Allergology International (2003) **52**: 97–103

Original Article

Induction of inducible nitric oxide synthase mRNA expression and nitric oxide production from macrophages stimulated with high-molecular size mite antigen HM1

Airo Tategaki, Takahide Okuda, Seiji Kawamoto, Kazuhisa Ono and Seiko Shigeta

Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima, Japan

Abstract

Background: High-molecular size mite antigen fraction (HM1) induced macrophage activation and prolonged airway inflammation from mite-sensitized mice. In the present study, we investigated the inflammatory factors in the HM1-activated macrophage in both non-immunized splenocytes and bronchoalveolar lavage (BAL) from mite-immunized and HM1-exposed mice.

Methods: *Dermatophagoides farinae* feces (Dff) extract was divided into HM1 and HM1-depleted fraction (DH) by size-exclusion chromatography. Transcriptional gene induction of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-12 and cytokine-inducible nitric oxide synthase (iNOS) in splenic macrophages stimulated with HM1 or DH was analyzed by using semiquantitative reverse transcription–polymerase chain reaction. Nitric oxide (NO) production was measured by using diaminofluoresceins (DAF), fluorescence indicators.

Results: Gene expression of TNF-α, IL-12 p40 and iNOS was observed with HM1 stimulation of splenic macrophages from non-immunized mice. In addition, the release of NO induced by HM1-stimulated splenic macrophages increased in a dose-dependent manner. However, splenic macrophages stimulated with DH induced TNF-α and IL-12 p40, but not iNOS, gene expression. Similarly, significant iNOS mRNA expression was detected in alveolar macrophages recovered from HM1-exposed mice, but not from DH-exposed mice.

Conclusion: In the present study, HM1 induced iNOS mRNA expression and NO production both *in vitro* and *in vivo*. The present results suggest that the ability of HM1 to induce NO production may be one of the causes aggravating airway inflammation in HM1-exposed mice.

Key words: airway inflammation, high-molecular size mite antigen, HM1, inducible nitric oxide synthase, nitric oxide.

Introduction

The inflammatory response in asthma is associated with airway hyperresponsiveness and the accumulation of various cells, including eosinophils, mast cells and, most importantly, CD4+ T helper (Th) 2 lymphocytes, which can be isolated from the lungs of asthmatic patients.[1,2] In contrast, clinical studies have demonstrated that the number of activated Th1 lymphocytes and macrophages, which usually suppress the ability of Th2 effector cells *in vitro*, was increased in bronchoalveolar lavage fluid (BALF) from human asthmatic patients.[3] Moreover, recent studies have demonstrated that Th1 lymphocytes are not protective, but lead to further problems in murine models of asthma.[4,5] The aggravation in asthma is usually considered to proceed in association with various cytokines, chemokines and enzymes that are released

Correspondence: Airo Tategaki, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Kagamiyama 1-3-1, Higashi-Hiroshima 739-8530, Japan. Email: lovero@hiroshima-u.ac.jp

Received 17 June 2002. Accepted for publication 20 January 2003.

from the various types of cells that infiltrate into the airway.

House dust mite, *Dermatophagoides farinae* (Df), is well recognized as a major source of bronchial asthma. Previously, we demonstrated that HM1 fractionated from Df feces extraction (Dff) by size-exclusion chromatography induced a proliferative response of T cells from non-immunized mice without major histocompatibility complex (MHC) class II restriction via macrophage activation.[6] In Dff-immunized mice, but not in non-immunized mice, HM1 developed and maintained airway hyperresponsiveness at least 120 h after final inhalation. The HM1-depleted fraction (DH)-exposed mice similarly demonstrated aggravated airway hyperresponsiveness, but the airway hyperresponsiveness in these mice was not maintained. We also demonstrated that aggravation of airway hyperresponsiveness was associated with the accumulation of macrophages in the lung.[6]

Clear evidence has been reported that alveolar macrophages in the inflammation are associated with allergic airway diseases such as asthma.[7] Alveolar macrophages have been identified as potent producers of several cytokines. These include macrophage inflammatory protein-1α and tumor necrosis factor (TNF)-α, which possess pro-inflammatory properties.[8,9] The cytokines were involved in the production of NO, which was upregulated in pulmonary inflammatory diseases.[10,11] In allergic patients[12] and in animal models (rats[13] and guiniea pigs[14,15]) of asthma, allergen provocation resulted in enhanced endogenous NO production during the late asthmatic response.

To further investigate the HM1-induced association between activated macrophages and enhancement of airway hyperresponsiveness, we investigated HM1-induced mRNA expression of the pro-inflammatory cytokine TNF-α and inducible NO synthase (iNOS). In the present study, we proved that HM1 is a potent inducer of iNOS.

METHODS

Mite antigens

Mite antigens were prepared according to previous reports.[16,17] Briefly, Df feces extracts from whole mite culture (Dff) was applied to a Superdex 75 column (Amersham Pharmacia Biothech, Uppsala, Sweden) and eluted with 0.9% NaCl. The highest peak, over 70 kDa, was collected as HM1. The content of HM1 in Dff was estimated to be approximately 20%. The HM1 fractions removed were dialyzed and lyophilized and used as the HM1-depleted Dff fraction (DH). Finally, 2.4 g DH was recovered from 3 g Dff.

Antigen exposure to Dff-immunized mice

Female BALB/c mice (6 weeks old) were sensitized twice with Dff (50 μg) adsorbed to 1 mg aluminum hydroxide by intraperitonaeal injections at 5 day intervals. Then, immunized mice were fixed and exposed to aerosols of 1% antigen (20 mL for 20 min) through their airways for 10 days.

Cell preparation

Splenic macrophages (CD11b+ cells) were prepared from non-immunized murine splenocytes using Magnetic Cell Sorting (MACS; Bergisch, Gladbach, Germany), according to previous reports.[18] Splenic macrophages were purified up to 98%.

Alveolar macrophages were isolated from the bronchoalveolar lavage (BAL) of Dff-immunized mice receiving daily exposure to aerosolized HM1, DH or phosphate-buffered saline (PBS) for 10 days. Bronchoalveolar lavage fluid was collected from four separate 1 mL PBS washes of the lungs. Approximately 3.6 mL of the total fluid was recovered and centrifuged (700 *g* for 10 min at 4°C). Alveolar macrophages were purified up to 97% by MACS.

Cell culture

Splenic macrophages (1×10^6 cells/mL) were cultured with 10 μg/mL HM1 in RPMI 1640 medium (Sigma Chemical, St Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (Sigma) and 0.05 mmol/L 2-merchaptoethanol in a flat-bottomed microtiter culture plate at 37°C for 2–24 h under 5% CO_2.

RNA isolation and reverse transcription–polymerase chain reaction

Total RNA was extracted from splenic macrophages cultured with respective mite antigens and alveolar macrophages from BAL of corresponding mite antigen-exposed mice. The method used was the Trizol method[19] and the mRNA was transcribed using 0.5 μg Oligo (dT) primer (Gibco BRL, Grand Island, NY, USA) and 200 U SUPERSCRIPT™II (Gibco BRL) reverse transecriptase in a final volume of 20 μL. Polymerase chain reaction (PCR)

was performed on a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA) in a reaction containing dNTP mix (20 pmol; Takara, Kyoto, Japan), forward and reverse primer (20 pmol each), ×10 EX Taq buffer (2 μL), optimum cDNA concentration and 0.5 U Taq polymerase, which was made up to 20 μL with distilled water. The PCR conditions were as follows: predenaturation at 94°C for 4 min, followed by 30–38 cycles of denaturation at 94°C for 30 s, optimal annealing temperature for 30 s and elongation at 72°C for 60 s. The amplimer sequences and lengths of the expected PCR products are given in Table 1.

Determination of NO from splenic macrophages stimulated with HM1 and DH

Splenic macrophages were cultured with several concentrations of HM1 or DH for 22 h and cells were then washed twice with PBS. Cells were incubated with 1 mmol/L DAF-2 and 100 mmol/L L-arginine for 6 h. The supernatants were transferred to 96-well microplates and the fluorescence was measured with a fluorescence microplate reader calibrated for excitation at 485 nm and emission at 538 nm.[20] The level of NO production was expressed as concentrations of the DAF-2T fluorescent compound, which was used for the fluorescent detection of NO using DAF-2.

RESULTS

HM1 induces iNOS gene expression

First, splenic macrophages from non-immunized mice were cultured with HM1 or DH, and the mRNA expression of MHC class II, functional activation markers TNF-α and IL-12 p40, pro-inflammatory cytokines and iNOS was analyzed by reverse transcription (RT)–PCR. To obtain comparable data on steady state levels of mRNA in the different samples, cDNA levels were normalized using GAPDH housekeeping gene expression levels. The PCR analyses were performed using equal amounts of cDNA with a non-saturating number of amplification cycles. Similar expression levels of mRNA encoding MHC class II, TNF-α and IL-12 p40 were observed in splenic macrophages cultured with either HM1 or DH. Splenic macrophages cultured with HM1 promoted efficient induction of iNOS mRNA in the presence or absence of polymixn B (10 μg/mL). However, the DH stimulus could not induce mRNA coding for iNOS (Fig. 1).

Release of NO from splenic macrophages cultured with HM1

The supernatant of macrophages cultured with HM1 produced fluorescent products upon incubation with DAF-2. The amount of the products that represented NO production was positively correlated with the concentration of HM1. We also found that the release of NO could not be detected in supernatants from macrophages cultured with DH (Fig. 2).

HM1 inhalation-induced iNOS expression of alveolar macrophages *in vivo*

Our previous report indicated that airway hyperresponsiveness was maintained in HM1-exposed mice for at least 120 h after final antigen inhalation by

Table 1 List of mRNA of interest, expected size of amplified cDNA and sequence of nucleotide primers

mRNA of interest and size of expected amplified cDNA	Primer sequences (sense and antisense 5′–3′)
GAPDH (306 bp)	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTGGTGAAGAC
MHC class II (229 bp)	TATGTGGACTTGGATAAGAAG ACAAAGCAGATAAGGGTGTTG
IL-12 p40 (384 bp)	CTGGCCAGTACACCTGCCAC GTGCTTCCAACGCCAGTTCA
TNF-α (692 bp)	ATGAGCACAGAAAGCATGATCCGC CCAAAGTAGACCTGCCCGGACTC
iNOS (481 bp)	AGCTCCTCCCAGGACCACAC ACGCTGAGTACCTCATTGGC

MHC, major histocompatibility complex; IL-12, interleukin-12; TNF-α, tumor necrosis factor-α; iNOS, inducible nitric oxide synthase.

Dff-immunized mice, although that of DH-exposed mice was reverted to normal levels.[18] To investigate the expression of mRNA for iNOS from alveolar macrophages under these conditions, BALF was collected at 120 h after final antigen exposure from Dff-immunized mice. The alveolar macrophages in the BALF were recovered. The cell numbers of alveolar macrophages were 3.3 ± 1.0, 11.9 ± 0.9 and 4.3 ± 2.3 (× 10^3 cells/mL BALF) for PBS-, HM1- and DH-exposed mice, respectively. The mRNA expression for MHC class II, TNF-α, IL-12 p40 and iNOS was analyzed by RT-PCR. The expression of iNOS mRNA was detected in alveolar macrophages from HM1-exposed, but not DH-exposed, mice. The MHC class II, TNF-α and IL-12 p40 mRNA was detected in both HM1- and DH-exposed mice (Fig. 3).

DISCUSSION

The main findings of the present *in vitro* and *in vivo* studies are that HM1 is a potent inducer of iNOS gene expression from splenic/alveolar macrophages. We demonstrated previously that HM1 exposure of Dff-immunized mice resulted in the development of airway hyperresponsiveness 24 h after inhalation and that the hyperresponsiveness was sustained even 120 h after inhalation. Although airway hyperresponsiveness was observed in DH-exposed mice, it returned to control levels as seen in mice that inhaled PBS mice within 120 h after final inhalation. It was found that HM1 was able to enhance and prolong airway hyperresponsiveness compared with DH. In addition, there were still significant amounts of alveolar macrophages accumulated

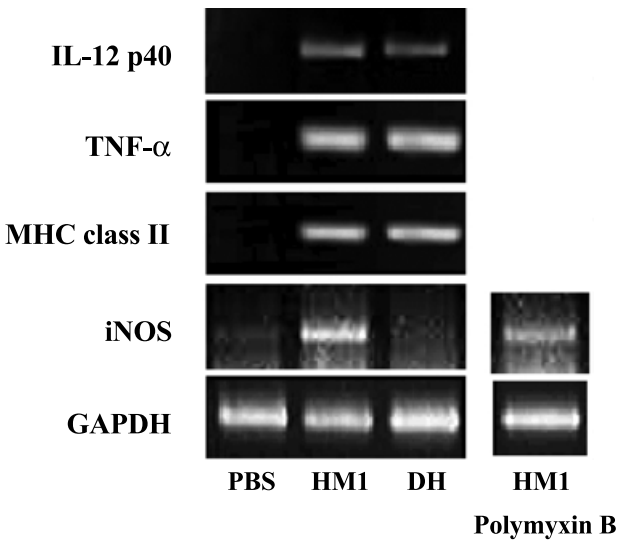

Fig. 1 High-molecular size mite antigen fraction (HM1) stimulus-induced inducible nitric oxide synthase (iNOS) mRNA expression from non-immunized murine macrophages. Splenic macrophages (2.0×10^5 cells/well) derived from non-immunized mice were cultured with 10 μg/mL mite antigen. Total RNA preparations were isolated after 24 or 2 h incubation for iNOS mRNA analysis or the analysis of other mRNA, respectively, and analyzed by reverse transcription–polymerase chain reaction. The polymerase chain reaction products were subjected to agarose gel electrophoresis. IL-12, interleukin-12; TNF-α, tumor necrosis factor-α; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; DH, HM1-depleted fraction.

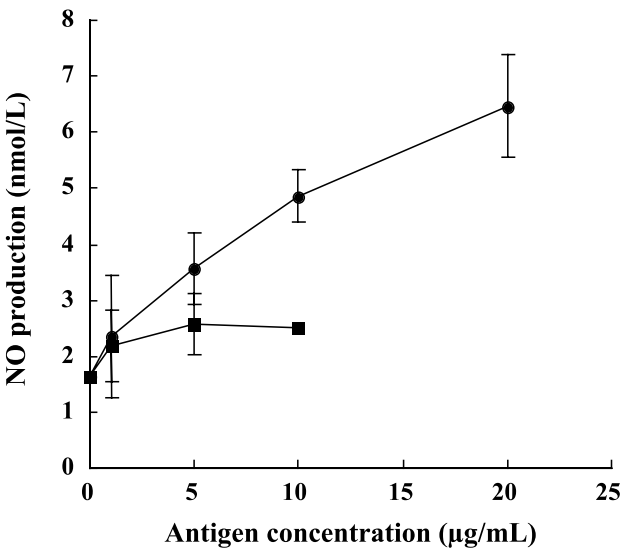

Fig. 2 Production of nitric oxide (NO) by macrophages stimulated with high-molecular size mite antigen fraction (HM1; ●) and the HM1-depleted fraction (DH; ■). Splenic macrophages were cultured with mite antigen for 22 h and washed with phosphate-buffered saline (PBS) and resuspended in PBS containing 1 μmol/L DAF-2 and 100 μmol/L L-arginine. After 6 h incubation, the fluorescence was measured with a fluorescence microplate reader calibrated for excitation at 485 nm and emission at 538 nm. The level of NO production is expressed as the concentration of the DAF-2T fluorescent compound used for the fluorescent detection of NO using DAF-2.

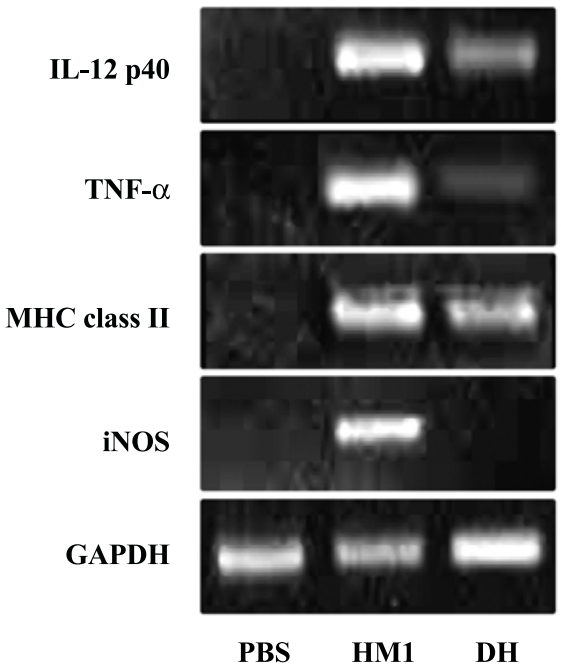

Fig. 3 Inducible nitric oxide synthase (iNOS) mRNA gene expression in alveolar macrophages from high-molecular size mite antigen fraction (HM1)-exposed mice. The BALB/c mice were immunized twice with 50 μg *Dermatophagoides farinae* feces at 5 day intervals and then exposed daily to HM1, the HM1-depleted fraction (DH) or phosphate-buffered saline (PBS) for 10 days. At 120 h after the final exposure, alveolar macrophages were collected and mRNA was analyzed for major histocompatibility complex (MHC) class II, tumor necrosis factor (TNF)-α, interleukin (IL)-12 p40 and iNOS by reverse transcription–polymerase chain reaction. The mRNA levels of each sample were normalized against GAPDH expression levels. Polymerase chain reaction products were analyzed by agarose gel electrophoresis.

in HM1-exposed murine lung 120 h after inhalation. In the present study, we focused on the effects of HM1 on macrophage activation and examined pro-inflammatory cytokine gene expression from macrophages stimulated with HM1.

Incubation of splenic macrophages from non-immunized mice for 24 h with HM1 indicated significant iNOS gene expression and NO production. However, no expression of the iNOS gene was observed following DH stimulation, although the treatment induced TNF-α and IL-12 p40 gene expression. Nitric oxide production and iNOS gene expression are known to be generated following stimulation with lipopolysaccharide (LPS), platelet-activating factor and immune complexes. Our results demonstrate that HM1 directly elicits iNOS mRNA expression and NO production, implying that the expression of iNOS mRNA was the result of the activation of a different pathway from that activated by immune complexes that interact with FcγR on the surface of macrophages. We also demonstrated that iNOS gene expression induced by HM1 was not affected by polymyxin B, a cyclic cationic peptide antibiotic that neutralizes the biological activity of LPS.

The concentration of NO in exhaled air is increased in asthmatic patients and experimental animals.[21] The high levels of NO could have detrimental effects by causing epithelial damage and airway hyperreactivity.[22,23] These observations suggest strong correlations between NO production in the lung and allergic asthma.

Airway inflammation was developed by IL-5-producing Th2 cells following eosinophil infiltration and was abrogated by interferon (IFN)-γ-producing Th1 cells and soluble IL-4 receptor.[24,25] In fact, Th1 cells and alveolar macrophages identified in the BALF of asthmatic patients were more active than those from healthy subjects.[26–28] The Th1-type T cell mainly mediates cellular immune responses and induces delayed-type hypersensitivity in humans and murine lung tissue.[29] These results explain the complexity of allergic asthma and indicate the difficulty of treating asthma. Recent studies using animal models of asthma have indicated that iNOS inhibitors block the infiltration of inflammatory cells in antigen-exposed mouse lung.[30] These studies also indicate that iNOS-deficient mice do not show allergic airway inflammation induced by allergen exposure.[31] In addition, inhalation of an iNOS inhibitor by asthmatic patients inhibited NO production in the airway.[32] These facts support the feasibility of an NO-targeting agent as a novel approach for the treatment of asthma.

The mRNA expression of IL-12 p40 and TNF-α seemed to decrease in DH-exposed mice compared with HM1-exposed mice. However, the mRNA expression of both IL-12 p40 and TNF-α was measured semiquantitatively. Therefore, accurate expression of the mRNA of both IL-12 p40 and TNF-α needs to be determined. In the future, we must measure mRNA expression of both IL-12 p40 and TNF-α quantitatively to determine the relationship between NO production and the expression of these cytokines.

In the present study, we demonstrated that HM1 directly induced iNOS gene expression and NO production both *in vitro* and *in vivo* in alveolar macrophages. In conclusion, we demonstrated the ability of HM1 to

induce NO production by analyzing iNOS gene expression and measuring the release of NO. Generally, it is well known that immune complexes induce iNOS gene expression and NO production from macrophages. High-molecular size mite antigen fraction is a candidate for the production of NO to aggravate airway hyperresponsiveness in mite-allergic asthma.

REFERENCES

1 Kirby JG, Hargreave FE, Gleich GJ, O'Byrne PM. Bronchoalveolar cell profiles of asthmatic and nonasthmatic subjects. *Am. Rev. Respir. Dis.* 1987; **136**: 379–83.

2 Bradley BL, Azzawi M, Jacobson M *et al*. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: Comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J. Allergy Clin. Immunol.* 1991; **88**: 661–74.

3 Krug N, Madden J, Redington AE *et al*. T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 1996; **14**: 319–26.

4 Randolph DA, Carruthers CJ, Szabo SJ, Murphy KM, Chaplin DD. Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma. *J. Immunol.* 1999; **162**: 2375–83.

5 Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* 1999; **103**: 175–83.

6 Tategaki A, Kawamoto S, Hashimoto K *et al*. Induction of a proliferative response of T cells by a high-molecular antigen in *Dermatophagoides farinae* feces. *Int. Arch. Allergy Immunol.* 2002; **127**: 276–84.

7 Chanez P, Vignola AM, Lacoste P, Michel FB, Godard P, Bousquet J. Increased expression of adhesion molecules (ICAM-1 and LFA-1) on alveolar macrophages from asthmatic patients. *Allergy* 1993; **48**: 576–80.

8 Alam R. Chemokines in allergic inflammation. *J. Allergy Clin. Immunol.* 1997; **99**: 273–7.

9 Ward PA. Role of complement, chemokines, and regulatory cytokines in acute lung injury. *Ann. N.Y. Acad. Sci.* 1996; **796**: 104–12.

10 Persson MG, Zetterstrom O, Agrenius V, Ihre E, Gustafsson LE. Single-breath nitric oxide measurements in asthmatic patients and smokers. *Lancet* 1994; **343**: 146–7.

11 Kharitonov SA, Rajakulasingam K, Oconnor B, Durham SR, Barnes PJ. Nasal nitric oxide is increased in patients with asthma and allergic rhinitis and may be modulated by nasal glucocorticoids. *J. Allergy Clin. Immunol.* 1997; **99**: 58–64.

12 Kharitonov SA, O'Connor BJ, Evans DJ, Barnes PJ. Allergen-induced late asthmatic reactions are associated with elevation of exhaled nitric oxide. *Am. J. Respir. Crit. Care Med.* 1995; **151**: 1894–9.

13 Yeadon M, Price R. Induction of calcium-independent nitric oxide synthase by allergen challenge in sensitized rat lung *in vivo*. *Br. J. Pharmacol.* 1995; **116**: 2545–6.

14 Yan ZQ, Hansson GK, Skoogh BE, Lotvall JO. Induction of nitric oxide synthase in a model of allergic occupational asthma. *Allergy* 1995; **50**: 760–4.

15 Martin S. Dual action of iNOS-derived nitric oxide in allergen-induced airway hyperreactivity in conscious, unrestrained guinea pigs. *Am. J. Respir. Crit. Care Med.* 1998; **158**: 1442–9.

16 Shigeta S, Tanaka T, Yoshizane T *et al*. High-molecular-size mite allergens with high carbohydrate content in mite extract containing mite feces of *Dermatophagoides farinae*. *Jpn. J. Allergol.* 1995; **44**: 170–81.

17 Fujii S, Ono K, Shigeta S, Oka S, Jyo T, Yamashita U. B cell mitogenic activity of house dust mite, *Dermatophagoides farinae*, antigens. *Immunol. Lett.* 1996; **49**: 37–42.

18 Tategaki A, Kawamoto S, Okuda T *et al*. A high-molecular-size mite antigen (HM1) fraction aggravates airway hyperresponsiveness of allergic mice in house dusts and whole mite cultures. *Int. Arch. Allergy Immunol.* 2002; **129**: 204–11.

19 Jander S, Pohl J, D'Urso D, Gillen C, Stoll G. Time course and cellular localization of interleukin-10 mRNA and protein expression in autoimmune inflammation of the rat central nervous system. *Am. J. Pathol.* 1998; **152**: 975–82.

20 Kojima H, Urano Y, Kikuchi K, Higuchi T, Hirata Y, Nagano T. Fluorescent Indicators for imaging nitric oxide production. *Angew Chem. Int. Ed. Engl.* 1999; **38**: 3209–12.

21 Gustafsson LE, Leone AM, Persson MG, Wiklund NP, Moncada S. Endogenous nitric oxide is present in the exhaled air of rabbits, guiniea pigs and humans. *Biochem. Biophys. Res. Commun.* 1991; **181**: 852–7.

22 Flak TA, Goldman WE. Autotoxicity of nitric oxide in airway disease. *Am. J. Respir. Crit. Care Med.* 1996; **154**: S202–6.

23 Sadeghi Hashjin G, Folkerts G, Henricks PA *et al*. Peroxynitrite induces airway hyperresponsiveness in guinea pigs *in vitro* and *in vivo*. *Am. J. Respir. Crit. Care Med.* 1996; **153**: 1697–701.

24 Lack G, Renz H, Saloga J. *et al*. Nebulized but not parenteral IFN-gamma decreases IgE production and normalizes airways function in a murine model of allergen sensitization. *J. Immunol.* 1994; **152**: 2546–53.

25 Renz H, Bradley K, Enssle K, Loader JE, Larsen GL, Gelfand EW. Prevention of the development of immediate hypersensitivity and airway hyperresponsiveness following *in vivo* treatment with soluble IL-4 receptor. *Int. Arch. Allergy Immunol.* 1996; **109**: 167–76.

26 Chanez P, Vignola AM, Lacoste P, Michel FB, Godard P, Bousquet J. Increased expression of adhesion molecules (ICAM-1 and LFA-1) on alveolar macrophages from asthmatic patients. *Allergy* 1993; **48**: 576–8.

27 Cembrzynska-Nowak M, Szklarz E, Inglot AD, Teodorczyk-Injeyan JA. Elevated release of tumor necrosis factor-alpha and interferon-gamma by bronchoalveolar leukocytes from patients with bronchial asthma. *Am. Rev. Respir. Dis.* 1993; **147**: 291–5.

28 Hessel EM, Van Oosterhout AJ, Van Ark I *et al*. Development of airway hyperresponsiveness is dependent on interferon-gamma and independent of eosinophil infiltration. *Am. J. Respir. Cell Mol. Biol.* 1997; **16**: 325–234.

29 Negro Alvarez JM, Hernandez Garcia J, Pagan Aleman JA *et al*. The role of rhinovirus in allergic airway inflammation. *Allergol. Immunopathol.* 1997; **25**: 302–9.

30 Trifilieff A, Fujitani Y, Mentz F, Dugas B, Fuentes M, Bertrand C. Inducible nitric oxide synthase inhibitors suppress airway inflammation in mice through down-regulation of chemokine expression. *J. Immunol.* 2000; **165**: 1526–33.

31 Xiong Y, Karupiah G, Hogan SP, Foster PS, Ramsay AJ. Inhibition of allergic airway inflammation in mice lacking nitric oxide synthase 2. *J. Immunol.* 1999; **162**: 445–52.

32 Rhodes CJ, Taylor KW. Effect of interferon and double-stranded RNA on β-cell function in mouse islets of Langerhans. *Biochem. J.* 1985; **228**: 87–94.