% in the present study. The small number of eosinophils in the other study might have obscured the inhibitory effect of anti-MIF Ab. Secondly, researchers in the other study used anti-MIF serum rather than anti-MIF Ab, and the total dose of anti-MIF serum given might not have been sufficient. Indeed, although the previous study also investigated the effect of anti-MIF serum on lipopolysaccharide-induced neutrophilic airway inflammation, the researchers could not demonstrate the effect of the anti-MIF serum either. In contrast, the present authors previously demonstrated that anti-MIF Ab significantly inhibited lipopolysaccharide-induced neutrophil accumulation in rat lungs [7]. Taken together, the anti-MIF serum used in the other study may not have had enough potency or may not have been given in a sufficient amount to exert a discernable effect. A less likely possibility for the discrepancy between the two studies is that the role of MIF in animal models of asthma may differ among species.

MIF is known to be constitutively expressed in bronchial epithelium [7, 9]. In the present study, the immunohistochemical experiment clearly demonstrated that expression of MIF was enhanced in airway epithelium after OA challenge in OA-sensitised rats. This is the first study to demonstrate that bronchial epithelium is a potent source of MIF in an asthma model. Previously, Rossi *et al.* [12] suggested that eosinophils might be a potential source of MIF in human asthma because even circulating eosinophils from normal volunteers were shown to produce MIF with phorbol myristate acetate stimulation. Indeed, in the present study, the majority of inflammatory cells in BALF were eosinophils. Accordingly, bronchial epithelium as well as eosinophils may jointly contribute to the increased levesl of MIF in BALF in the present rat asthma model.

Because 60–70% of total cells in BALF in OA-sensitised rats were eosinophils, the attenuation of the number of total cells by treatment with anti-MIF Ab is mostly attributed to the attenuation of the number of eosinophils. It has been reported that the eotaxin levels are highly elevated in BALF from patients with asthma [22] and that eotaxin is associated with airway hyperresponsiveness [23]. Eotaxin may therefore play an important role in the pathogenesis of bronchial asthma. As a result, the present authors wondered whether the effect of the anti-MIF Ab on airway inflammation might be at least in part explained by its effect on eotaxin. It was found that the level of eotaxin in BALF was certainly elevated after OA challenge compared with that in naive rats. However, no significant difference was observed in the levels of eotaxin in BALF between the OA group and the OA+anti-MIF Ab group. In animal models, IL-13 has been shown to induce airway hyperresponsiveness and airway eosinophilia [24, 25]. It is also possible that IL-13-dependent airway hyperresponsiveness occurs *via* mechanisms that are independent of airway eosinophilia [26]. In the present study, the levels of IL-13 in BALF were elevated after OA challenge; however, there was no significant difference between the OA group and the OA+anti-MIF Ab group. The authors also measured the expression of IL-5 mRNA and macrophage inflammatory protein (MIP)-1α mRNA using tissue homogenates after OA challenge. These chemokines are known to have a role in the recruitment of eosinophils to airways in asthma. However, the level of mRNA for MIP-1α did not increase after antigen challenge and that of IL-5 was under the detection limits, even after antigen challenge in this model (data not shown). A previous study reported that MIF significantly delayed spontaneous neutrophil apoptosis *in vitro*, as well as eosinophil apoptosis to some extent [27]. Thus, the anti-MIF Ab might reduce the number of eosinophils and neutrophils in BALF by enhancing apoptosis of those cells.

The anti-MIF Ab dramatically reduced the number of neutrophils in BALF in the present study. Neutrophils are known to be increased in the airways of patients with status asthmaticus [28], during exacerbations of asthma [29] and in sputum from subjects with severe asthma [30]. However, the role of neutrophils in asthma is not fully understood. The attenuation of the number of neutrophils may be partially attributed to the anti-inflammatory effect of anti-MIF Ab in the present model. The authors have previously reported that anti-MIF Ab inhibits lipopolysaccharide-induced neutrophil accumulation in rat lungs *via* its suppressive effect on MIP-2, a powerful neutrophil chemokine [7]. Therefore, the suppression of MIP-2 might cause attenuation of the number of neutrophils in the rat asthma model.

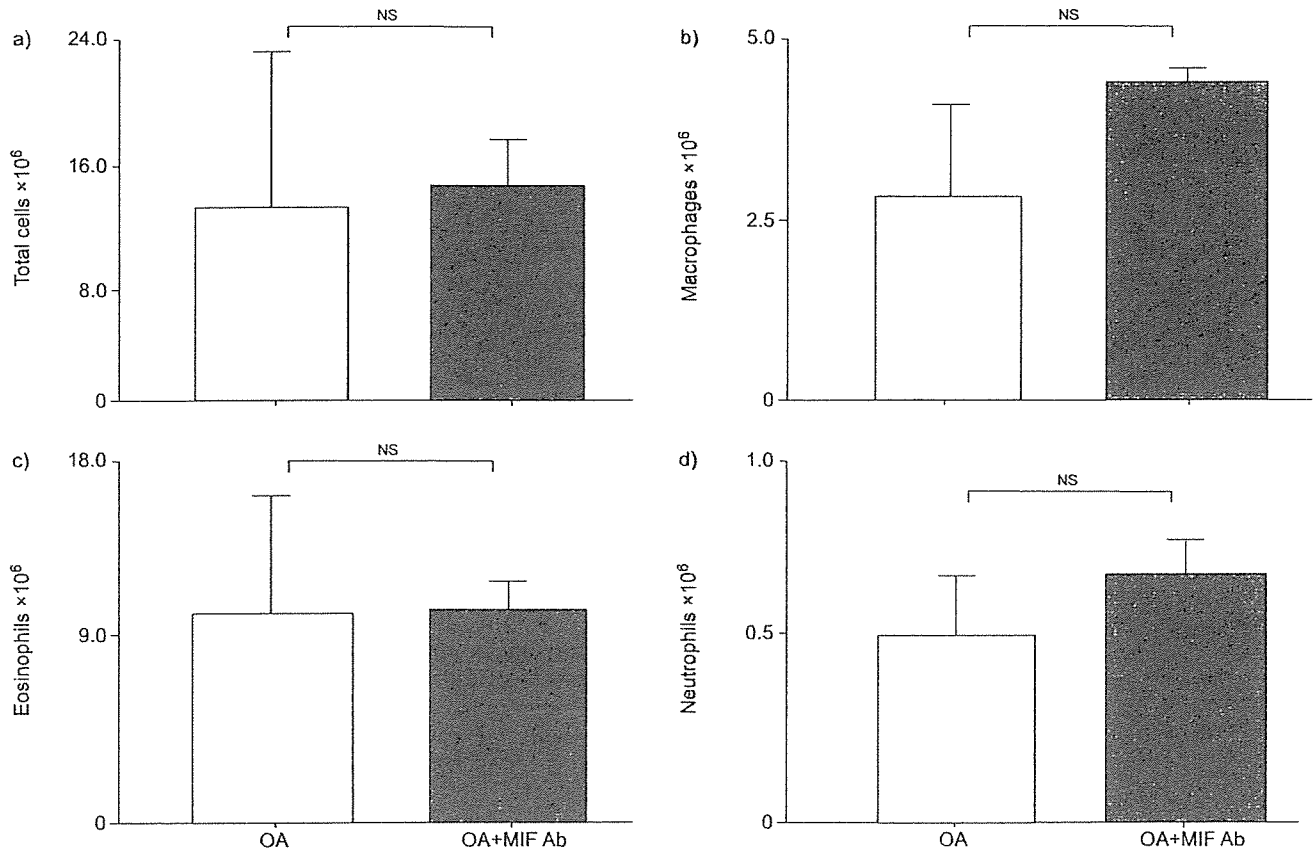

FIGURE 6. Effect of single administration of anti-macrophage migration inhibitory factory antibody (MIF Ab) on a) total cells, b) macrophages, c) eosinophils and d) neutrophils in bronchoalveolar lavage fluid (BALF). A 2-mg aliquot of anti-MIF Ab or rabbit IgG was injected once, 2 h before ovalbumin (OA) challenge and bronchoalveolar lavage was performed 3 days after OA challenge. A single administration of anti-MIF Ab did not reduce the number of total and differential cell counts in BAL fluid (n=3).

In the present study, the anti-MIF Ab did not affect antigen-specific IgE in serum, which led to investigation of whether a single dose of anti-MIF Ab could exert its effect before OA inhalation. A single administration of anti-MIF Ab did not reduce the number of total cells and differential cell counts in BALF, suggesting that the serial injection of the anti-MIF Ab from OA sensitisation to 2 days after OA inhalation are necessary for its suppressive effect to be exerted. The total amount of anti-MIF Ab might be important for exertion of its effect. It was therefore concluded that anti-MIF Ab suppressed OA-induced airway inflammation by an independent mechanism of OA-sensitisation.

Glucocorticoids are currently the most effective anti-inflammatory agent in the treatment of asthma [31]. However, it is widely recognised that a small proportion of patients, who are often named as steroid-resistant asthmatics, fail to respond to glucocorticoids. MIF might play a role in the blunt response to endogenous or exogenous steroids [5, 10]. This consideration leads to the speculation that anti-MIF therapy may not only have direct anti-inflammatory effects, but also act by recovering the function of endogenous and/or exogenous glucocorticoids.

Finally, some comments should be made on the weakness of the experimental protocol in this study. First, quantitative assessment of airway hyperresponsiveness was not performed, particularly for naive rats and OA+anti-MIF Ab rats; this meant it was unclear how much anti-MIF Ab attenuated airway hyperresponsiveness in the OA-immunised lungs. Such assessment was not performed because the authors' specific interest lay in assuring that enhanced airway hyperresponsiveness by OA immunisation and inhalation was actually attenuated by anti-MIF Ab. Secondly, airway pressure was used to assess airway hyperresponsiveness, which is influenced by changes in both airway resistance and lung compliance. As the increased airway pressure was confirmed to return to baseline in a short time, the change of compliance, which is likely to be caused by lung parenchymal injury, could be negligible in the present study (data not shown).

In summary, the present manuscript has demonstrated that macrophage migration inhibitory factor is involved in the asthmatic response in the ovalbumin-sensitized rat asthma model. It has also been shown that bronchial epithelium is a potent source of macrophage migration inhibitory factor in this asthma model. The anti-macrophage migration inhibitory factor antibody also significantly attenuated ovalbumin-induced airway inflammation and airway hyperresponsiveness. Although these data support the concepts that macrophage migration inhibitory factor plays an important

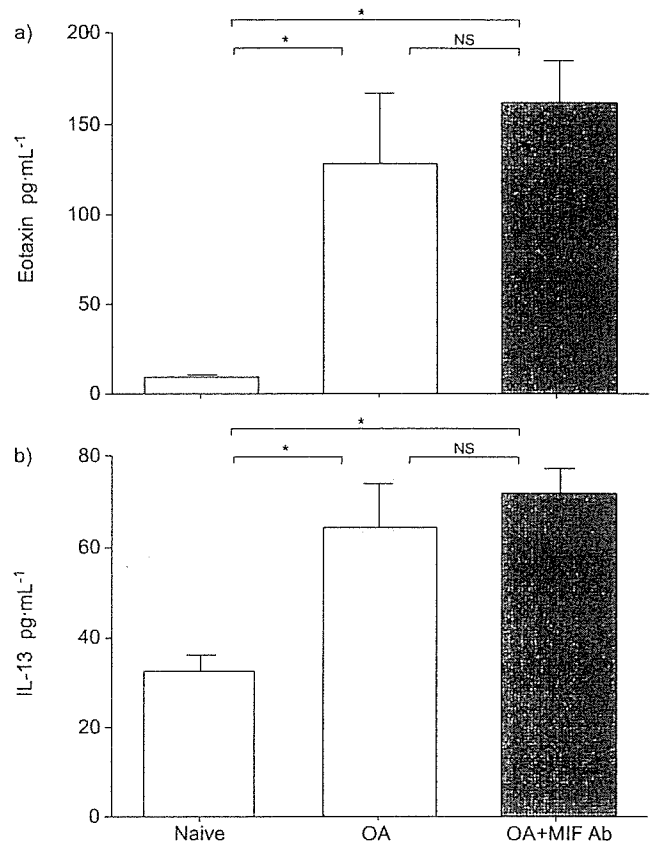

FIGURE 7. Effects of anti- macrophage migration inhibitory factory antibody (MIF Ab) on a) eotaxin and b) interleukin (IL)-13 levels in bronchoalveolar lavage fluid (BALF). The levels of eotaxin and IL-13 in BALF were significantly elevated in the ovalbumin (OA) group compared with the Naive group 8 h after the OA challenge. However, there were no significant differences in both chemokines between the OA group and the OA+anti-MIF Ab group (n=3 for each).

role in asthma and anti-macrophage migration inhibitory factor antibody may have a therapeutic potential for asthma, further investigations are necessary to fully understand the mechanism of the effect of anti-macrophage migration inhibitory factor antibody on asthma pathology and to examine the therapeutic potential of the anti-macrophage migration inhibitory factor antibody in human asthma.

REFERENCES

1 Bloom BR, Bennett B. Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity. *Science* 1996; 153: 80–82.

2 David JR. Delayed hypersensitivity *in vitro*: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proc Natl Acad Sci USA* 1966; 56: 72–77.

3 Weiser WY, Temple PA, Witek-Giannotti JS, Remold HG, Clark SC, David JR. Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor. *Proc Natl Acad Sci USA* 1989; 86: 7522–7526.

4 Baumann R, Casaulta C, Simon D, Conus S, Yousefi S, Simon HU. Macrophage migration inhibitory factor delays apoptosis in neutrophils by inhibiting the mitochondria-dependent death pathway. *FASEB J* 2003; 17: 2221–2230.

5 Bucala R. MIF rediscovered: cytokine, pituitary hormone, and glucocorticoid-induced regulator of the immune response. *FASEB J* 1996; 10: 1607–1613.

6 Donnelly SC, Haslett C, Reid PT, *et al.* Regulatory role for macrophage migration inhibitory factor in acute respiratory distress syndrome. *Nat Med* 1997; 3: 320–323.

7 Makita H, Nishimura M, Miyamoto K, *et al.* Effect of anti-macrophage migration inhibitory factor antibody on lipopolysaccharide-induced pulmonary neutrophil accumulation. *Am J Respir Crit Care Med* 1998; 158: 573–579.

8 Tanino Y, Makita H, Miyamoto K, *et al.* Role of macrophage migration inhibitory factor in bleomycin-induced lung injury and fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 2002; 283: L156–L162.

9 Bacher M, Meinhardt A, Lan HY, *et al.* Migration inhibitory factor expression in experimentally induced endotoxemia. *Am J Pathol* 1997; 150: 235–246.

10 Calandra T, Bernhagen J, Metz CN, *et al.* MIF as a glucocorticoid-induced modulator of cytokine production. *Nature* 1995; 377: 68–71.

11 Bacher M, Metz CN, Calandra T, Mayer K, Chesney J, Lohoff M. An essential regulatory role for macrophage migration inhibitory factor in T-cell activation. *Proc Natl Acad Sci USA* 1996; 93: 7849–7854.

12 Rossi AG, Haslett C, Hirani N, *et al.* Human circulating eosinophils secrete macrophage migration inhibitory factor (MIF). Potential role in asthma. *J Clin Invest* 1998; 101: 2869–2874.

13 Yamaguchi E, Nishihira J, Shimizu T, *et al.* Macrophage migration inhibitory factor (MIF) in bronchial asthma. *Clin Exp Allergy* 2000; 30: 1244–1249.

14 Korsgren M, Kallstrom L, Uller L, *et al.* Role of macrophage migration inhibitory factor (MIF) in allergic and endotoxin-induced airway inflammation in mice. *Mediators Inflamm* 2000; 9: 15–23.

15 Elwood W, Barnes PJ, Chung KF. Airway hyperresponsiveness is associated with inflammatory cell infiltration in allergic Brown-Norway rats. *Int Arch Allergy Immunol* 1992; 99: 91–97.

16 Haczku A, Chung KF, Sun J, Barnes PJ, Kay AB, Moqbel R. Airway hyperresponsiveness, elevation of serum-specific IgE, and activation of T cells following allergen exposure in sensitized Brown-Norway rats. *Immunology* 1995; 85: 598–603.

17 Waserman S, Olivenstein R, Renzi P, Xu LJ, Martin JG. The relationship between late asthmatic responses and antigen-specific immunoglobulin. *J Allergy Clin Immunol* 1992; 90: 661–669.

18 Nishihira J, Kuriyama T, Nishino H, Ishibashi T, Sakai M, Nishi S. Purification and characterization of human macrophage migration inhibitory factor: evidence for specific binding to glutathione and formation of subunit structure. *Biochem Mol Biol Int* 1993; 31: 841–50.

19 Mizue Y, Nishihira J, Miyazaki T, *et al.* Quantitation of macrophage migration inhibitory factor (MIF) using the one-step sandwich enzyme immunosorbent assay:

elevated serum MIF concentrations in patients with autoimmune diseases and identification of MIF in erythrocytes. *Int J Mol Med* 2000; 5: 397–403.

20 Ohnuma N, Yamaguchi E, Oguri M, Kawakami Y. Hyposensitization attenuates airway inflammation and antigen-induced proliferative response by lymphocytes in a rat model of bronchial asthma. *Respiration* 1998; 65: 469–475.

21 Holgate ST, Robinson C, Church MK. Mediators of immediate hypersensitivity. *In*: Middleston E Jr, Reed CE, Regis EF, eds. Allergy Principles and Practice. 4th Edn. St Louis, Mosby, 1993; pp. 267–301.

22 Lamkhioued B, Renzi PM, Abi-Younes S, *et al.* Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J Immunol* 1997; 159: 4593–4601.

23 Ying S, Robinson DS, Meng Q, *et al.* Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant colocalization of eotaxin mRNA to bronchial epithelial and endothelial cells. *Eur J Immunol* 1997; 27: 3507–3516.

24 Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 1998; 282: 2258–2261.

25 Grunig G, Warnock M, Wakil AE, *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998; 282: 2261–2263.

26 Walter DM, McIntire JJ, Berry G, *et al.* Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *J Immunol* 2001; 167: 4668–4675.

27 Baumann R, Casaulta C, Simon D, Conus S, Yousefi S, Simon HU. Macrophage migration inhibitory factor delays apoptosis in neutrophils by inhibiting the mitochondria-dependent death pathway. *FASEB J* 2003; 17: 2221–2230.

28 Lamblin C, Gosset P, Tillie-Leblond I, *et al.* Bronchial neutrophilia in patients with noninfectious status asthmaticus. *Am J Respir Crit Care Med* 1998; 157: 394–402.

29 Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 1995; 95: 843–852.

30 Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care Med* 2000; 161: 9–16.

31 Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention; NHLBI/WHO Workshop Report. Bethesda, National Institutes of Health, National Heart, Lung, and Blood Institute, 2002. Publication No. 02–3659..

Polymorphisms in the Muscarinic Receptor 1 Gene Confer Susceptibility to Asthma in Japanese Subjects

Yukiko Maeda, Nobuyuki Hizawa, Eisei Jinushi, Ayumi Honda, Daisuke Takahashi, Yoshinobu Fukui, Satoshi Konno, Tadamichi Shimizu, Hiroshi Shimizu, Etsuro Yamaguchi, and Masaharu Nishimura

First Department of Medicine and Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Rationale: The human cholinergic receptor muscarinic-1 (CHRM1) is widely distributed in the lungs. In patients with asthma, CHRM1 may be involved in airway constriction, airway epithelial cell proliferation, and airway inflammation. The *CHRM1* gene is located on chromosome 11q13, which is one of the candidate loci for asthma and atopy.

Objectives: To determine the role of the *CHRM1* gene polymorphisms in asthma.

Methods: We studied nine single-nucleotide polymorphisms (−18379G > A, −9697C > T, −6965T > C, −4953A > G, +267A > C, +1353C > T, +3970C > G, +5418C > G, and +5455G > T) in a case-control study using 326 patients with asthma and 333 healthy control subjects. We also examined functional consequences of the −9697C > T and −4953A > G polymorphisms at the regulatory region using an mRNA reporter assay.

Measurements and Main Results: Two common single-nucleotide polymorphisms (−9697C > T and −4953A > G) were associated with asthma. The odds ratio for the TT homozygotes at the −9697C > T polymorphism was 0.29 compared with the CC homozygotes (95% confidence interval, 0.12–0.73; p = 0.008), and the odds ratio for the GG homozygotes at the −4953A > G polymorphism was 1.86 compared with the AA homozygotes (95% confidence interval, 1.04–3.34; p = 0.038). Haplotype analysis showed that the −9697T/−6965T/−4953A haplotype was associated with a lower risk of asthma (p = 0.00055) and the −9697C/−6965T/−4953G haplotype was associated with an increased risk of asthma (p = 0.020). The −9697T/−4953A haplotype was also associated with lower luciferase activity *in vitro* compared with the −9697C/−4953G haplotype.

Conclusions: This study, together with an *in vitro* functional study, suggests that the *CHRM1* gene is an important susceptibility locus for asthma on chromosome11q13.

Keywords: case-control studies; IgE; muscarinic cholinergic receptor-1; single-nucleotide polymorphism

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Genetic studies repeatedly have linked asthma and asthma-related phenotypes to chromosome 11q13, on which several biological candidate genes are located.

What This Study Adds to the Field

Gene coding the human cholinergic receptor muscarinic-1 (CHRM1) is an important susceptibility locus for asthma at chromosome 11q13.

The cholinergic nerves are the dominant neural bronchoconstrictor pathway in humans (1). They release acetylcholine onto muscarinic receptors causing cholinergic bronchoconstriction (2), mucous hypersecretion, and edema in the airways. Increases in cholinergic nerve activity and cholinergic hypersensitivity are associated with asthma, and patients with asthma are hypersensitive to the bronchoconstricting actions of muscarinic agonists (3). The human cholinergic receptor muscarinic 1 (CHRM1; Online Mendelian Inheritance of Man database no. 118510) is widely localized in the human lung, including the alveolar walls, bronchial epithelial cells, parasympathetic ganglia, neuromuscular junction, and submucosal glands (4). Studies using pirenzepine, a muscarinic antagonist selective for M1 receptors, have shown that M1 muscarinic receptors are involved in vagally induced bronchoconstriction (5–7). M1 receptor–deficient mice showed increased bronchoconstriction in response to 10^{-8} M muscarine in peripheral airways (8), suggesting the existence of an M1 receptor–dependent pathway counteracting cholinergic bronchoconstriction. M1 receptors also play a role in mast cell function (9), epithelial cell proliferation in the trachea (10), release of neutrophil and monocyte chemotactic activity from epithelial cells (11), acetylcholine-induced relaxation of the human pulmonary veins (12), and regulation of water and electrolyte secretion on submucosal glands (13). Taken together, CHRM1 is critically involved in the pathophysiology of asthma.

The gene encoding CHRM1 exists on chromosome 11q13, which has been linked to asthma and asthma-related phenotypes in several genomewide searches (14–17). Given the important role of muscarinic cholinergic mechanisms in asthma, the *CHRM1* gene is biologically an excellent candidate for asthma susceptibility in the region of chromosome 11q13. Thus, in the current study, we examined whether genetic variations in the *CHRM1* gene are associated with asthma. To gain insight into the possible molecular basis of the disease association, we also examined functional consequences of single-nucleotide polymorphisms (SNPs) at the regulatory region of the *CHRM1* gene.

METHODS

See online supplement for additional details.

Study Subjects

A total of 659 unrelated Japanese adults were enrolled in the study (Table 1). Asthma was defined on the basis of recurrent episodes of at least two of three symptoms (cough, wheeze, and dyspnea) that are associated with demonstrable reversible airflow limitation (15% variability in FEV_1 or in peak expiratory flow rate either spontaneously or with an inhaled, short-acting β_2-agonist), or increased airway responsiveness to methacholine, or both, as described elsewhere (18).

(Received in original form January 19, 2006; accepted in final form August 24, 2006)

Supported by the Japan Society for the Promotion of Science (grant 17590771).

Correspondence and requests for reprints should be addressed to Nobuyuki Hizawa, M.D., First Department of Medicine, Hokkaido University Graduate School of Medicine, N-15 W-7, Kita-Ku, Sapporo 060-8638, Japan. E-mail: nhizawa@med.hokudai.ac.jp

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 174. pp 1119–1124, 2006
Originally Published in Press as DOI: 10.1164/rccm.200601-081OC on August 24, 2006
Internet address: www.atsjournals.org

TABLE 1. BASIC CHARACTERISTICS OF 659 JAPANESE SUBJECTS

	Healthy Control Subjects (n = 333)	Subjects with Asthma (n = 326)	p Value*
Age, yr, median (range)	41 (18–72)	45 (16–79)	< 0.0001
Sex, n (male/female)	208/125	148/178	< 0.0001
Smoking, n (never/ex-/current)	226/12/95	190/69/67	< 0.0001
Atopy, n (%)	170 (51)	237 (73)	< 0.0001
Total serum IgE, log IU/ml, mean (SD)	1.84 (0.627)	2.40 (0.622)	< 0.0001
FEV_1, % predicted, mean (SD)	—	69.2 (13.5)	
% Reversibility in FEV_1, median (range)	—	16.9 (0–211)	

* One-way analysis of variance or χ^2 test was used where appropriate.

Identification of Polymorphisms

Genomic DNA from Japanese subjects was genotyped for +267A > C (rs2067477) and +1353C > T (rs2067480), because an association of these polymorphisms at the muscarinic M1 receptor gene with cognitive function in schizophrenic patients has been reported (19). We selected an additional seven SNPs for genotyping based on the frequency and location of SNPs and the linkage disequilibrium (LD) structure in and around the *CHRM1* gene. We initially obtained genotyping data of 26 HapMap SNPs (spanning 31.6 kb around the gene) from 45 unrelated Japanese subjects at the International HapMap Project (available online at http://hapmap.org/). To select tagSNPs in this region, we used the multimarker predictor method implemented in the Tagger program (20). Tag set was generated (using a threshold r^2 of 0.8) using 14 common SNPs with a minor allele frequency of more than 0.05 in the Japanese population.

As the +267A > C and +1353C > T polymorphisms were in a complete LD, we genotyped a total of eight SNPs (−18379G > A [rs1938677], −9697C > T [rs2075748], −6965T > C [rs542269], −4953A > G [rs1942499], +1353C > T [rs2067480], 3970C > G [rs4963323], 5418C > G [rs11601597], and 5455G > T [rs11605665]) for all individuals (n = 659). These SNPs were typed using the assay that combines kinetic (real-time quantitative) polymerase chain reaction (PCR) with allele-specific amplification, as described elsewhere (18). The PCR products were detected using the ABI 7700 Sequence Detection System with the dsDNA-specific fluorescent dye SYBR Green I (Applied Biosystems, Foster City, CA). The −4953A > G polymorphism was typed using TaqMan assay (Applied Biosystems).

Statistical Analysis

The association of the *CHRM1* gene polymorphism was measured by odds ratio (OR) with 95% confidence intervals (CI) as estimates of relative risk for the development of asthma. We used the Hardy-Weinberg equilibrium (HWE) program (21) to compare observed numbers of genotypes with the numbers of genotypes expected under HWE. For haplotype analyses, we used the Haplo.score program, which adjusts for covariates and calculates simulation p values for each haplotype (22).

Luciferase Reporter Gene Assay

We constructed two promoter reporter plasmids by placing two haplotypes (−9697C/−4953G and −9697T/−4953A) into the pGL3-Basic vector. Human neuroblastoma IMR32 cells (1×10^6) were transiently transfected with 9.5 μg of one of the two constructs and 0.5 μg of the pRL-TK vector, an internal control for transfection efficiency. After 24 h, we measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Tokyo, Japan). Results were expressed as means ± SEM and were compared by paired t test.

Electrophoretic Mobility Shift Assay

Transcription factor (nuclear factor [NF]-κB or upstream stimulating factor [USF]-1)-DNA binding activity was analyzed using the electrophoretic mobility shift assay (EMSA) kit (Panomics, Redwood, CA), according to the manufacturer's instructions.

RESULTS

Characteristics of the 333 healthy control subjects and 326 subjects with asthma are shown in Table 1. The median age of subjects with asthma was significantly higher than in healthy control subjects ($p < 0.0001$). There were significantly more females in the asthma group than in the control group ($p < 0.0001$). Subjects with asthma were more likely to be atopic and had higher levels of total serum IgE than did healthy control subjects (χ^2 test or analysis of variance, $p < 0.0001$). More than 50% of the control subjects were atopic, which is consistent with

TABLE 2. COMPARISONS OF ALLELE AND GENOTYPE FREQUENCIES OF EIGHT *CHRM1* SINGLE-NUCLEOTIDE POLYMORPHISMS BETWEEN PATIENTS WITH ASTHMA AND CONTROL SUBJECTS

SNP	Allele/Genotype	HC n (%)	BA n (%)	p Value*
−18379 (rs1938677)	G	361 (54.5)	335 (48.1)	0.33
	A	301 (45.5)	311 (51.9)	
	GG	107 (32.3)	92 (28.5)	0.56
	GA	146 (44.1)	151 (46.7)	
	AA	78 (23.6)	80 (24.8)	
−9697 (rs2075748)	C	508 (76.3)	533 (81.7)	0.015
	T	158 (23.7)	119 (18.3)	
	CC	195 (58.6)	216 (66.3)	0.039
	CT	118 (35.4)	101 (31.0)	
	TT	20 (6.0)	9 (2.7)	
−6965 (rs542269)	T	491 (73.9)	472 (73.1)	0.72
	C	173 (26.1)	174 (26.9)	
	TT	184 (55.4)	175 (54.2)	0.94
	TC	123 (37.1)	122 (37.8)	
	CC	25 (7.5)	26 (8.0)	
−4953 (rs1942499)	A	477 (71.6)	434 (66.6)	0.047
	G	189 (28.4)	218 (33.4)	
	AA	174 (52.3)	147 (45.1)	0.15
	AG	129 (38.7)	140 (42.9)	
	GG	30 (9.0)	39 (12.0)	
+1353 (rs2067480)	C	615 (92.3)	608 (93.3)	0.52
	T	51 (7.7)	44 (6.7)	
	CC	284 (85.3)	287 (88.0)	0.19
	CT	47 (14.1)	34 (10.5)	
	TT	2 (0.6)	5 (1.5)	
+3970 (rs4963323)	C	533 (80.3)	524 (80.9)	0.78
	G	131 (19.7)	124 (19.1)	
	CC	216 (65.1)	211 (65.1)	0.75
	CG	101 (30.4)	102 (31.5)	
	GG	15 (4.5)	11 (3.4)	
+5418 (rs11601597)	C	414 (62.3)	385 (59.6)	0.31
	G	250 (37.7)	261 (40.4)	
	CC	132 (39.7)	113 (35.0)	0.44
	CG	150 (45.2)	159 (49.2)	
	GG	50 (15.1)	51 (15.8)	
+5455 (rs11605665)	G	538 (81.5)	496 (77.5)	0.074
	T	122 (18.5)	144 (22.5)	
	GG	218 (66.1)	192 (60.0)	0.19
	GT	102 (30.9)	112 (35.0)	
	TT	10 (3.0)	16 (5.0)	

Definition of abbreviations: BA = bronchial asthma; HC = healthy controls.
* χ^2 Test.

recent findings that the prevalence of atopy (as indicated by specific IgE against common inhaled allergens) among Japanese subjects is increasing (23, 24). Prebronchodilator baseline FEV_1 at an initial visit to our hospital was examined for 293 subjects with asthma (89.9%), and improvement of FEV_1 after bronchodilator therapy (400 μg salbutamol) or after a course of standard asthma medications (inhaled corticosteroids, long-acting β_2-agonists, theophylline, or leukotriene modulators) was recorded for 214 (65.6%) subjects with asthma (Table 1).

All eight of the SNPs investigated were within the HWE in the control group ($p > 0.05$). The overall success rate for genotyping was 99.6%. Of the eight SNPs, two common SNPs (−9697C > T [rs2075748] and −4953A > G [rs1942499]) in the regulatory region of the *CHRM1* gene had a significant association with asthma (Table 2). Both of these SNPs were significantly associated with asthma when the analysis was adjusted for age, sex, smoking status, and atopic status (Table 3). The OR for the TT homozygotes of the −9697C > T polymorphism was 0.29 compared with the CC homozygotes (95% CI, 0.12–0.73; $p = 0.008$), and the OR for the GG homozygotes of the −4953A > G polymorphism was 1.86 compared with the AA homozygotes (95% CI, 1.04–3.34; $p = 0.038$).

We analyzed data from the eight SNPs with the Haploview program (25) and identified two haplotype blocks (Figure 1) in our case-control population. Haplotype block I comprised three SNPs in the regulatory region (−9697C > T [rs2075748], −6965T > C [rs542269], −4953A > G [rs1942499]), and haplotype block II comprised three SNPs (+1353C > T [rs2067480], +3970C > G [rs4963323], +5418C > G [rs11601597]) in the coding exon and the 3′-UTR. Haplotype analyses were performed in both blocks I and II. The frequency of *CHRM1* haplotypes is shown in Table 4. In block I, the −9697T/−6965T/−4953A haplotype was associated with a significantly lower risk of asthma ($p = 0.00055$) and the −9697C/−6965T/−4953G haplotype was associated with a significantly increased risk of asthma ($p = 0.020$). Inspection of specific haplotypes revealed that this association is most likely due to 9697C > T and 4953A > G, because the same allele for −6965T > C is part of both risk and protective haplotypes. In contrast, none of the haplotypes in block II was associated with asthma.

In the case-only study, associations between asthma-related phenotypes, such as total serum IgE levels and atopy, and the polymorphisms of *CHRM1* were also investigated. We could not find any significant association between the genotypes of the eight SNPs and total serum IgE levels or atopy (*see* Tables E1 and E2 in the online supplement).

The transcriptional activity of the *CHRM1* SNPs at the regulatory region was compared between the −9697C/−4953G haplotype and the −9697T/−4953A haplotype transiently transfected into human neuroblastoma IMR32 cells. Luciferase activity in cell extracts was assessed 24 h after transfection, and was expressed as fold increase in the activity of the *CHRM1* reporter constructs compared with the pRL-TK vector. Figure 2 shows that the reporter plasmid carrying the −9697T/−4953A promoter displayed 37% lower transcriptional activity compared with the plasmid carrying the −9697C/−4953G promoter ($p = 0.019$).

TABLE 3. GENETIC IMPACT ON ASTHMA OF EIGHT SINGLE-NUCLEOTIDE POLYMORPHISMS IN AND AROUND THE CHRM1 *GENE*

		OR (95% CI)	
SNP	Genotype	Adjustments (−)	Adjustments (+)*
18379G > A (rs1938677)	GG	1 (Reference)	1 (Reference)
	GA	1.20 (0.84–1.72)	1.14 (0.77–1.71)
	AA	1.19 (0.79–1.81)	1.33 (0.84–2.12)
−9697C > T (rs2075748)	CC	1 (Reference)	1 (Reference)
	CT	0.77 (0.56–1.07)	0.73 (0.51–1.06)
	TT	0.41 (0.18–0.91)†	0.29 (0.12–0.73)†
6965T > C (rs542269)	TT	1 (Reference)	1 (Reference)
	TC	1.04 (0.75–1.44)	1.00 (0.70–1.44)
	CC	1.09 (0.61–1.97)	1.04 (0.54–2.02)
−4953A > G (rs1942499)	AA	1 (Reference)	1 (Reference)
	AG	1.28 (0.93–1.78)	1.38 (0.96–1.98)
	GG	1.54 (0.91–2.60)	1.86 (1.04–3.34)†
+1353C > T (rs2067480)	CC	1 (Reference)	1 (Reference)
	CT	0.72 (0.45–1.15)	0.61 (0.37–1.02)
	TT	2.47 (0.48–12.9)	1.96 (0.31–12.5)
+3970C > G (rs4963323)	CC	1 (Reference)	1 (Reference)
	CG	1.03 (0.74–1.44)	1.12 (0.78–1.62)
	GG	0.75 (0.34–1.67)	0.78 (0.33–1.86)
+5418C > G (rs11601597)	CC	1 (Reference)	1 (Reference)
	CG	1.24 (0.88–1.73)	1.29 (0.89–1.88)
	GG	1.19 (0.75–1.89)	1.16 (0.70–1.93)
+5455G > T (rs11605665)	GG	1 (Reference)	1 (Reference)
	GT	1.25 (0.90–1.74)	1.28 (0.89–1.85)
	TT	1.82 (0.81–4.1)	1.73 (0.73–4.09)

Definition of abbreviations: CI = confidence interval; OR = odds ratio.

* Adjustment for matching factors and potential confounding factors, including sex, age, smoking status, and atopic status, was performed by unconditional logistic regression analysis.

† $p < 0.05$.

‡ $p = 0.01$.

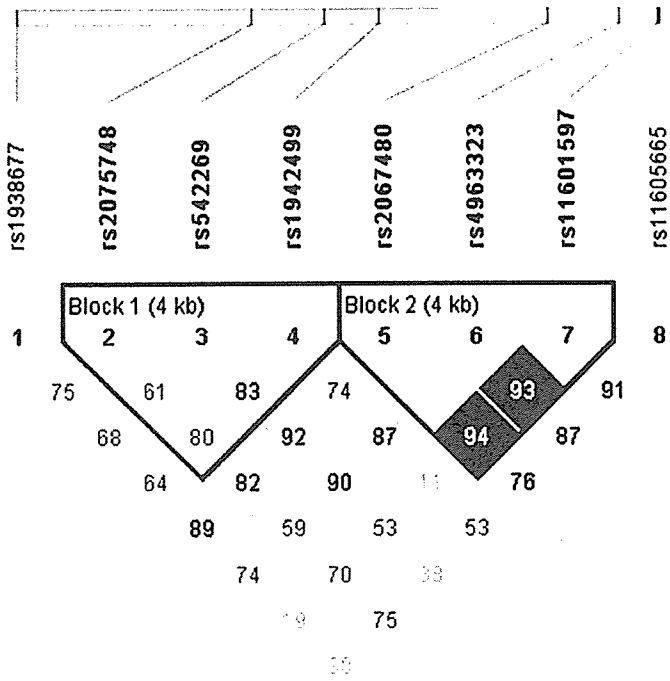

Figure 1. Locations and linkage disequilibrium (LD) map structure of single-nucleotide polymorphisms (SNPs) in and around the *CHRM1* Haploview plot shows pairwise LD (D' values) for 8 SNPs based on genotypes of 659 individuals of the case-control study. The eight SNPs include −18379G > A [rs1938677], −9697C > T [rs2075748], −6965T > C [rs542269], −4953A > G [rs1942499], +1353C > T [rs2067480], +3970C > G [rs4963323], +5418C > G [rs11601597], and +5455G > T [rs11605665]. LD blocks are framed in *black* and were classified according to the "solid spine" option (25). Each *square* plots the level of LD (D' values) between a pair of SNPs.

This difference in transcriptional activity was consistent in eight independent experiments.

EMSA failed to show a robust difference in binding affinity of NF-κB to the −9697T or the −9697C allele, or in binding affinity of USF-1 to the −4953A or the −4953G allele (data not shown).

DISCUSSION

Given a high *a priori* biological plausibility for asthma, we tested the hypothesis that the allelic variants at the regulatory region of the *CHRM1* confer susceptibility to asthma by conducting a case-control study in a relatively large population of unrelated Japanese subjects. In accordance with our primary hypothesis, we found that the presence of the −9697CC genotype, −4953GG genotype, or the −9697C/−4953G haplotype at the regulatory region was significantly associated with a diagnosis of asthma. Our genetic association study had several strengths: first, muscarinic receptors, including M1, have been biologically implicated in the pathogenesis of asthma; second, the gene encoding the CHRM1 is located on chromosome 11q13, a genomic region

TABLE 4. ESTIMATED HAPLOTYPE FREQUENCIES OF THE *CHRM1* GENE POLYMORPHISMS

				Haplotype Frequency			
				Control (*n* = 333)	Asthma (*n* = 326)	Haplotype-specific Score	p Value* (*Empirical*)
Block I							
Haplotype	−9697/	−6965/	−4953				
1	T	T	A	0.207	0.158	−3.03	0.00055
2	C	C	A	0.239	0.234	−0.23	0.827
3	C	T	A	0.250	0.254	0.057	0.959
4	C	T	G	0.268	0.303	2.15	0.020
Block II							
Haplotype	+1353/	+3970/	+5418				
1	T	C	C	0.371	0.391	0.120	0.131
2	C	C	C	0.353	0.349	0.808	0.814
3	C	G	C	0.192	0.179	0.926	0.918
4	C	C	G	0.371	0.391	0.454	0.481

Haplotype frequencies were estimated using the Haplo.Stats program. In Block I (regulatory region of the gene), haplotype analyses showed that the −9697T/−6965T/−4953A haplotype was associated with a significantly lower risk of asthma (p = 0.00055) and the −9697C/−6965T/−4953G haplotype was associated with a significantly increased risk of asthma (p = 0.020). In contrast, in Block II, none of haplotypes showed a significant association with asthma. Note that haplotype-specific scores give effect estimates; negative haplotype-specific scores are associated with a protective effect, and positive haplotype-specific scores are associated with an increased risk. Haplotypes with frequencies less than 0.05 were excluded from the analyses.

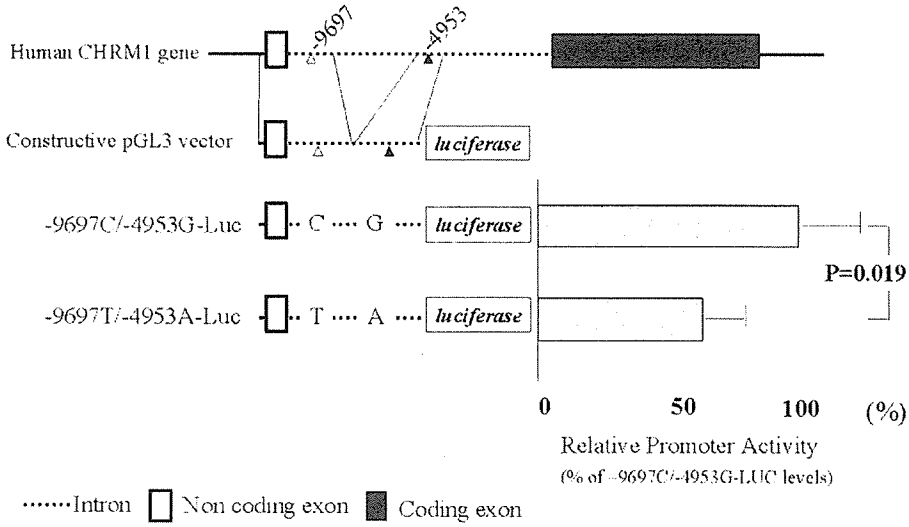

Figure 2. Transcription activity analysis of the promoter region of *CHRM1* IMR32 cells were transiently transfected with 9.5 μg of *CHRM1* luciferase reporter constructs (schematically depicted to the *left*) plus 0.5 μg of pRL-TK vector. Luciferase activities were normalized against the internal control Renilla values. The data represent means ± SD for the entire dataset (four independent experiments, each in duplicate), and are expressed as a percentage of the −9697C/−4953G LUC activity. The difference between these constructs was significant at p = 0.019 (paired *t* test; n = 8).

linked to the diagnosis of asthma and atopy in several genome-wide scans (14–17); third, *in vitro* functional analyses have shown that the haplotype at the regulatory region has an effect on a basal promoter function in IMR32 cells, with the −9697T/4953A haplotype associated with 37% decreased promoter activity compared with the −9697C/−4953G haplotype. Accordingly, our data suggest that the −9697/−4953 haplotype may influence the affinity of a particular nuclear protein to the regulatory region of the *CHRM1* gene, resulting in altered transcriptional activity and ultimately leading to a higher or lower risk of asthma.

Although the exact mechanisms underlying the involvement of the *CHRM1* gene in the pathogenesis of asthma remain to be identified, several reports indicate that the cholinergic pathway has an important role in the pathogenesis of asthma, in particular in the regulation of bronchoconstriction, airway inflammation, and airway remodeling. An M1 receptor-dependent pathway counteracts cholinergic bronchoconstriction, possibly via the release of a relaxing agent (8); both respiratory epithelia and sympathetic nerve terminals within bronchial smooth muscle are equipped with M1 receptors (7, 26) and releasable bronchodilating agents, such as nitric oxide and prostaglandin E_2 (27). Studies with the M1 receptor–preferring antagonist, pirenzepine, have also suggested the existence of pulmonary M1 receptors modulating airway diameter (28). Furthermore, Jones and colleagues (29) demonstrated that stable expression in RBL-2H3 mast cells of the M1 muscarinic acetylcholine receptor leads to carbachol-stimulated mast cell degranulation. An animal model of asthma showed that anticholinergic agents protect against allergen-induced airway remodeling (30). Together with these *in vivo* and *in vitro* findings, our findings support the contention that CHRM1 plays an important role in the pathogenesis of asthma. Our findings may be of considerable relevance to asthma treatment, providing an important basis for identification of individuals for whom the cholinergic pathway could be targeted.

Sequence analysis indicated that the T allele at the −9697C > T polymorphism creates a potential NF-κB binding site and that the A allele at the −4953A > G polymorphism creates a potential USF-1 transcription factor binding site by reference to the MatInspector or TFSEARCH database (31). We, however, failed to see any difference in binding intensities of these nuclear factors to the −9697C > T or −4953A > G polymorphism. Therefore, we cannot exclude the possibility that these SNPs might not be causative in nature, but are in LD with a true susceptibility allele in the regulatory region of the *CHRM1* gene. Population stratification may influence the observed associations (32). However, our population is racially homogeneous, as all subjects recruited in the study were from the Japanese population, which is considered monoracial; thus, our subjects had a relatively low risk of population stratification effects. Furthermore, we recruited all participants in the current study from a single institute to minimize the chance of mixing populations with inherently diverse allele frequencies of a susceptibility gene. In addition, all SNPs were in HWE in a set of unrelated healthy subjects. Therefore, we believe that the usual problems associated with population stratification may be of limited importance in the present study. Nevertheless, we acknowledge that population stratification may have influenced the present findings, and that the findings of the current study are preliminary and do not, by themselves, conclusively confirm an etiologic relationship. A more comprehensive approach that examines the functional consequences of the *CHRM1* promoter polymorphisms and identifies the possible promoter-dependent mechanism for an association between *CHRM1* and asthma is required.

In conclusion, given the important role of muscarinic cholinergic mechanisms in pulmonary disease, this case–control study, together with an *in vitro* functional analysis, suggests that the *CHRM1* gene is an important susceptibility locus for asthma at chromosome 11q13. The −9697/−4953 haplotype at the regulatory region of the gene may contribute to the development of asthma by altering the human lung muscarinic receptor system in ways that could account for the increased *in vivo* lung cholinergic hyperresponsiveness found in patients with asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank all of the subjects of this study for their participation. They also thank Yoshiko Obata and Tomoko Akiyama for their excellent technical assistance, and Takeshi Sawazaki at the Pharmaceutical Research Laboratory, Hitachi Chemical Co., Ltd., for kindly measuring Ag-specific IgE antibody levels (MAST).

References

1. Barnes PJ. Neural control of human airways in health and disease. *Am Rev Respir Dis* 1986;134:1289–1314.

2. Watson N, Magnussen H, Robe KF. Pharmacological characterization of the muscarinic receptor subtype mediating contraction of human peripheral airway. *J Pharmacol Exp Ther* 1995;274:1293–1297.
3. Jacoby DB, Fryer AD. Anticholinergic therapy for airway diseases. *Life Sci* 2001;68:2565–2572.
4. Zaagsma J, Meurs H, Roffel AF. Muscarinic receptors in airways diseases. Basel, Switzerland: Birkhäuser Verlag; 2001.
5. Bloom JW, Yamaura HI, Baumgartener C, Halonen M. A muscarinic receptor with high affinity for pirenzepine mediates vagally induced bronchoconstriction. *Eur J Pharmacol* 1987;133:21–27.
6. Lammers JW, Minette P, McCusker M, Barnes PJ. The role of pirenzepine-sensitive (M1) muscarinic receptors in vagally mediated bronchoconstriction in humans. *Am Rev Respir Dis* 1989;139:446–449.
7. Maclagan J, Fryer AD, Faulkner D. Identification of M1 muscarinic receptors in pulmonary sympathetic nerves in the guinea-pig by use of pirenzepine. *Br J Pharmacol* 1989;97:499–505.
8. Struckmann N, Schwering S, Wiegand S, Gschnell A, Yamada M, Kummer W, Wess J, Haberberger RV. Role of muscarinic receptor subtypes in the constriction of peripheral airways: studies on receptor-deficient mice. *Mol Pharmacol* 2003;64:1444–1451.
9. Reinheimer T, Mohlig T, Zimmermann S, Hohle KD, Wessler I. Muscarinic control of histamine release from airways: inhibitory M1-receptors in human bronchi but absence in rat trachea. *Am J Respir Crit Care Med* 2000;162:534–538.
10. Metzen J, Bittinger F, Kirkpatrick CJ, Kilbinger H, Wessler I. Proliferative effect of acetylcholine on rat trachea epithelial cells is mediated by nicotinic receptors and muscarinic receptors of the M1-subtype. *Life Sci* 2003;72:2075–2080.
11. Koyama S, Rennard SI, Robbins RA. Acetylcholine stimulates bronchial epithelial cells to release neutrophil and monocyte chemotactic activity. *Am J Physiol* 1992;262:L466–L471.
12. Walch L, Gascard JP, Dulmet E, Brink C, Norel X. Evidence for a M1 muscarinic receptor on the endothelium of human pulmonary veins. *Br J Pharmacol* 2000;130:73–78.
13. Yang CM, Farley JM, Dwyer TM. Muscarinic stimulation of submucosal glands in swine trachea. *J Appl Physiol* 1988;64:200–209.
14. Daniels SE, Bhattacharrya S, James A, Leaves NI, Young A, Hill MR, Faux JA, Ryan GF, le Souef PN, Lathrop GM, *et al.* A genome-wide search for quantitative trait loci underlying asthma. *Nature* 1996;383:247–250.
15. Los H, Koppelman GH, Postma DS. The importance of genetic influences in asthma. *Eur Respir J* 1999;14:1210–1227.
16. Malerba G, Pignatti PF. A review of asthma genetics: gene expression studies and recent candidates. *J Appl Genet* 2005;46:93–104.
17. Hizawa N, Yamaguchi E, Furuya K, Ohnuma N, Kodama N, Kojima J, Ohe M, Kawakami Y. Association between high serum total IgE levels and D11S97 on chromosome 11q13 in Japanese subjects. *J Med Genet* 1995;32:363–369.
18. Hizawa N, Yamaguchi E, Takahashi D, Nishihira J, Nishimura M. Functional polymorphisms in the promoter region of macrophage migration inhibitory factor and atopy. *Am J Respir Crit Care Med* 2004;169:1014–1018.
19. Liao DL, Hong CJ, Chen HM, Chen YF, Lee SM, Chang CY, Chen H, Tsai SJ. Association of muscarinic M1 receptor genetic polymorphisms with psychiatric symptoms and cognitive function in schizophrenic patients. *Neuropsychobiology* 2003;48:72–76.
20. de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. *Nat Genet* 2005;37:1217–1223.
21. Terwillinger JD, Ott J. Handbook of human genetic linkage. Baltimore, MD: Johns Hopkins University Press; 1994.
22. Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet* 2002;70:425–434.
23. Nakagomi T, Itaya H, Tominaga T, Yamaki M, Hisamatsu S, Nakagomi O. Is atopy increasing? *Lancet* 1994;343:121–122.
24. Kusunoki T, Hosoi S, Asai K, Harazaki M, Furusho K. Relationships between atopy and lung function: results from a sample of one hundred medical students in Japan. *Ann Allergy Asthma Immunol* 1999;83:343–347.
25. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263–265.
26. Shapiro MS, Gomeza J, Hamilton SE, Hille B, Loose MD, Nathanson NM, Roche JP, Wess J. Identification of subtypes of muscarinic receptors that regulate Ca^{2+} and K^+ channel activity in sympathetic neurons. *J Life Sci* 2001;68:2481–2487.
27. Spicuzza L, Basile L, Belvisi MG, Bellofiore S, Matera MG, Cazzola M, Di Maria GU. The protective role of epithelium-derived nitric oxide in isolated bovine trachea. *Pulm Pharmacol Ther* 2002;15:357–362.
28. Bloom JW, Baumgartener-Folkerts C, Palmer JD, Yamamura HI, Halonen M. A muscarinic receptor subtype modulates vagally stimulated bronchial contraction. *J Appl Physiol* 1988;65:2144–2150.
29. Jones SV, Choi OH, Beaven MA. Carbachol induces secretion in a mast cell line (RBL-2H3) transfected with the m1 muscarinic receptor gene. *FEBS Lett* 1991;289:47–50.
30. Gosens R, Bos IS, Zaagsma J, Meurs H. Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. *Am J Respir Crit Care Med* 2005;171:1096–1102.
31. Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 1995;23:4878–4884.
32. Freedman ML, Reich D, Penney KL, McDonald GJ, Mignault AA, Patterson N, Gabriel SB, Topol EJ, Smoller JW, Pato CN, *et al.* Assessing the impact of population stratification on genetic association studies. *Nat Genet* 2004;36:388–393.

천식 및 알레르기 제 26 권 제 3 호
Vol. 26, No. 3, Sep., 2006

□ Review Article □

Management of Food Allergy: "Food Allergy Management Guideline 2005" by National Food Allergy Research Group Supported by the Ministry of Health, Welfare, and Labor

Motohiro Ebisawa

Department of Allergy Clinical Research Center for Allergology and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Kanagawa, Japan

In October 2005, we released "Food Allergy Management Guideline 2005". To cover food allergy from infancy to adulthood, the project committee included not only pediatric researchers, but also internists, dermatologists, and otolaryngologists. The guideline concept was to utilize the data accumulated by the National Food Allergy Research Group, to be plain and as short as a pamphlet, and to be released on internet. The most glowing argument was about relation between infantile atopic dermatitis and food allergy, and how it should be treated in the guideline. To avoid neither overvaluation nor undervaluation, fastidious care was given to the denotation. With the definition of "infantile atopic dermatitis associated with food allergy", both dermatologic and pediatric members of the project committee finally came to agreement, which was a landmark between dermatologists and pediatricians in Japan. The guideline explains fundamentals with the least paragraphs and with tables and figures as many as possible. Flowcharts are made largely as a composition in the parts of diagnosis and treatment. I really hope that this guideline is useful for Korean doctors involved in food allergy and that quality of life of food allergy patients and their parents are improved. **(Korean J Asthma Allergy Clin Immunol 2006;26:177-185)**

Key words: Anaphylaxis, Food allergy, Food provocation test, Referral relationship

INTRODUCTION

In October 2005, we released on internet "Food Allergy Management Guideline 2005",[1] the most important subject we had worked on in the course of "the research on determination of causative agent (allergen) for anaphylaxis caused by food and other factors, and on establishment of its prevention and prognosis" (principal investigator: Motohiro Ebisawa, M.D., Ph.D., 3 years project since 2003), which is the national research project on prevention and treatment of immunological and allergic diseases, supported by the Ministry of Health, Welfare, and Labor by Japanese government.

The guideline was created for general physicians to improve diagnosis and treatment of food allergy and for food-allergy patients to improve their quality of life. Its concept was decided in 2003 that it should utilize the data accumulated by the Food Allergy Research Group supported by the Ministry of Health, Welfare, and Labor, that it should be plain and as short as a pamphlet, and that it should be released on internet so that anyone is free to download it. In 2004 the draft was written by staffs of the Department of Pediatric Allergy, National Hospital Organization, Sagamihara National Hospital, and then in January 2005 it was discussed for three hours in the public symposium held at the 5th Workshop on Food Allergy, (which was combined with the meeting of the Food Allergy Research Group supported by the Ministry of Health, Welfare, and Labor.) To cover food allergy from infancy to adulthood, the project committee included not only pediatric researchers, but also internists, dermatologists, and otolaryngologists (Table 1). The guideline finally came to completion through three times tough arguments for about 10 hours altogether in June, July and September 2005, and through further communication by e-mail.

The most glowing argument was about relation between infantile atopic dermatitis and food allergy, and how it should be

Correspondence : Motohiro Ebisawa, Department of Allergy, Clinical Research Center for Allergology and Rheumatology, National Hospital Organization, Sagamihara National Hospital, 18-1, Sakuradai, Sagamihara, Kanagawa 228-8522, Japan. Tel: +81-42-742-8311, Fax: +81-42-742-5314, E-mail: m-ebisawa@sagamihara-hosp.gr.jp

Table 1. "Food Allergy Management Guideline 2005" Project Committee

(Principal investigator)	
Motohiro Ebisawa	Department of Allergy, National Hospital Organization, Sagamihara National Hospital, Clinical Research Center for Allergology and Rheumatology
(Pediatricians)	
Yukou Aihara	Yokohama City University Municipal General Medical Center, Pediatric General Medical Center
Akira Akasawa	General Medical Care Department, National Center for Child Health and Development, Childhood Care Service
Setsuko Ito	Department of Food Studies and Nutrition, Doshisha Women's College of Liberal Arts
Atsuo Urisu	Department of Pediatrics, Fujita Health University Banbuntane Hotokukai Hospital
Naomi Kondo	Department of Pediatrics, Gifu University School of Medicine
Rumiko Shibata	Department of Pediatrics, National Hospital Organization Fukuoka National Hospital
Mitsuhumi Mayumi	Department of Pediatrics, Fukui University School of Medicine
Hiroshi Tachimoto	Department of Pediatrics, National Hospital Organization, Sagamihara National Hospital
Takanori Imai	Department of Pediatrics, National Hospital Organization, Sagamihara National Hospital
(Otolaryngologists)	
Kimihiro Okubo	Department of Otolaryngology, Nippon Medical School
(Internists)	
Kazuo Akiyama	Clinical Research Center for Allergoloy and Rheumatology, National Hospital Organization, Sagamihara National Hospital
Naohito Suzuki	Department of Allergy and Respiratory Disease, Fraternity Memorial Hospital,
Takemasa Nakagawa	Department of Internal Medicine, St. Mariannna University School of Medicine
(Dermatologists)	
Zenro Ikezawa	Department of EnvironmentalImmunopathology and Dermatology, Yokohama City University School of Medicine
Masutaka Furue	Department of Dermatology, Kyushu University Graduate School of Medicine
(Collaborator for document preparation)	
Chizuko Sugizaki	Department of Allergym, Clinical Research Center for Allergoloy and Rheumatology, National Hospital Organization, Sagamihara National Hospital,

treated in the guideline. The point whether to admit dermatitis as a symptom of food allergy or not, was on focus. The committee members of dermatologists insisted that generally eczema can not be developed by the allergic stimuli absorbed through digestive system. In contrast, the committee members of pediatricians insisted that infantile food allergy can be diagnosed in the subjects among infantile atopic dermatitis. The committee members from pediatrics explained the onset situation of infantile food allergy to the members from dermatology in order to achieve agreement.[2] The guideline tried to include clinical classifications of types of food allergy as it was thought out to place emphasis basically on comprehensibility.

Another big argument was about definition of "complicated cases of infant atopic dermatitis and food allergy." To avoid neither overvaluation nor undervaluation, fastidious care was given to the denotation. With the definition of "infantile atopic dermatitis associated with food allergy", both dermatologic and pediatric members of the project committee finally came to agreement, which was a landmark between dermatologists and pediatricians in Japan.

The guideline explains fundamentals with the least paragraphs and with tables and figures as many as possible. Flowcharts are made the most of as a composition in the parts of diagnosis and treatment. In the following sections, I explain summary of the guideline using actual tables, figures and flowcharts.

THE GENERAL

1. Definition

It is based on food allergy committee report from the Japanese Pediatric Allergy Society.[3] In that report, food allergy is defined as "Phenomenon of harmful symptoms (on skin, in the mucosa, in the digestive system, in the respiratory system or anaphylaxis) triggered by immunologic mechanisms after consuming a causative food. (not including food poisoning, nor response to toxic foods, nor food intolerance; pseudo-allergen, enzyme disorders)."