Shortening of the Induction Period of Allergic Asthma in Cynomolgus Monkeys by *Ascaris suum* and House Dust Mite

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Abstract. The development of non-human primate models of asthma requires a period of time (e.g., 0.5 – 1 year). To develop the models in a short period, male cynomolgus monkeys were sensitized with dinitrophenyl-*Ascaris suum* (DNP-As) allergen by intraperitoneal and intramuscular injection and by intratracheal inhalation. All sensitized animals developed positive intradermal skin reaction to DNP-As. Sensitization elevated allergen-specific IgE levels in serum, the number of CCR4-positive T helper lymphocytes in peripheral blood, and IL-4 and IL-5 releases from phorbol 12-myristate 13-acetate- and ionomycin-stimulated peripheral blood. In addition, allergen challenge induced increases in lung resistance, airway inflammation, and hyperresponsiveness to inhaled methacholine. Next, animals were sensitized with house dust mite extracts (HDM) under the similar procedure. In these animals sensitized with DNP-As or HDM, inhaled fluticasone propionate and oral prednisolone inhibited the allergen-induced airway hyperresponsiveness. Taken together, monkey asthma models were successfully developed by sensitization with DNP-As or HDM under a short-term protocol (within 7 weeks). These models should be useful for the evaluation of anti-inflammatory drugs for asthma treatment.

Keywords: airway hyperresponsiveness, bronchoalveolar lavage, CCR4-positive cell, cytokine, methacholine

Introduction

Allergic asthma is a disease characterized by early and late responses, and airway hyperresponsiveness related to pulmonary inflammation. To better understand the mechanisms involved in the pathogenesis of allergic asthma, several species of laboratory animals have been used as asthma models. However, most animal models are inadequate to achieve an understanding of the mechanisms involved because of species differences and differences in the gene sequences in the immune and respiratory systems. Non-human primate models could reduce these concerns (1 – 9). Thus far, although wild-caught cynomolgus monkeys (10 – 13) and squirrel monkeys (14) sensitive to inhaled *Ascaris suum* allergen had been used, these wild-caught monkeys are not currently available. Recently, several studies showed that sensitization of monkeys with allergens could elicit allergen-specific IgE production (9) and allergic asthma symptoms upon allergen provocation (15, 16). However, a long period of time is required for the development of these models (15, 16). Development of a non-human primate model of asthma in a short period would be useful for target validation and evaluation of therapeutic candidates. For this purpose, we sensitized cynomolgus monkeys by periodic injection of dinitrophenyl-*Ascaris suum* (DNP-As) allergen and then inhaling the aerosolized allergen for 7 weeks. The allergen-induced immune response, airway inflammation, and dysfunction were characterized.

A limited number of animal models of allergy are
based on aeroallergens present in daily environment such as ragweed (17). Although the majority of asthmatic patients is sensitized with mites, very few investigators used mites or mite extracts as immunizing agents (9, 15, 16). Therefore, we sensitized cynomolgus monkeys by house dust mite extracts (HDM) under the similar protocol. In these sensitized animals, the effect of inhaled fluticasone propionate and oral prednisolone against the airway hyperresponsiveness were evaluated.

Materials and Methods

Animals

The animals used in this study were twelve male cynomolgus monkeys (Macaca fascicularis) weighing between 2.4 and 7.0 kg. They were individually kept in stainless-steel cages placed in an air-conditioned room maintained at 26 ± 3°C and at 55 ± 20% relative humidity with controlled illumination (light on 7.00 to 19.00) and fed a commercial diet and tap water ad libitum. The experiments were conducted in accordance with “Pharmaceutical Research Center Policy on the Care and Use of Laboratory Animals” of Kyowa Hakko Kogyo Co., Ltd. Every treatment, such as sensitization, challenge, pulmonary function test, skin test, and bronchoalveolar lavage (BAL), was conducted under anesthesia by intramuscular injection of 8 mg/kg ketamine (Sankyo Lifetech Co., Ltd., Tokyo) and 1 mg/kg xylazine (Bayer Medical, Ltd., Tokyo).

Sensitization

Monkeys were sensitized with DNP-As or HDM. The sensitization procedure is shown in Fig. 1a. A group of animals was sensitized three times by intraperitoneal (3.6 ml/kg) and intramuscular injection (0.4 ml/kg) weekly and boosted by intramuscular injection (0.4 ml/kg) of 5 mg DNP-As in 50 mg/ml Al(OH)₃ saline suspension 5 weeks after the first sensitization (a). In a similar manner, another group of animals was sensitized three times by intraperitoneal (2.4 ml/kg), intramuscular (0.8 ml/kg) and subcutaneous (0.8 ml/kg) injection weekly and then boosted by intramuscular (0.4 ml/kg) injection of 2.5 mg HDM in 50 mg/ml Al(OH)₃ saline suspension 5 weeks after the first sensitization (a). In addition, the animals were further sensitized by inhalation (30 breaths) of aerosolized DNP-As (4 mg/ml in saline) or HDM (5 mg/ml in saline) 4 and 7 weeks after the first sensitization (a). Animals sensitized with DNP-As were challenged by inhalation of the allergen (4, 40, 400, and 4000 µg/ml) on weeks 12 (Control trial, b) and 18. Then, fluticasone propionate was inhaled for 6 days and challenged on weeks 18 (Treatment trial, b). Animals sensitized with HDM were challenged by inhalation of the allergen (50, 500, and 5000 µg/ml) on weeks 12 (Control trial, b) and 21. Then, the animals were treated with prednisolone on week 21 (Treatment trial, b). The pulmonary function test and BAL were conducted before and after the allergen challenge.

Challenge

Sensitized animals were challenged by inhalation of the allergen on weeks 12 and 18 (DNP-As) or on weeks 12 and 21 (HDM). The challenge procedure is shown in Fig. 1b. Respiratory response to the allergen was tested immediately and that to methacholine was tested before and 24 h after the allergen challenge.
Respiratory response to methacholine and allergen

Animals were intubated a cuffed endotracheal tube (internal diameter = 5.0 mm) and mechanically ventilated with a volume-type respirator (Model 665; Harvard Apparatus, Holliston, MA, USA). Spontaneous breathing was stopped by inducing hyperventilation (50 breaths/min), and then lung resistance ($R_L$) was measured by standard computer analyses of the flow and pressure signals, digitized by BioSystem XA (Buxco Electronics, Inc., Wilmington, NC, USA) at a ventilation frequency of 30 breaths/min. Firstly, saline mist aerosolized by an ultrasonic nebulizer (NE-U07; Omron, Kyoto) was inhaled (15 breaths) via a flow pump (Flow rate: 1200 ml/min, Model 16-4; Encynova, Broomfield, CO, USA) connected to the inspiratory line of the respirator. Such setting of animals and equipment is common for allergen sensitization, challenge, and respiratory response test. Pulmonary function was monitored for 3 min to establish the baseline parameter. After that, aerosolized methacholine was inhaled (15 breaths), and then the response to methacholine was monitored until the maximum $R_L$ was confirmed. Cumulative doses were exposed until a 50% increase of baseline response in $R_L$ was observed, and the provocative concentration (PC$_{50}$) of methacholine was calculated by linear regression (Pre PC$_{50}$). One week later, animals were challenged by inhalation of aerosolized allergens (DNP-As: 4, 40, 400, and 4000 $\mu$g/ml; HDM: 50, 500, and 5000 $\mu$g/ml) until a 100% increase of baseline response in $R_L$. The immediate asthmatic response (IAR) was expressed as a percent of the saline control. Twenty-four hours after the allergen challenge, the methacholine responses were repeatedly assessed (Post PC$_{50}$). The value (%: Post PC$_{50}$/Pre PC$_{50}$) obtained by dividing the Post PC$_{50}$ with the Pre PC$_{50}$ was used for evaluation of airway hyperresponsiveness (AHR) in asthma models. This series of assessments is defined as “Control trial”. Approximately 5 weeks after the “Control trial”, the animals sensitized with DNP-As were treated with inhaled fluticasone propionate by a matching metered dose inhaler (MDI) and a cuffed endotracheal tube at a dose of 100 $\mu$g once daily for 6 days before allergen challenge. This series of assessments is defined as the “Treatment trial”. In the animal sensitized with HDM, approximately 8 weeks after the “Control trial”, the “Treatment trial” was done to evaluate the effect of prednisolone. Prednisolone was administered orally at a dose of 10 mg/kg once daily for 4 days before allergen challenge.

Bronchoalveolar lavage (BAL)

After the assessment of methacholine responses, a bronchoscope (BF-3C30 or BF-XP40; Olympus, Tokyo) was inserted through a cuffed endotracheal tube. A 15-ml aliquot of phosphate-buffered saline (PBS) was infused and retrieved after washing. The BAL fluid (BALF) was centrifuged, and the supernatant fraction was discarded. The pellet was stained with Turk stain solution (Nacalai Tesque, Inc., Kyoto), and the number of total cells was counted. Cytospin slides were prepared and stained with Diff-Quik stain solution (Sysmex Corporation, Hyogo). Differential cell counts were performed by counting 300 cells per sample.

Intradermal skin test

An intradermal skin test was performed 12 weeks after the first sensitization in order to check the sensitization condition easily. Hair of the thorax area was shaved, and allergens (DNP-As or HDM, 100 $\mu$g/ml) and diluent (physiological saline) were injected at the two different sites in a volume of 100 $\mu$l. The diameters of wheal at the site of injection were measured 20 min after the injection. When the diameter (either vertical diameter or transversal diameter) of the wheal was more than 15 mm, it was considered positive.

DNP-As-specific IgE ELISA

Peripheral blood was collected from each animal sensitized with DNP-As before and 12 weeks after the first sensitization. Ninety-six well plates were coated with DNP-As at a concentration of 10 $\mu$g/ml (50 $\mu$l/well, overnight at 4°C). The plate was washed and blocked with 1% BSA-PBS. After the blocking, serum samples were added to the wells (50 $\mu$l/well). The plate was incubated overnight at 4°C. IgE was detected by incubating the plate with peroxidase-labeled anti-human-IgE antibody (1:1000 dilution, 50 $\mu$l/well) for 1 h at room temperature. The plate was developed by using tetramethyl benzidine (TMB; Sigma-Aldrich Co., Tokyo). The reaction was stopped by addition of 1 mol/l sulfuric acid (Wako Pure Chemical Industries, Ltd., Osaka), and the absorbance at 450 nm was determined.

Cytokine assay

Peripheral blood was collected from each animal sensitized with DNP-As before and 3 weeks after the first sensitization. Peripheral blood was cultured in culture medium (RPMI 1640 plus 10% FBS plus 1% penicillin-streptomycin) for 24 h in the presence of phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and ionomycin (IOM, 1 $\mu$g/ml). From each well, the culture medium containing blood was each recovered and centrifuged at 4°C. The concentrations of interleukin (IL)-4, IL-5, and interferon (IFN)-γ in the supernatants were determined by using ELISA kits: human IL-4 BD OptEIA™ ELISA Set (BD Biosciences, San Diego, CA, USA).
Flow cytometer analysis of CCR4-positive T lymphocytes

Peripheral blood was collected from each animal sensitized with DNP-As before and 3 weeks after the first sensitization. Smear slide of peripheral blood was simultaneously stained with PE-labeled anti-human-CCR4 antibody (Becton Dickinson, Flanklin Lakes, NJ, USA; protein concentration: 0.2 mg/ml) and FITC-labeled anti-human-CD4 antibody (Becton Dickinson). The lysing solution (Becton Dickinson) was added and the mixture was left for 12 min at room temperature in the dark. After centrifugation (430 × g; 5 min; 4°C), the supernatant was discarded. The cell pellet was tapped and then added with washing buffer. The mixture was stirred and filtered with a nylon mesh. The stained cells were analyzed by an EPICS XL-MCL (Beckman Coulter, Fullerton, CA, USA).

Statistical analysis

All values are expressed as the mean ± S.E.M. A paired t-test was used for the analysis of cellular composition of the airway and flow analysis. A Sign-Wilcoxon test was used for the analysis of AHR, cytokine production and specific IgE. P<0.05 was considered statistically significant. Statistical analysis was not done in small experiments, because of the number of animals (n = 3).

Results

Intradermal skin test

All twelve sensitized animals developed positive intradermal skin reaction to DNP-As or HDM. Wheal size was greater than the negative diluent, and the diameter (either vertical diameter or transversal diameter) of the wheal was more than 15 mm (Table 1). Skin reactivity to HDM was a little stronger than that of DNP-As.

DNP-As-specific IgE in serum

Specific IgE was produced in all sensitized animals. Even though the levels varied among the animals, specific IgE was clearly and significantly increased by the sensitization (Fig. 2).

CCR4-positive T lymphocytes and cytokine production in peripheral blood

The number of CCR4-positive T lymphocytes in peripheral blood was significantly (P<0.05) increased 3 weeks after the first sensitization in all the animals (Fig. 3).

IL-4, IL-5, and IFN-γ were produced in peripheral blood by PMA (phorbol 12-myristate 13-acetate) and IOM (ionomycin) stimulation (Fig. 4: a, b, and c). Three weeks after the first sensitization, IL-4 and IL-5 levels were significantly (P<0.05) increased from the Pre-sensitization levels. In contrast, IFN-γ level was significantly (P<0.05) decreased compared with the Pre-sensitization level.

Airway responsiveness to allergen and methacholine

Airway responses to inhaled methacholine were measured before and 24 h after exposure to DNP-As aerosols. There was no change in baseline Rl, measured before and after allergen challenge (data not shown). The mean Pre and Post PC50 was 8.1 ± 0.9 and 2.1 ± 0.6 mg/ml, respectively (Fig. 5). Sensitized animals exhibited a statistically significant decrease in the Post PC50 (P<0.05). Animals were exposed to various concentrations of aerosolized DNP-As or HDM until a 100% increase in Rl (Fig. 6: a and b). Inhalation of increasing concentrations of allergen aerosol produced a dose-responsive increase in Rl and resulted in

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<tr>
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<tr>
<td>9</td>
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Table 1. Skin reactivity to DNP-As or HDM in sensitized cynomolgus monkeys

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Twelve weeks after the first sensitization, diluent (PBS) and allergens (100 µg/ml) were intradermally injected into the thorax area in a volume of 100 µl. Swelling developing at the site of injection was measured 20 min after the injection. When the diameter (either vertical diameter or transversal diameter) of the wheal was more than 15 mm, it was considered positive.
moderate airway obstruction. The immediate increase (IAR) in $R_L$ was $81 \pm 318\%$ and $174 \pm 520\%$ in DNP-As and HDM sensitized animals, respectively.

**Allergen-induced airway inflammation**

In animals sensitized with DNP-As, the mean volume of BAL fluid retrieved did not significantly differ between Pre-challenge ($9.2 \pm 0.6 \text{ ml}$) and Post-challenge ($7.7 \pm 0.9 \text{ ml}$). Thus, all BALF cell data were compared on per ml basis (Fig. 7a). The majority of the cells in Pre-challenge BALF was macrophages. Allergen challenge induced an increase in the number of total cells, but non-significantly. This may be caused by a big difference among animals. Concomitant with the increase in total cells, there was marked increase in the number of eosinophils by allergen challenge. In contrast, there were no significant differences in the numbers of neutrophils or lymphocytes between Pre- and Post-challenge. Allergen-induced airway inflammation in animals sensitized with HDM showed the similar reaction as the animals sensitized with DNP-As, but the inflammatory reaction to HDM (Fig. 7b) was a little stronger than that of DNP-As.

**Effects of steroids on the AHR**

In the Control trial in the animals sensitized with DNP-As, AHR values averaged $32 \pm 6\%$ (Fig. 8). When
these same animals were treated with fluticasone propionate, AHR values in the Treatment trial averaged 91 ± 7%. Fluticasone propionate markedly inhibited allergen-induced AHR in the asthma model sensitized with DNP-As. In the Control trial in the animals sensitized with HDM, AHR values averaged 26 ± 6% (Fig. 8). When these same animals were treated with prednisolone, AHR values in the Treatment trial averaged 91 ± 5%. Prednisolone markedly inhibited allergen-induced AHR in the asthma model sensitized with HDM.

**Discussion**

There are many papers about monkey asthma models that used cynomolgus monkeys. Cynomolgus monkeys exhibit 95% homology with humans (18), making this a candidate species suitable for genomic studies. Pre-
the model sensitized with DNP-As (n = 3). Values are means ± S.E.M.

![Graph](image_url)

**Fig. 8.** Effects of steroids on the AHR in the asthma models sensitized with DNP-As or HDM. AHR was assessed before and at approximately 5 weeks (DNP-As) and 8 weeks (HDM) after the Control trial. The value (%: Post PC_{50}/Pre PC_{50}) obtained by dividing the Post PC_{50} with the Pre PC_{50} was used for evaluation of AHR. In the model sensitized with DNP-As (n = 3), fluticasone propionate was inhaled at a dose of 100 µg once daily for 6 days by matching MDI. In the model sensitized with HDM (n = 3), prednisolone was orally administered at a dose of 10 mg/kg once daily for 4 days.

Previously, we have also developed an *Ascaris*-induced asthma model by using wild-caught cynomolgus monkeys naturally sensitized with *Ascaris*. The model could reflect not all, but several aspects of the symptoms in asthma patients. However, this naturally sensitized model has some undesirable issues, including the big variance among monkeys. In addition, wild-caught cynomolgus monkeys are not available for experiments now because of the Convention on International Trade in Endangered Species of Wild Fauna and Flora. For these reasons, actively immunized monkeys should be used for the development of asthma models. Several groups published articles about such models (15, 16). The methods reported required a long term such as a half-year or a year. If we can develop an asthma model in shorter periods of time, it would be useful for drug evaluation and investigation of the pathogenesis of asthma. This study was done to develop such models.

Allergic asthma can be defined as a disease characterized by a chronic inflammation disorder of the airway. Based on this definition, we have checked four physiological and immunological parameters before evaluating drugs in this model. First, it was confirmed whether the sensitized monkeys really became sensitive to the allergen. This was confirmed by positive skin reactions against sensitized allergens (Table 1) and increase in allergen-specific IgE level in the serum (Fig. 2). Second, it was confirmed whether the animals developed airway obstruction after allergen challenge (Fig. 6). This was confirmed by the increase in the airway resistance associated with cough and rapid shallow breathing.

These airway obstructions were reversed by the treatment with aerosolized albuterol and epinephrine injection (data not shown). This meant that the increased airway resistance was based on the airway obstruction. Third, it was confirmed whether the animals developed airway inflammation. There was an increase in the number of inflammatory cells in the bronchoalveolar lavage after allergen challenge (Fig. 7). The sensitized animals exhibited increased number of CCR4-positive T helper lymphocytes in peripheral blood (Fig. 3). In addition, IL-4 and IL-5 release from peripheral blood was increased by allergen challenge (Fig. 4). Fourth, it was confirmed whether the sensitized animals developed non-specific airways responsiveness. This was confirmed by the methacholine inhalation test (Fig. 5). Allergen challenge induced a four-fold reduction in the dose of methacholine aerosol required for producing a 50% increase in the airway resistance. The evidence, such as sensitivity to the allergen, Th2-dominant inflammation in the airway, acute reversible bronchoconstriction and airway hyperresponsiveness assured that this model is an appropriate monkey asthma model.

In this study, two allergens were used. One was DNP-As and the other was HDM. Both allergens are known to induce Th2-type immunological responses in many animal species. First, we tried to induce asthmatic responses by DNP-As because naturally sensitized monkey asthma models were developed by *Ascaris* exposure. It was a good model, but we wanted more stable and stronger responses. We first succeeded to induce asthmatic responses by the sensitization with DNP-As. Then, we tried to induce asthmatic responses by the sensitization with mites, because dust mites are commonly associated with human asthma (19) and has been shown to be effective in inducing symptoms of asthma in common laboratory animals, including mice (20, 21), rats (22), rhesus monkeys (9, 15), and rabbits (23). In addition, natural sensitivity to dust mites had been documented in monkeys (24), increasing the relevance of the allergen. We added the subcutaneous injection in the sensitization by HDM because several studies showed that sensitization by subcutaneous injection with HDM could elicit allergen-specific IgE production (9) and allergic asthma symptoms (15, 16). As we expected, the sensitization with HDM succeeded to induce asthmatic responses as well as DNP-As. Although we did not measure the HDM-specific IgE in the animals sensitized with HDM, skin reaction and IAR were as strong as, or rather stronger, than those caused by DNP-As. These 2 parameters, antigen-induced immediate skin reaction and IAR, are mainly considered to be dependent on antigen-specific IgE. Accordingly, HDM-specific IgE should be increased at
the same level as DNP-As, it is speculated.

Finally, we tested whether this model was sensitive to oral and inhaled corticosteroids (Fig. 8). Administration of prednisolone and fluticasone propionate at clinically relevant doses improved airway hyperresponsiveness.

In conclusion, we have developed a cynomolgus monkey asthma model with two different allergens by a short-period protocol. This model should be useful for the evaluation of anti-asthma drugs. Moreover, it should be useful for the investigation of the pathogenesis of asthma.

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References