0 pg/ml   500 pg/ml   1000 pg/ml

Fig. 2 Detection of fluorescence-linked beads in the wells with various concentrations of hIFN-γ. The intensity of the bead-bound fluorescence increased according to the hIFN-γ concentration.
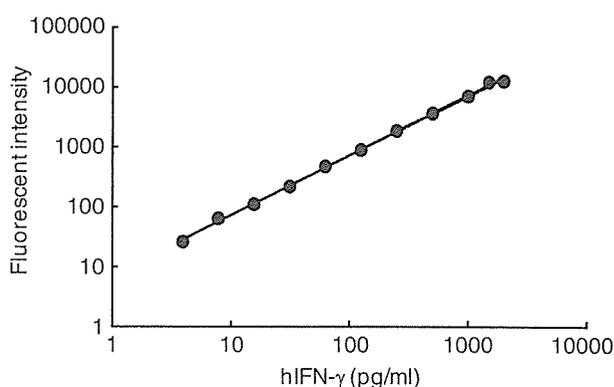

Fig. 3 The standard curve for hIFN-γ obtained with FLISA. The linear dynamic range for hIFN-γ was 3.9–1,500 ng/ml. Each data point represents the average of triplicate assays.

into a 96-well plate. 50 μl of beads mixture (antibody beadss : allophycocyanin (APC) conjugated mouse anti-hIFN-γ mAb (554702, Pharmingen, California, USA)s:FLISA buffer (PBS, 1% BSA, 0.35 M NaCl, 0.1% Tween-20, 0.01% NaN3) =100:6.4:6500) was then mixed with the sample or standard in each well and incubated for 4 hours overnight at room temperature in the dark. After incubation, the 96-well plate was scanned using the FMAT® scanner. The average fluorescence per bead was recorded.

RESULTS

FMAT® 8100 HTS includes a bead-based assay system and is designed to negate the unbound fluorescent dye-labeled antibody. At measurement, FMAT® scans for fluorescent-bound beads resting on the bottom of multiwell trays (Fig. 1). No beads are found in the upper portions of the wells and non-specific fluorescence is not involved in background fluorescence intensity. In the lower 100 μm of the wells, the signals of unbound fluorescent dye-labeled antibody are deleted due to the limited size and form of the beads. Actually, the fluorescence intensity of negative con-

trol wells is 31.6 + / − 2.1 and that of the wells, which have 3.9 pg/ml of the minimum IFN-γ is 77.2 + / − 4.9. This is an obvious difference between negative control wells and minimum IFN-γ wells.

A standard solution of hIFN-γ was two-fold serially diluted in RPMI-1640 + 10% FCS. The intensity of the bead-bound fluorescence increased according to hIFN-γ concentration (Fig. 2). A standard curve for hIFN-γ was generated using the FMAT® scanner (Fig. 3). The linear dynamic range of hIFN-γ by FLISA was 3.9–1500 pg/ml. These results indicate that our system can recognize minute changes of hIFN-γ values. With previously established methods for IL-6 and IL-8, the linear dynamic ranges were 15.6–1000 pg/ml and 15.6–2000 pg/ml, respectively. Our FLISA system has an equivalent dynamic range comparable to these results,[9] a range that is sufficient to be applied in clinical use.

To test whether FLISA could accurately quantify hIFN-γ in a biological sample, inhibition assays of hIL-18 were performed.[15,16] We measured about 150 samples using FLISA and about 90 samples using ELISA. Sample preparation time of FLISA was equal to that of ELISA. hIFN-γ produced by KG-1 cells was measured using ELISA and FLISA (Figs. 4A, 4B). hIL-18 was neutralized by anti-hIL-18mAb or IL-18BP before stimulation. The IC50s for anti-hIL-18mAb and hIL-18BP using FLISA were 19.7 ng/ml and 5.2 ng/ml, respectively (Fig. 4B). Similarly, those using ELISA were estimated as 27 ng/ml and 5.4 ng/ml, respectively (Fig. 4A). However, as shown in Figure 4, the results of ELISA did not show a regression curve suitable for estimating precise IC50 values. The means of the coefficient of variation using the same samples measured by ELISA and FLISA were respectively 11.1% and 3.8%, suggesting that the edge effect often giving non-specific results may be smaller in FLISA than in ELISA.

Our proposed FLISA method was modified to use a combination of capture antibody and fluorescent dye-labeled detection antibody. As shown in Figure 5,
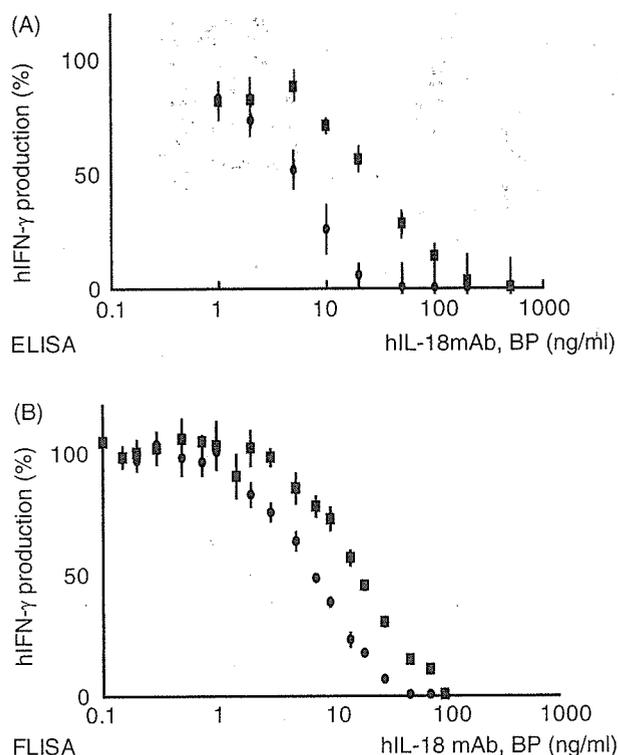
(A)

(B)

Fig. 4 Inhibition assay of hIL-18 by hIL-18BP or anti-hIL-18 mAb. hIL-18 (final concentration 2 ng/ml) was incubated with two-fold dilutions of anti-hIL-18 mAb or hIL-18BP for 1 hour at 37°C. After this incubation, the mixtures were added to KG-1 cells, and after 24 hours hIFN-γ was measured. The stimulation control of hIL-18 (2 ng/ml) was set at 100%, and the percent change was calculated for each concentration of anti-hIL-18mAb or hIL-18BP. Each data point represents an average of triplicate assays. **(A)** The inhibition curve measured by ELISA. **(B)** The inhibition curve measured by FLISA. The solid circle shows the inhibition of hIL-18 by hIL-18BP. The solid square shows the inhibition of hIL-18 by anti-hIL-18 mAb.

streptavidin beads and a biotinylated mouse anti-hIFN-γ mAb were used as IFN-γ capture particles and APC conjugated mouse anti-hIFN-γ mAb was used as the IFN-γ detection reagent. Previously, goat anti-mouse IgG (Fc) beads and mouse anti-hIFN-γ mAb have been used as the IFN-γ capture particles and Cy 5.5 conjugated mouse anti-hIFN-γ mAb as the IFN-γ detection reagent. Figure 6 shows our standard curve for IFN-γ estimated IFN-γ values in detail compared with that from the previously proposed FLISA method.

DISCUSSION

When measuring various cytokines, the effect of the environmental temperature is one of the factors influencing inter-experimental accuracy. ELISA requires washing and application of a detection antibody fol-
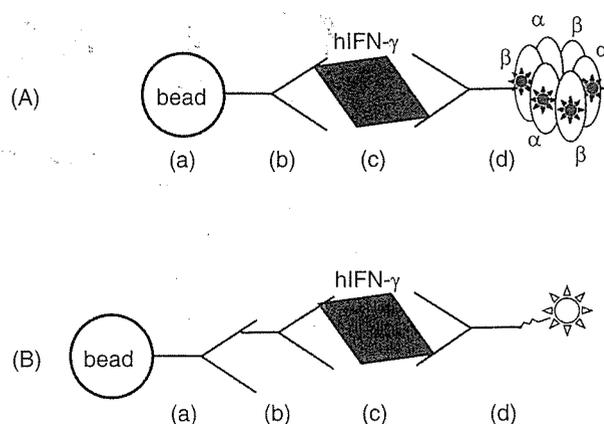

Fig. 5 Schematic diagram of our FLISA method **(A)** and a previous method **(B)**. **(A)**: **(a)** streptavidin beads. **(b)** biotinylated mouse monoclonal anti-hIFN-γ mAb. **(c)** hIFN-γ sample. **(d)** APC conjugated mouse monoclonal anti-hIFN-γ mAb beads. **(B)**: **(a)** goat anti-mouse IgG(Fc) beads. **(b)** mouse anti-hIFN-γ mAb. **(c)** hIFN-γ sample. **(d)** Cy5.5 conjugated mouse anti-hIFN-γ mAb.
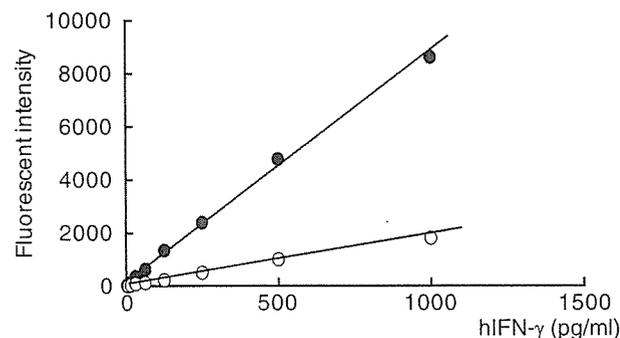

Fig. 6 The standard curve for hIFN-γ obtained with our newly proposed FLISA method (solid circles) and the previously reported FLISA method (open circles). The sensitivity of our method was about four-fold higher than that of the previously reported method.

lowing the first incubation, and washing and application of a detecting reagent following the second incubation. On the other hand, with FLISA, measurements can be performed following the first incubation. In all ELISA steps it is therefore very easy to expose the samples to environmental temperatures. The edge effect, which is the difference in temperature between the outer and inner wells, is therefore one of the factors resulting in inter-experimental errors.[17,18] Accurate screenings are very much required in working toward drug discoveries.[19,20]

We used two kinds of mouse hIFN-γ mAb in our FLISA method;one a capture antibody, the other an APC conjugated antibody as a detection antibody. Measurements of hIFN-γ in a previous report used a

52

goat anti-mouse IgG antibody as the bead-coating antibody and two kinds of mouse anti-hIFN-γ mAb, a capture antibody and a Cy5.5 conjugated antibody (Fig. 5).[21] The sensitivity of our proposed FLISA method was about four-fold higher than that of previous methods (Fig. 6). That is, the minimum unit for measurement in our method is four times smaller;0.5 pg/ml/fluorescence (FL) in the previous method by Komatsu et al., 0.125 pg/ml/FL in our method. Therefore, we were able to obtain detailed IFN-γ values. The sensitivity of the FLISA method proposed in this paper allowed the discrimination between differences.

The sensitivity of each method may depend on the structural difference between APC and Cy5.5. One APC molecule bounds to one antibody. The APC is a trimer that consists of α and β subunits $(\alpha\beta)_3$. Each subunit has one fluorophore;so as a result an APC conjugated antibody contains six fluorophores (Fig. 5A).[22] However, one Cy5.5 binds to one antibody, and the Cy5.5 is a single fluorophore itself. Thus an antibody conjugated Cy5.5 has one fluorophore (Fig. 5B). Although the fluorescent dye/antibody ratio of APC is equal to that of Cy5.5, an APC conjugated antibody has six times as many fluorophores as a Cy5.5 conjugated antibody. The difference in fluorescence intensity may also be a factor increasing sensitivity.

To quantify hIFN-γ in large amounts of sample, we developed a more efficient assay method than conventional ELISA, which can involve many incubation and washing steps, and requires large amounts of antibodies.[9] In contrast, our FLISA method is a homogeneous bead-based immunoassay that requires no wash steps. Over 100-fold less capture antibody is needed in FLISA than in conventional ELISA.[7] However, the most striking difference is the time required for the assay. In our study, the occupation time of the FLISA method was about 1 hour, demonstrating that FLISA can be performed in a much shorter time than conventional ELISA, which requires about 5 hours.[23] A minimal time requirement is a crucial factor for applications such as HTS in drug discovery research. FLISA is thus an attractive method as it involves less hands-on time and lower running costs.[21]

In immunological studies using FLISA, measurements of IL-6 and IL-8 and CD3+/CD4+ lymphocyte counts in whole blood have been reported.[9,24] The bead-based system of the FLISA is readily applicable to any other plate-based assay, such as non-radioactive kinase, phosphatase, and protease assays. A multiplexed bead-based receptor-ligand binding assay has already been demonstrated using FLISA.[25,26] In addition, FLISA is capable of detecting and quantifying fluorescence on live cells, allowing for such diverse assays as apoptosis and cytotoxicity, and cellular immunoassays, and receptor ligand binding assays.[25-28] The FLISA system can be adapted for HTS

of large libraries of chemical and natural products, thus having a place in any laboratory that routinely performs multiple, repetitive assays.

REFERENCES

1. Kelso A. Cytokines and their receptors;an overview. Ther. Drug Monit. 2000;**22**:40-43.
2. Zhu H, Yang J, Murphy TL et al. Unexpected characteristics of the IFN-gamma reporters in non-transformed T cells. J. Immunol. 2001;**167**:855-865.
3. Tran EH, Prince EN, Owens T. IFN-gamma shapes immune invasion of the central nervous system via regulation of chemokines. J. Immunol. 2000;**164**:2759-2768.
4. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu. Rev. Immunol. 1997; **15**:749-795.
5. Ohnishi H, Kato Z, Watanabe M et al. Interleukin-18 is associated with the severity of atopic dermatitis. Allergol. Int. 2003;**52**:123-130.
6. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. Cytokine Growth Factor Rev. 2001;**12**:53-72.
7. Shikano H, Kato Z, Kaneko H et al. IFN-gamma production in response to IL-18 or IL-12 stimulation by peripheral blood mononuclear cells of atopic patients. Clin. Exp. Allergy 2001;**31**:1263-1270.
8. Kondo N, Matsui E, Kaneko H et al. Genetic defects in downregulation of IgE production and a new genetic classification of atopy. Allergol. Int. 2004;**53**:77-85.
9. Swartzman EE, Miraglia SJ, Mellentin-Michelotti J, Evangelista L, Yuan PM. A homogeneous and multiplexed immunoassay or high-throughput screening using fluorometric microvolume assay technology. Anal. Biochem. 1999;**271**:143-151.
10. Kato Z, Jee J, Shikano H et al. The structure and binding mode of interleukin-18. Nat. Struct. Biol. 2003;**10**:966-971.
11. Omoya K, Kato Z, Matsukuma E et al. Systemic optimization of active protein expression using GFP as a folding reporter. Protein Expr. Purif. 2004;**36**:327-332.
12. Li A, Kato Z, Ohnishi H et al. Optimized gene synthesis and high expression of human interleukin-18. Protein Expr. Purifi. 2003;**32**:110-118.
13. Konishi K, Tanabe F, Taniguchi M et al. A simple and sensitive bioassay for the detection of human interleukin-18/interferon-γ-inducing factor using human myelomonocytic KG-1 cells. J. Immunol. Methods 1997;**209**:187-191.
14. Wu C, Sakorafas P, Miller R et al. IL-18 receptor beta-induced changes in the presentation of IL-18 binding sites affect ligand binding and signal transduction. J. Immunol. 2003;**170**:5571-5577.
15. Okamura H, Tsutsui H, Komatsu T et al. Cloning of a new cytokine that induces IFN-γ production by T-cells. Nature 1995;**378**:88-91.
16. Ushio S, Namba M, Okura T et al. Cloning of the cDNA for human IFN-γ-inducing factor, expression in Escherichia coli,and studies on the biologic actives of the protein. J. Immunol. 1996;**156**:4274-4279.
17. Oliver DG, Sanders AH, Hogg RD, Hellman JW. Thermal gradients in microtitration plates. Effects on enzyme-linked immunoassay. J. Immunol. Methods 1981;**42**:195-201.
18. Shekarchi IC, Sever JL, Lee YJ, Castellano G, Madden DL. Evaluation of various plastic microtiter plates with

measles, toxoplasma, and gamma globulin antigens in enzyme-linked immunosorbent assays. *J. Clin. Microbiol.* 1984;**19**:89-96.

19. Matthieu S, Bruce MR, Sharmistha D *et al.* Discovery of diverse thyroid hormone receptor antagonists by high-throughput docking. *Proc. Natl. Acad. Sci. U. S. A.* 2003; **100**:7354-7359.

20. Morokata T, Ida K, Yamada T. Characterization of YM-90709 as a novel antagonist which inhibits the binding of interleukin-5 to interleukin-5 receptor. *Int. Immunopharmacol.* 2004;**4**:873-883.

21. Komatsu N, Shichijo S, Maeda Y, Itoh K. Measurement of interferon-γ by high-throughput fluorometric microvolume assay technology system. *J. Immunol. Methods* 2002; **263**:169-176.

22. Brejc K, Ficner R, Huber R, Steinbacher S. Isolation, crystallization, crystal structure analysis and refinement of allophycocyanin from the cyanobacterium Spirulina platensis at 2.3A resolution. *J. Mol. Biol.* 1995;**249**:424-440.

23. Ölschläger P, Srikant-lyer S, Lange S, Schmitt J, Schmid RD. Fluorophor-linked immunosorbent assay;a time- and cost-saving method for the characterization of antibody fragments using a fusion protein of a single-chain antibody fragment and enhanced green fluorescent protein. *Anal. Biochem.* 2002;**309**:27-34.

24. Dietz LJ, Dubrow RS, Manian BS, Sizto NL. Volumetric capillary cytometry;a new method for absolute cell enumeration. *Cytometry* 1996;**23**:177-186.

25. Melletin-Michelotti J, Evangelista LT, Swartzman EE, Miraglia SJ, Werner WE, Yuan PM. Determination of ligand binding affinities for endogenous seven-transmembrane receptors using fluorometric microvolume assay technology. *Anal. Biochem.* 1999;**272**:182-190.

26. Martens C, Bakker A, Rodriguez A *et al.* A generic particle-based nonradioactive homogeneous multiplex method for high-throughput screening using microvolume fluorometry. *Anal. Biochem.* 1999;**273**:20-31.

27. Miraglia S, Swartman EE, Mellentin-Michelotti J *et al.* Homogeneous cell- and bead-based assays for high throughput screening using fluorometric microvolume assay technology. *J. Biomol. Screen* 1999;**4**:193-204.

28. Lee JY, Miraglia S, Yan X *et al.* Oncology drug discovery applications using the FMAT 8100 HTS system. *J. Biomol. Screen* 2003;**8**:81-88.

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

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 cmH₂O 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·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 (Pao) was continuously measured. An aerosol of Mch or OA was administered through a reservoir box connected to the ventilator system. After measurement of baseline Pao, 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·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·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·mL^{-1}; IL-13 was 1.5 pg·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·mL^{-1} versus 1.3±1.1 ng·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
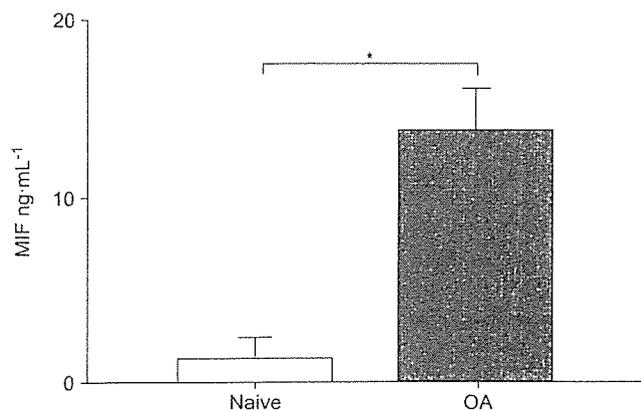

FIGURE 1. Levels of macrophage migration inhibitory factor (MIF) in bronchoalveolar lavage fluid (BALF). MIF in BALF significantly increased 3 days after the ovalbumin (OA) challenge in the OA group (n=6) compared with the Naive group (n=3). *: p<0.05.

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.

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±3.5 ×10^6 in the OA group versus 10.5±2.4 × 10^6 in the OA+anti-MIF Ab group, p<0.01; eosinophils: 10.5±2.7 ×10^6 in the OA group versus 6.2±2.7 ×10^6 in the OA+anti-MIF Ab group, p<0.01;

− 53 −

neutrophils: $1.4\pm1.2 \times 10^6$ in the OA group *versus* 0.16 ± 0.27 $\times10^6$ 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·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·ml^{-1}). The Naive group did not respond to either 5% OA or Mch (up to 16.0 mg·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·mL^{-1} in the OA group *versus* 18.6 ± 5.7 U·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·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
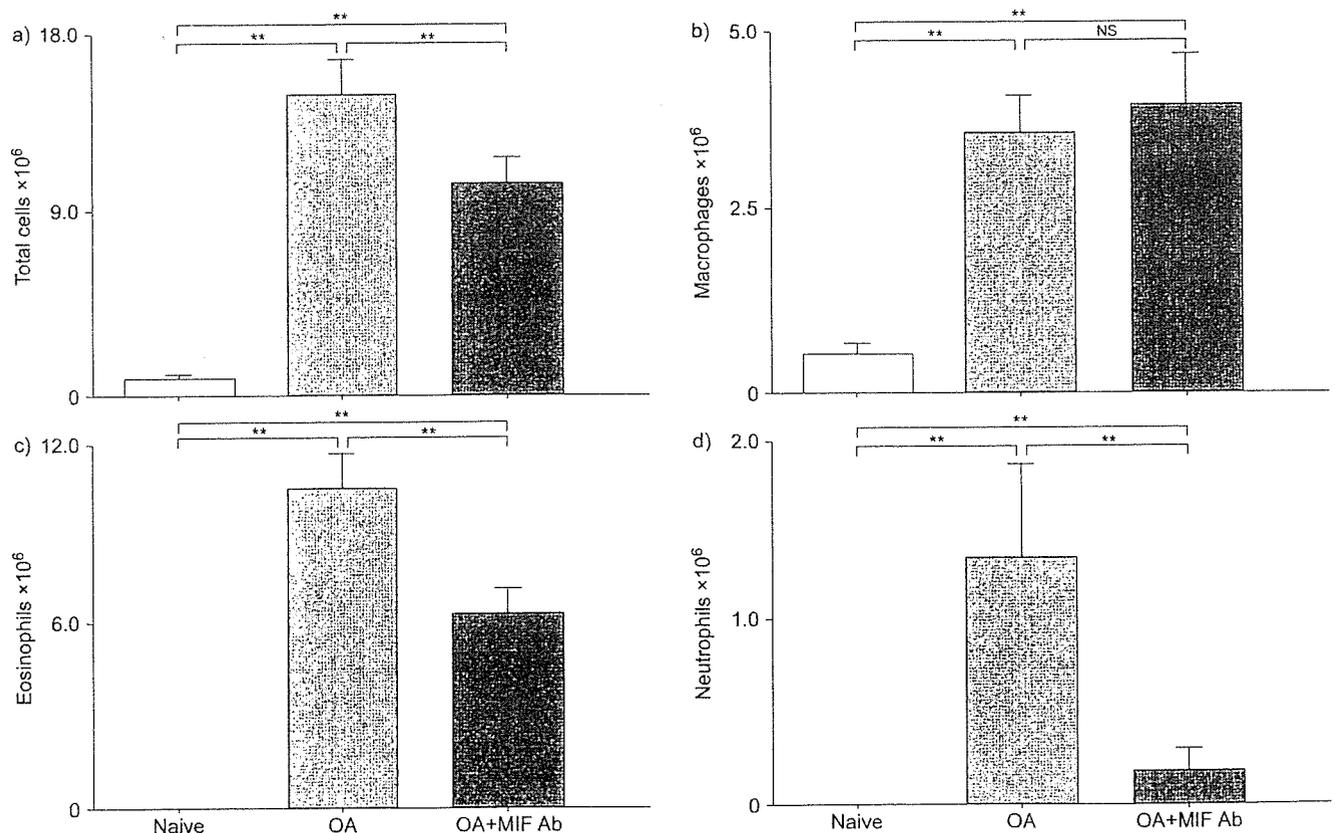

FIGURE 3. Total and differential cell counts in bronchoalveolar lavage fluid. Bronchoalveolar lavage was performed 3 days after ovalbumin (OA) challenge. The numbers of a) total cells, b) macrophages, c) eosinophils d) and neutrophils were significantly elevated in the OA group in comparison with the 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.
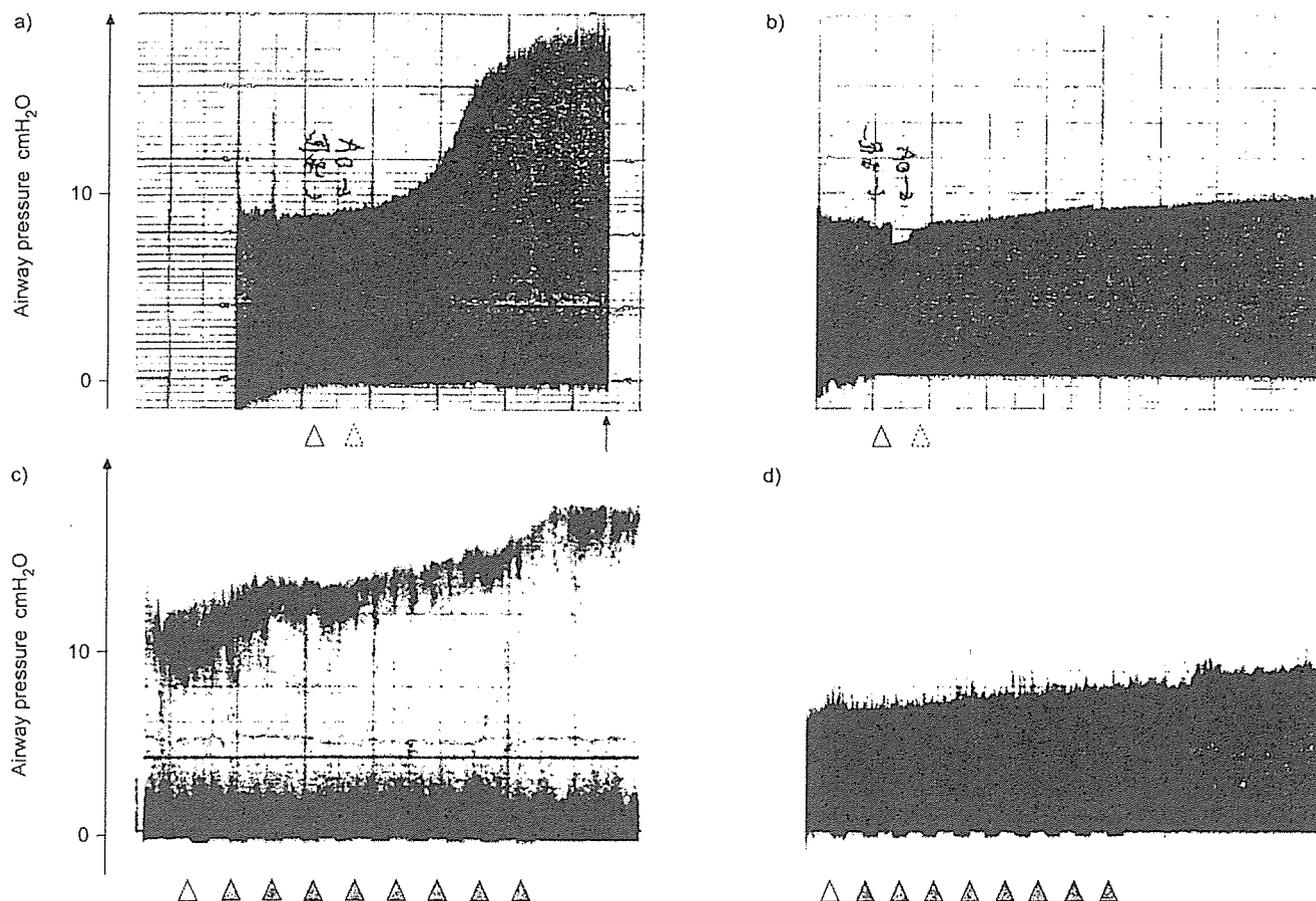
FIGURE 4. The effect of anti-macrophage migration inhibitory factor antibody (MIF Ab) on airway contraction. Both ovalbumin (OA)-specific and non-specific airway responsiveness were examined 3 days after OA challenge. After measurement of baseline pressure, an aerosol of 5% OA (indicated by the dotted 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 $mg \cdot mL^{-1}$ (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 $mg \cdot mL^{-1}$. 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 $pg \cdot mL^{-1}$ in the Naive group, 127.3 ± 38.0 $pg \cdot mL^{-1}$ in the OA group, and 160.0 ± 23.3 $pg \cdot mL^{-1}$ 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 $pg \cdot mL^{-1}$ in the Naive group, 63.0 ± 16.9 $pg \cdot mL^{-1}$ in the OA group, and 72.4 ± 8.1 $pg \cdot mL^{-1}$ 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
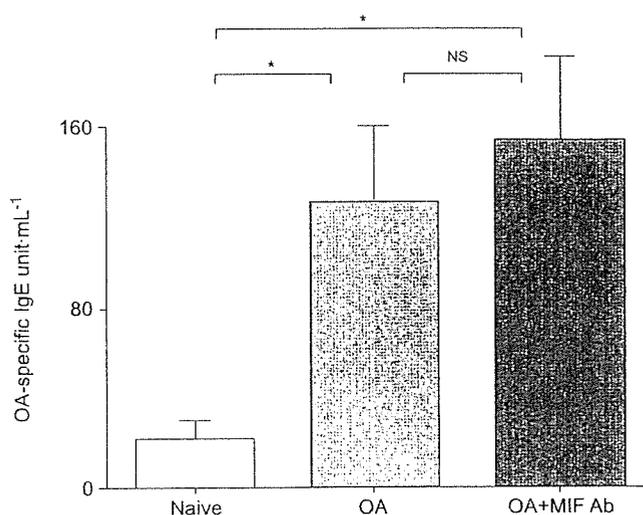
FIGURE 5. Effect of anti-macrophage migration inhibitory factor antibody (MIF Ab) on ovalbumin (OA)-specific immunoglobulin (Ig)E levels in serum. Serum was removed from the inferior vena cava 3 days after the OA challenge (n=6). The levels of OA-specific IgE in serum were significantly elevated in the 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 $64.9 \pm 3.7\%$ 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.
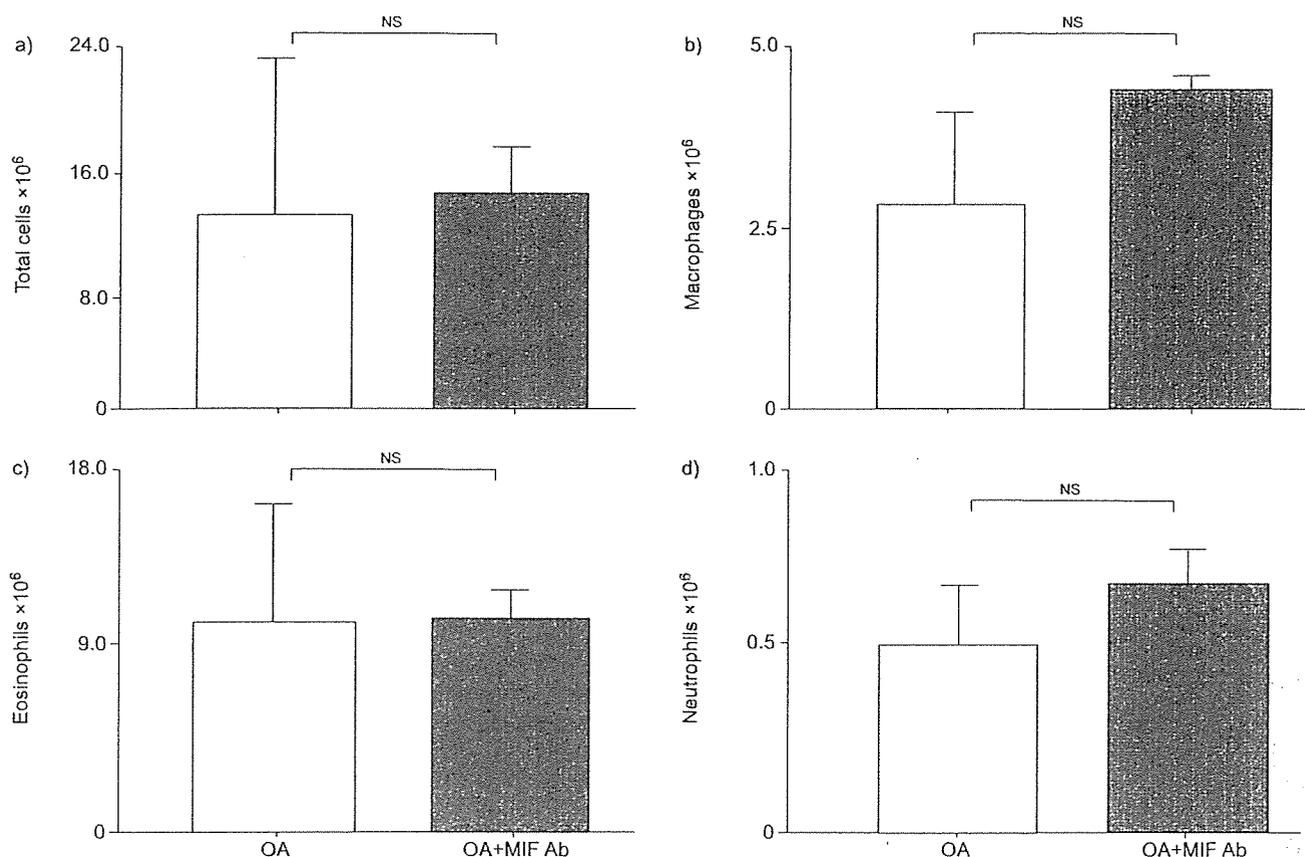
FIGURE 6. Effect of single administration of anti-macrophage migration inhibitory factory antibody (MIF Ab) on a) total cells, b) macrophages, c) eosinophils and d) neutrophils in bronchoalveolar lavage fluid (BALF). A 2-mg aliquot of anti-MIF Ab or rabbit IgG was injected once, 2 h before ovalbumin (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
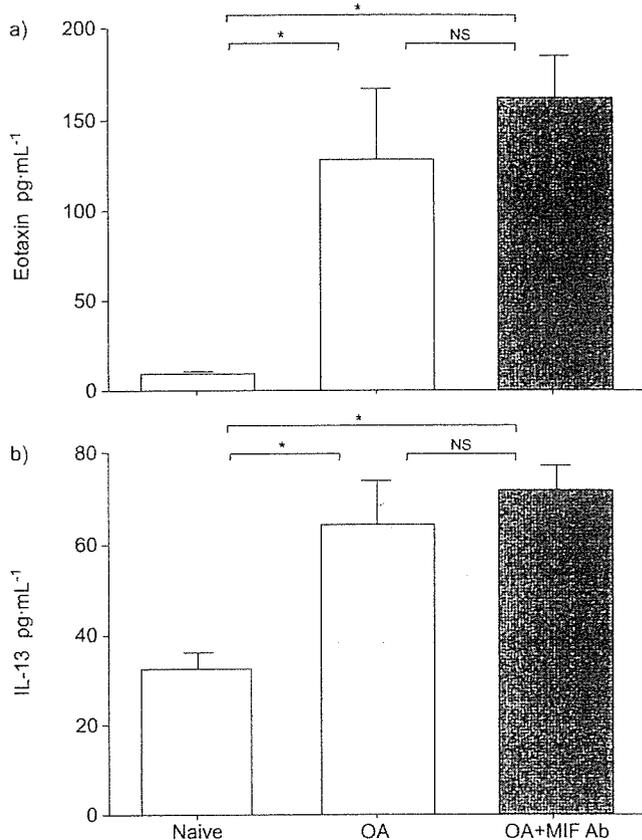
FIGURE 7. Effects of anti- macrophage migration inhibitory factory antibody (MIF Ab) on a) eotaxin and b) interleukin (IL)-13 levels in bronchoalveolar lavage fluid (BALF). The levels of eotaxin and IL-13 in BALF were significantly elevated in the ovalbumin (OA) group compared with the Naïve group 8 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..

衛 生 仮 説
-喘息・アレルギーにおける意義と検証-

Naomi Kondo ＜Chairman＞
Professor, Gifu University Graduate School of Medicine,
Department of Pediatrics, JAPAN
岐阜大学大学院医学研究科小児病態学 教授　近藤　直実

Shuichi Kaminogawa
Professor, Nihon University College of Bioresource
Science Department of Food Science and Technology, JAPAN
日本大学生物資源科学部食品科学工学科 教授　上野川 修一

Monica Kraft
Associate Professor of Medicine, Duke University Medical Center
Division of Pulmonary, Allergy and Critical Care Medicine, USA
デューク大学医療センター，肺病・アレルギー・救命医療部 準教授

衛生仮説とは

近藤

今日は「衛生仮説 – 喘息・アレルギーにおける意義と検証 –」をテーマにご討議いただきたいと思います．ゲストとして日本大学生物資源科学部食品科学工学科 教授の上野川修一先生，デューク大学肺病・アレルギー・救命医療部 準教授の Monica Kraft 先生にご参加いただきました．上野川先生はプロバイオティクスをご専門に研究され，Kraft 先生は喘息とアレルギー学のご専門でいらっしゃいます．よって「衛生仮説」について理論と治療への応用の両面から，幅広いお話をお聞きできるものと期待しております．

上野川・Kraft

よろしくお願いします．

近藤

まずはじめに，私から衛生仮説の歴史について簡単な説明をしたいと思います．近年，豊かな西欧産業国において，結核，A 型肝炎，流行性耳下腺炎（おたふくかぜ）などの感染性疾患の罹患率が低下する一方，自己免疫性疾患・アレルギー性疾患の罹患率がますます高くなるという現象がみられています．

1989年に英国の Strachan 博士が，こうした現象に対し「生活水準や衛生環境の向上による幼少時の感染の減少が，ア

レルギー疾患の増加の原因である」という考えを提唱しました[1]．この仮説は当初，免疫学的な裏付けが不十分であったことから広く受け入れられることはありませんでしたが，Th1細胞，Th2細胞に関する理解の深まりとともに現在では非常に注目される説となっています．その後，微生物の曝露がアレルギー性疾患の発症を予防するという考え，すなわち「衛生仮説」へと発展したわけです．

Kraft 先生はこの「衛生仮説」を非常に熱心に研究されていらっしゃいますが，いつ頃からご研究を始められたのですか．

Kraft

私がこの仮説について真剣に取り組むきっかけになったのは，2002年に New England Journal of Medicine に掲載された Braun-Fahrländer 教授の研究です[2]．Braun-Fahrländer 教授は，ヨーロッパにおいて，農場に住む子供は都会育ちの子供と比較して，ウシやウマの糞などに含まれる細菌あるいはそれに含まれるエンドトキシン LPS（リポポリサッカライド）の曝露量が有意に高く，さらに生後1年の間に曝露を受けた小児は，その時期に曝露を受けなかった小児と比べて，その後の喘鳴，花粉症，喘息の発症率が有意に低いことを示しました（図 1）．

その後，Braun-Fahrländer 教授らは，末梢血単核球を解析することにより，この曝露の細胞レベルでの影響を調べました．彼らは細胞に LPS を添加し，いくつかの炎症性メディエーターを観察しています．このとき LPS 添加量が多いほど，観察された炎症反応は軽度でした．このように，LPS 曝露は

WHAT IS HYGIENE HYPOTHESIS?

Kondo

Today, we would like to discuss "Hygiene hypothesis - significance and verification in asthma/allergy-". We have invited Professor Shuichi Kaminogawa from Nihon University College of the Bioresource Science Department of Food Science and Technology and Dr. Monica Kraft, associate professor at Duke University Division of Pulmonary, Allergy and Critical Care Medicine. Professor Kaminogawa specializes in probiotics and Dr. Kraft is a specialist in asthma and allergy. Today, we hope to hear a variety of opinions on "hygiene hypothesis" regarding aspects of the theory and its application to treatment.

Kaminogawa/Kraft

Nice to meet you.

Kondo

First, I would like to briefly explain the history of the hygiene hypothesis. Recently, in rich Western industrial countries, while the prevalence of infectious diseases such as tuberculosis, hepatitis A, and mumps is decreasing, the prevalence of autoimmune diseases and allergic diseases continues to increase.

In 1989, Strachan DP proposed the hypothesis that "The decrease in the incidence of infection in infancy and childhood as a result of improved living standards and more hygienic living conditions is equivalent to an increase in allergic diseases".[1] This hypothesis was not widely accepted due to lack of supporting data, but as understanding of Th1 cells and Th2 cells deepened, it started to draw more attention. Later, this theory evolved as "exposure to microorganisms prevents allergic diseases," namely "hygiene hypothesis".

Dr. Kraft, you actively study this "hygiene hypothesis." When did you start studying it?

Kraft

I think a study that really made me start to think about this hypothesis in earnest was the Braun-Fahrländer C study in the New England Journal of Medicine in 2002.[2] Braun-Fahrländer C showed that in Europe, children living on farms had significant exposure to LPS (lipopolysaccharide: bacteria contained in cow and horse dung or endotoxins contained therein) if you look at the exposure during the first year of life and then the subsequent development of wheezing, hay fever, and asthma, there is a significant decrease compared to children that have not been exposed in that period of life (**Fig. 1**).

Braun-Fahrländer C looked at celluler effects of this exposure by evaluation of peripheral blood mononuclear cells. They exposed the cells to LPS and looked at several inflammatory mediators. The greater the LPS exposure in fact, the less inflammation they saw. Therefore, LPS exposure reduced the Th2 inflammatory response.

Kondo

I see. Professor Kaminogawa, how did you get interested in "hygiene hypothesis"?

Kaminogawa

I am very interested in the hygiene hypothesis from the viewpoint of intestinal microorganisms. Antibiotics are widely used for the treatment of many infectious diseases and many people who readily take antibiotics contract allergic diseases such as asthma and atopic dermatitis. So I think from the viewpoint of microbiology, intestinal bacteria are very important in explaining the hygiene hypothesis.

EPIDEMIOLOGICAL VERIFICATION OF HYGIENE HYPOTHESIS

Kondo

There are many epidemiological data concerning the hygiene hypothesis. In an Ethiopian study, it was reported that the prevalence of asthma is higher in urban areas than in rural areas. There is a similar report from Germany. In Western countries, too, it has been reported that children on farms have a lower frequency of developing allergy or asthma.

Kaminogawa

Another concept of hygiene hypothesis is that for example, it has been reported that children with many brothers and sisters are less at risk of having an allergic reaction bronchial asthma and eczema. Do you think that infections from brothers and sisters prevent the development of allergy?

Kraft

I think so. There is a similar study about day care. It was reported that children that enter day care in infancy are less at risk of developing asthma later on in life. In other words, early communal living increases the chances of infection and decreases the development of atopy. Infection from siblings is one route and day care is another route. Children are

Hygiene Hypothesis
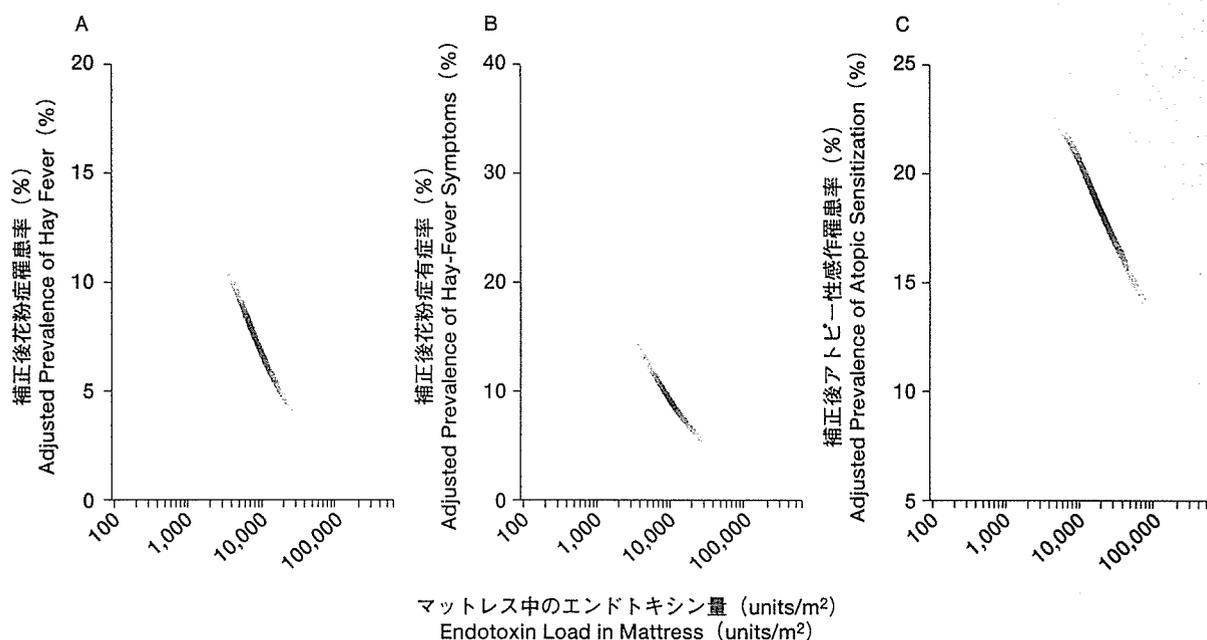# −Significance and Verification in Asthma/Allergy−

9

図1 対数変換したエンドトキシン量に対する花粉症罹患率(A)，花粉症有症率(B)，アトピー性感作罹患率(C)のスムースプロット
Fig. 1 Smoothed Plots of the Prevalence of Hay Fever (A), Hay-Fever Symptoms (B), and Atopic Sensitization (C). In Relation to the Log-Transformed Endotoxin-Load Values

この解析では，年齢，性別，研究地域，喘息・花粉症家族歴，親の教育レベル，兄弟の数に関して補正してある．それぞれのアウトカムに関して，エンドトキシン量の増加にしたがって一様な減少が観察される．すべてのグラフに関して，スムージングスパンは0.9とした．
The analyses controlled for age, sex, study area, family history of asthma and hay fever, educational level of the parents, and number of siblings. For each outcome, there was a monotonic decrease with increasing endotoxin load, A smoothing span of 0.9 was used for all three graphs.

(文献2より引用)

Th2炎症性反応を抑制するのです．

近藤

なるほど．では，上野川先生はどういった観点から「衛生仮説」に興味をもたれていますか．

上野川

私は自身の専門である腸内細菌との関連で，興味をもつに至りました．抗生物質は現在多くの感染症の治療に用いられていますが，抗生物質を服用する人の多くが，喘息やアトピー性皮膚炎などのアレルギー性疾患に罹患しているようです．微生物学の観点からみても，衛生仮説を証明する上で腸内細菌は非常に重要ではないでしょうか．

衛生仮説に関する疫学的検証

近藤

現在までに，衛生仮説を支持する疫学的データがいくつか

報告されています．エチオピアでの成績では，喘息は農村地区より都市部に多いことが報告されました．ドイツでも同様の報告があります．また，西欧の工業国においても，農家の子供はアレルギーや喘息の発症頻度が低いという報告がありましたね．

上野川

それと，衛生仮説のもうひとつのポイントだと思うのですが，多くの兄弟姉妹をもつ子供はアレルギー反応(気管支喘息，湿疹)を起こしにくいという報告もあります[1]．やはり兄弟姉妹からの感染がアレルギー発症の予防につながっているのでしょうか．

Kraft

そう思います．同じ主旨の論文で，デイ・ケア(保育園，託児所)を扱ったものがあります．乳幼児期にデイ・ケアに預けられた子供では，後に喘息を発症する率が低いという報告です．つまり，早い時期に集団生活を始めることが感染の機会を多くし，アトピーの発症率を下げるとする説ですね．兄弟

born with an immune system that is skewed towards Th2. It takes infections to skew the immune system to Th1. And if you don't have infection in infancy, then you may potentially continue along this Th2 pathway and develop allergic disease. This is the main concept of hygiene hypothesis (**Fig. 2**).

Kondo

Yes. Some reports say that having a pet such as a dog or cat in the house prevents allergy. What do you think about this?

Kraft

As for the issue about having dogs and cats, Ownby DR et al showed that exposure to animal allergens during infancy reduces subsequent allergic sensitization at age 6 to 7.

Ownby DR et al looked at 835 children in Detroit Michigan and followed about half of them to age 6 or 7 years. They evaluated atopy by skin prick test positivity to common aerial allergens.

Kaminogawa

Very, very interesting.

Kraft

As a result, the skin prick test positivity was lower in children with 2 or more dogs. However, current asthma was not affected.

Kondo

This shows only sensitization is decreased.

Kraft

Right. This study only shows the effect on allergic sensitization.[3] Perzanowski MS et al looked at the association between pets and the development of allergic diseases. They found that children exposed to pets during infancy had lower risk of developing atopic dermatitis and asthma. We do not know about asthma, but it seems that exposure to animal allergens from pets is really related to predicting the development of atopy. One mechanisms may be through immunological tolerance (condition marked by the absence of an immune reaction to specific antigens).

IMMUNOLOGICAL/MOLECULAR BIOLOGICAL VERIFICATION OF HYGIENE HYPOTHESIS

Kondo

Okay, since we are now talking about the mechanisms of hygiene hypothesis, we should probably move on to the next topic, molecular biological/molecular genetic data.

The papers we have talked about so far only show a co-relation between the frequency of allergic disorders and environmental factors; they do not show a causal relationship

between them. We need to clarify the causal relationship in future studies. For that, we need molecular biological data and molecular genetic data. Dr. Kraft, could you tell us about basic research reports?

Kraft

Well, I can show you a little bit of our work when I worked at the National Jewish Medical and Research Center in Denver, Colorado. In this study, which was led by Professor Richard Martin, the effects of mycoplasma infection on the development of Th2 inflammation in a mouse model of asthma was investigated. Airway hyper responsiveness (BHR), the number of inflammatory cells in the lung, and protein levels of interferon (IFN) -γ (Th1 cytokine) and interleukin (IL) -4 (Th2 cytokine) were evaluated.[4]

In the mice that were infected by *Mycoplasma pneumoniae* before ovalbumin (OVA) sensitization, although BHR was lower than the control group (saline) at 3 days after the infection, both BHR and lung inflammatory markers increased after that and IL-4 increased on Day 7 and 21. The IFN-γ /IL-4 ratio increased at 3 days after the infection and decreased on Day 7.

On the other hand, in the mice that were infected by Mycoplasma after sensitization with OVA, airway hyperresponsiveness decreased and infiltration of inflammatory cells to the lung was also mild compared to the control group. In BALF (bronchoalveolar lavage fluid), IFN-γ significantly increased and IL-4 decreased, suggesting a Th1 response.

What is important is that infection after sensitization increase BHR and Th2 inflammation, and infection before sensitization favors Th1 inflammatory response.

Gifu University Graduate School of Medicine,
Department of Pediatric Pathology
Professor **Naomi Kondo**

11

姉妹からの感染もひとつの感染ルートとしてあるでしょうし，こうしたデイ・ケアのルートもあると思います．もともとヒトは Th2優位の免疫系をもって生まれてきます．それが感染により Th1優位の免疫系へと変化するわけです．その途中，つまり幼少期に感染がないまま進んでしまうと，胎児の頃からもっている Th2優位の状態が継続し，アレルギー性疾患を発症する可能性が高くなるのではないかというのが衛生仮説の主な考え方ですよね（図2）．

近藤

そうですね．ペットとしてイヌやネコを屋内で飼うことがアレルギー発症の予防になるという報告がありますが，これについてはどのようにお考えですか．

Kraft

イヌ，ネコ飼育の問題については，米国 Georgia 医科大学小児科の Ownby 博士らが，乳児期の動物アレルゲンの曝露がその後の6～7歳時のアレルギー感作のリスクを減少することを示しています．

Ownby 博士らはミシガン州デトロイト市の乳児835人のうち約半数について，6～7歳まで経過観察することに成功しました．一般的な空中アレルゲンに対する皮膚プリックテストを行い，アトピーの有無を確認しています．

上野川

とても興味深いですね．

Kraft

その結果，2匹以上のイヌがいる場合，皮膚テストの陽性率が低下していました．ただし，すでに発症してしまっている喘息は何ら影響を受けなかったようです．

近藤

感作だけが減少したということですか．

Kraft

そのとおりです．この研究では，アレルゲン感作への影響までしかわかりません．ペット所有とアレルギー性疾患発症との関連については，スウェーデンの Perzanowski 博士ら[3]が行った研究で検討されています．乳幼児期にペットに接触していた子供においてアトピー性皮膚炎と喘息の発症率の減少がみられています．ペットによる動物アレルゲンの曝露がその後の喘息の発症に真に影響するのかどうかは明らかではありませんが，アトピーの発症に関しては何らかの関連があるようです．メカニ

ズムのひとつは，免疫寛容（特定の抗原に免疫反応を起こさない状態）かもしれません．

ぜひこれらのメカニズムについて，先生方のご意見をお聞きしたいところです．

衛生仮説に関する免疫学的・分子学的検証

近藤

ちょうど衛生仮説のメカニズムに話が移ったようなので，分子免疫学的・分子遺伝学的なデータの話題に入りましょう．

今まで話題にあがった論文は，アレルギー性疾患の頻度と環境因子の相関を示しているに過ぎず，その間の因果関係については示していません．われわれは今後の研究によってこの因果関係を明らかにしていく必要があるわけです．そのためには，いわゆる分子生物学レベルのデータ，遺伝子レベルのデータが必要になってきます．Kraft 先生，現在まで報告されている基礎研究について教えていただけますか．

Kraft

それでは，私が米国ユダヤ医学研究センター（コロラド州デンバー，National Jewish Medical and Research Center）に在籍していたときの研究を少し紹介したいと思います．Richard Martin 教授が中心となって行っていた研究の一部なのですが，喘息モデルマウスを用いて，マイコプラズマ感染が，Th2炎症の発症にどのように影響を与えているのかが検討されました．気道過敏性（BHR），肺の炎症細胞数，Th1サイトカインである IFN（インターフェロン）-γ，Th2サイトカインである IL（インターロイキン）-4の蛋白レベルを指標として検討しました[4]．

OVA（卵白アルブミン）感作・誘発の前にマイコプラズマ（*mycoplasma pneumoniae*）感染を行ったマウスでは，感染3日後にはコントロール群（生理食塩水）に比べ BHR が低値を示していたものの，その後 BHR，肺の炎症指標ともに増加し，IL-4は7日目，21日目で上昇しています．IFN-γ/IL-4比でみると，感染群で3日目に上昇し，7日目で低下しています．

一方，OVA で感作・誘発した後にマイコプラズマに感染させた群では，コントロール群に比べて，BHR が低下し，肺への炎症細胞の浸潤も軽度でした．このとき，BALF（気管支肺胞洗浄液）中の IFN-γ 値が有意に増加し，IL-4値が減少していることとあわせて考えると，Th1優位の反応が起きていることがわかります．

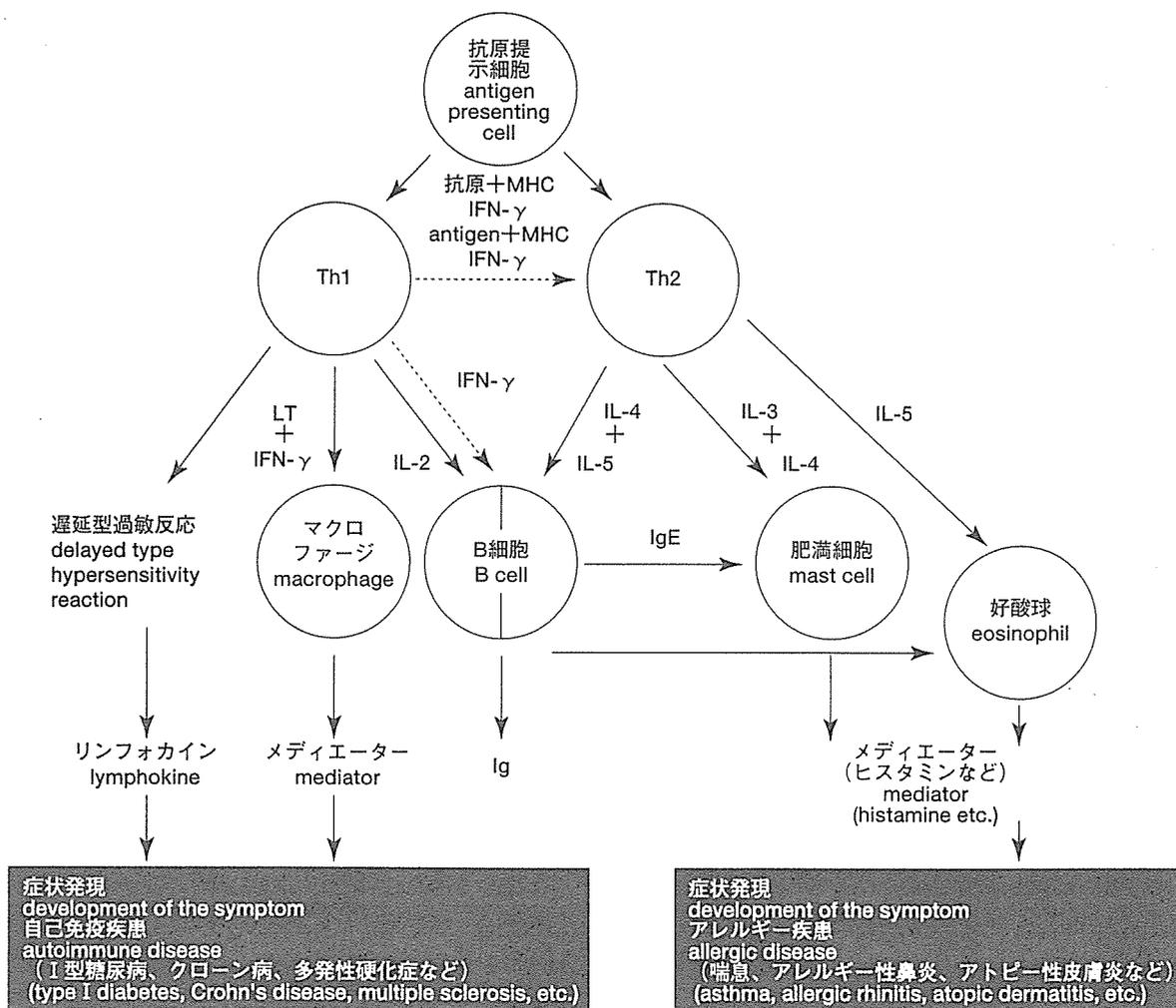重要なのは，感作後の感染では BHR と Th2が悪化するのに対し，感作前の感染では Th1炎症反応が優勢になるということです．

図2 2種類のヘルパーT細胞の反応系と発現する疾患
Fig. 2 Responses of 2 different types of helper T cells and developing diseases
Th1細胞とTh2細胞のアンバランスにより疾患が発症する.
Imbalance between Th1 cells and Th2 cells causes diseases.

(Asthma Frontier 2 : 21, 2003)

The importance of the timing of infection was confirmed by the animal experiment (**Fig. 3, 4**).

Kondo

These are very interesting results. It means that in humans, sensitization to allergens and the state of infection in the perinatal period, neonatal period, and infancy is important.

Kaminogawa

Very interesting. I am very interested in the cellular/ molecular differences in the body.

Kraft

There is another important study by Eisenbarth SC and colleagues published in the Journal of Experimental Medicine in 2002.[5] They gave LPS to mice. It turned out that the dose of LPS was important. Low-dose LPS induced a Th2 response and high-dose LPS induced a Th1 response. So, it is not only timing, but also dose that is important (**Fig. 5**).

Kaminogawa

What is the mechanism behind it? Why does low-dose LPS induce the Th1 reaction and high dose LPS induce the Th2 reaction?

13

近藤

感染の「時期」が重要であることが，動物実験によって確認されたのです（図3, 4）.

近藤

大変，興味深い成績ですね．ヒトに適用して考えると，周産期，新生児期，乳児期におけるアレルゲン感作と感染の状況が重要ということですね.

上野川

非常に興味深い現象ですね．さらに，体内において細胞レベル，分子レベルでどのような違いになっているのか知りたいですね.

Kraft

もうひとつ，重要な研究に米国の Eisenbarth 博士らが2002年に the Journal of Experimental Medicine に発表したものがあります[5]．Eisenbarth 博士らはマウスに菌体成分エンドトキシンの LPS を投与しました．その結果，LPS 量が重要であることがわかりました．少量の LPS では Th2反応が誘発され，多量の LPS では Th1反応が誘発されることがわかりました．感染にはその「時期」だけでなく，病原微生物の「量」も重要だということです（図5）.

上野川

LPS 量が多いと Th1反応が誘発され，少ないと Th2反応が誘発されるという現象のメカニズムは何でしょうか.

Kraft

Eisenbarth 博士らは，何らかの形で新しい経路を介して働いているのではないかと推測しています．まだメカニズムは完全には解明されていませんが，感染時期だけでなく「量」も重要であるという概念を提唱した点で，評価できると思います.

上野川

メカニズムに関して，すでにデータや論文をお持ちなのですか.

Kraft

いいえ，Eisenbarth 博士らの論文からの引用です．TLR（トールライクレセプター）4欠損マウスはこの反応を示しません．TLR4はこの経路に必須であり，MyD88非依存的シグナル伝達経路を介して作用します．これは新しい情報です.

近藤

ありがとうございます．多くの研究者が Th1/Th2のバランスに注目しているわけですが，われわれもこれらに影響を与える因子に着目した検討を行っていますので，少し紹介させて下さい.

まずわれわれは IL-10のアレルギーにおける役割について検討しました．IL-10は1989年に Mosman らにより Th1細胞からのサイトカイン産生を抑制する因子として同定されました．その後の研究によって，その他の種々の細胞からも産生され，いろいろな作用があることが明らかになりました．ただ，この時点では，アレルギーにおけるサイトカインネットワークに関与する作用は十分には解明されていませんでした.

そこでわれわれはこれらの点について検討したのです．IL-10は，末梢血単核球分画を用いて IL-12や IFN-γ など Th1サイトカインを in vitro において強発現させた場合，濃度依存的にこれらのサイトカインの産生を抑制しました（図6）．一方，T 細胞クローンを用いて調べると，Th2サイトカインである IL-4の産生を濃度依存的に抑制しました．以上のことから，IL-10は in vitro において強発現させた Th1サイトカインの IL-12，IFN-γ に対し，抑制効果を有する一方，Th2サイトカインをも抑制することがわかったのです．したがって，IL-10は Th1と Th2のバランスの調節作用をもっていると考えられます.

なお in vitro においてコントロール群とアレルギー群とで，これらサイトカインの産生状況を調べてみたところ，アレルギー群では IL-10の産生が有意に減少していました．さらに，2歳以上の小児と2歳未満の小児で比較をしてみると，2歳以上のアレルギー疾患児で，IL-10産生が低下していました.

Kraft

この Th1/Th2バランスにおいて，IL-10は非常に重要な役割を果たしていると思います．しかもその役割は生後間もない頃から始まるのだと思います．IL-10の機能制御の遺伝学的側面を評価する研究が行われるべきでしょう.

衛生仮説を基盤にした治療予防法とその開発 ～プロバイオティクスの可能性～

近藤

それでは，将来的に衛生仮説がアレルギーの治療・予防にどのように役立てられるのか，このあたりについてお話していきたいと思います.

今までのお話のなかで，アレルギーの予防には Th1免疫応答反応を促進して，過剰な Th2反応を抑制することでアレル