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Experimental Monkey Model Sensitized with Mite Antigen

Abstract

Background: Monkeys are considered to have an immune system very similar to that of humans, as compared with mice, rats, and guinea pigs. Although primate allergic models to several pollen allergens have been developed, no model of house dust mite allergy has been reported. In this study, we attempted to induce type I allergy to mite allergens in rhesus monkeys. **Methods:** Six rhesus monkeys were immunized subcutaneously with crude mite extract adsorbed on aluminum hydroxide for 4 months. Then 5 monkeys positive for IgE production to mite extract were further immunized subcutaneously and conjunctivally with recombinant Der f 2 (rDer f 2). The status of sensitization to mite extract and rDer f 2 in monkeys was examined before and after the immunization. Plasma antigen-specific IgE and IgG levels, cutaneous reaction, and histamine release from peripheral blood leukocytes were measured. After conjunctival immunization, immediate conjunctivitis and leukocyte influx into conjunctiva after rDer f 2 challenge were examined. **Results:** After immunization with crude mite extract, 5 of 6 sensitized monkeys showed IgE response to the mite, and 4 out of 5 rDer f 2-sensitized monkeys exhibited IgE production to rDer f 2. Three monkeys sensitized with rDer f 2 showed immediate conjunctivitis and conjunctival eosinophilia after applying rDer f 2 to their eyes. Sensitized animals also showed IgG response to mite antigens. **Conclusion:** Four rhesus monkeys were positive for IgE production and allergic reactions to both mite extract and rDer f 2. These monkeys could represent a useful model for studying the development and regulation of house dust mite allergy.

Key Words

Mite
Recombinant Der f 2
Rhesus monkey
IgE

Introduction

In industrialized countries, a considerable number of people has allergic symptoms caused by type I allergies, and recently the number of allergic patients needing medical care has increased remarkably [1, 2]. Among the many aeroallergens, house dust mites are the most common [3], and any approach to the experimental study of house dust mite allergy to improve medical treatment requires a suitable animal model, such as the mouse or guinea pig [4, 5].

However, it is suspected that the entire mechanism of histamine release from basophils or mast cells and the characteristics of IgE antibody of these animals differ somewhat to those of humans.

In the 1960s to 1970s, primates were used as a model of immediate hypersensitivity in humans through passive sensitization of the monkeys with human reaginic sera and bronchial asthma after *Ascaris* infection [6, 7]. Recently, several studies showed that active immunization of monkeys with pollen allergens could elicit allergen-specific IgE

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production and allergic symptoms upon antigen provocation [8, 9]. However, there have been no reports on the immune response to mite allergens in monkeys.

In the present study, we attempted the induction of sensitization in rhesus monkeys with mite allergens as seen in the atopic phenotype in man. We used crude extract from a house dust mite, *Dermatophagoides farinae*, and recombinant Der f 2 (rDer f 2) which has been shown to exhibit allergenic activity in humans comparable with native Der f 2 prepared from crude mite extract [10, 11]. Monkeys were subcutaneously and conjunctivally immunized with crude mite extract and rDer f 2, and examined for allergic reactions after antigen challenge.

Materials and Methods

Animals

Six male *Macaca mulatta* monkeys weighing 4–6 kg were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were individually kept in stainless steel cages placed in an air-conditioned room maintained at $24 \pm 2^\circ\text{C}$ and $55 \pm 15\%$ relative humidity with controlled illumination (light on from 7.00 to 19.00), and fed a commercial diet and tap water ad libitum. All 6 monkeys were negative for mite allergens before the immunization, as determined by the cutaneous reactivity test and histamine release assay of peripheral blood leukocytes.

Antigen

rDer f 2, clone 1 [10], was kindly supplied by the Nikka Whisky Distilling Co., Ltd. (Chiba, Japan). Crude body extract from house dust mite, *D. farinae* (mite extract), was kindly supplied by Torii Co., Ltd. (Chiba, Japan). This mite extract contained about 0.3% (w/w) native Der f 2, as determined by enzyme-linked immunosorbent assay (ELISA). Allergens were dissolved in PBS before use.

Immunization

Monkeys were immunized according to the schedule shown in figure 1. Allergen solution containing 10 μg of crude mite extract adsorbed in 9 mg of aluminium hydroxide was injected subcutaneously into 6 monkeys 8 times at intervals of 2 weeks. After this, 5 of the 6 immunized monkeys, which showed IgE production against mite extract, were subcutaneously immunized with either 10 μg of crude mite extract + 10 μg of rDer f 2 or 10 μg of rDer f 2 alone once a month for 5 months, then subjected to conjunctival sensitization with mite extract and rDer f 2. For conjunctival sensitization, 0.05 ml of antigen solution containing 0.25 mg of crude mite extract and 0.25 mg of rDer f 2 was applied to both eyes for 4 sequential days using a microsyringe. Conjunctival sensitization was performed twice at intervals of 3 weeks.

Determination of Plasma Antigen-Specific IgE and IgG Levels

Heparinized blood was collected from the saphenous veins of individual monkeys. Plasma was separated from the whole blood and frozen until we measured antigen-specific IgE and IgG levels. Anti-mite and anti-rDer f 2 IgE were determined by ELISAs following the recommendations of the manufacturers of the antibodies. Plasma was 30-fold diluted with PBS containing 0.05% (w/v) Tween 20 and 5% bo-

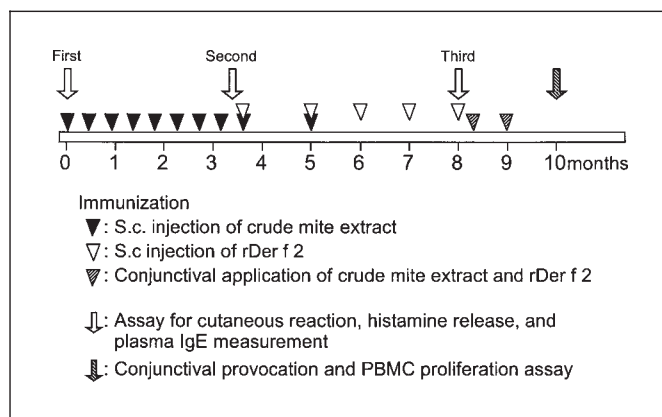


Fig. 1. Experimental schedule. 6 rhesus monkeys, No. 1101–1106, were immunized with crude mite extract for 4 months, and 5 of the 6 monkeys, 1101, 1102, 1104, 1105, and 1106 were further immunized with mite extract + rDer f 2 for 5 months. Status of sensitization with mite allergens was examined with 3 (first to third) assays and a conjunctival provocation test.

vine serum albumin fraction V before IgE determination. In the assays, we used mite extract or rDer f 2 as a capturing antigen and biotinylated goat anti-human IgE (No. BA-3040, Vector, Calif., USA) as a detecting antibody. The Vector antibody was chosen from several commercial antihuman IgE antibodies because of its high affinity to monkey IgE. Biotinylated antibody was labeled with avidin-conjugated peroxidase (No. A-2004, Vector, Calif., USA) for color development using 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) and H_2O_2 as substrates. Diluted plasmas were reacted in the well (1) with capturing antigen or (2) without antigen. Change in absorbance at 405 nm (ABS 405 nm), that is the difference between ABS 405 nm of (1) and (2), was calculated, and IgE levels were expressed by change in ABS 405 nm. Antimite extract and anti-rDer f 2 IgGs were determined by the same method as IgE measurement with the following modifications. Plasma was 10,000-fold diluted for determining anti-mite IgG, and 1,000-fold for anti-rDer f 2 IgG, respectively. Biotinylated goat anti-human IgG (No. BA-3000, Vector, Calif., USA) was used as a detecting antibody.

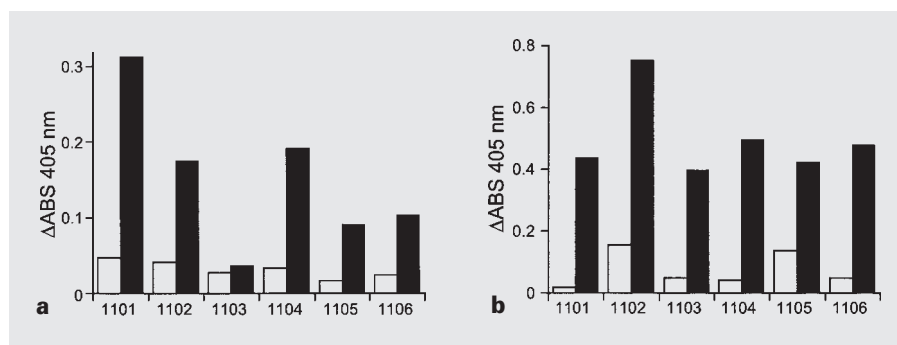
Assay of Histamine Release from Peripheral Blood Leukocytes

Peripheral blood leukocytes were separated from whole blood by dextran sedimentation as described [12]. Leukocytes were incubated for 40 min at 37°C in the presence or absence of mite allergens (0.01–10 $\mu\text{g}/\text{ml}$ of mite extract or rDer f 2). Histamine released into the supernatant or remaining in the cells was measured with a commercial ELISA kit (HRT[®], Miles, Wash., USA). All data reflect means of duplicate determinations.

Cutaneous Reactivity Tests

Animals were anesthetized by intramuscular injection of ketamine chloride (10 mg/kg), then intradermally injected with 0.1 ml of antigen solution (0.01–100 $\mu\text{g}/\text{ml}$ of mite extract or rDer f 2) into sites in the abdomen. After 15–20 min, the minimum concentration of antigen inducing positive erythema wheals was determined.

Fig. 2. Anti-mite extract IgE (a) and IgG (b) levels in plasma as determined by an ELISA in the first (□) and second (■) assay, which corresponded to before and after the immunization with mite extract.



Plasma IgE measurement, histamine release assay, and cutaneous reactivity test were performed 3 times (first, second, and third assay) as shown in figure 1. rDer f 2 was not used in the first assay.

Conjunctival Provocation Tests

Allergic conjunctivitis was provoked under anesthesia by two applications of rDer f 2 solution to the eyes at 24-hour intervals. The development of erythema and wheals on the eyelids, and changes in the distance between the upper and lower eyelids were examined. During anesthetization, the eyes remained open and the distance between the upper and lower eyelids was almost constant in our monkeys. Since this distance was decreased by conjunctivitis correlating with the degree of wheals of the eyelids, we used it as an index of the reaction. We also determined leukocyte numbers and β -hexosaminidase activities in the conjunctival lavage fluid (CLF).

After anesthetization with ketamine chloride, the distance between the upper and lower eyelids of both eyes was measured, and 0.05 ml of 10 mg/ml rDer f 2 or saline was applied to the eyes. After 20 min, the distance between the eyelids of both eyes was remeasured, the eyes washed with 0.1 ml of saline, and CLF recovered. After separation of cells and supernatant of CLF by centrifugation, hexosaminidase activity in the supernatant, possibly released from mast cells in the conjunctiva [13–15], was determined by using *p*-nitrophenyl N-acetyl- β -*d*-glucosaminide as a chromogenic substrate. Hexosaminidase activity was expressed by ABS 405 nm. Cells were resuspended in an equal volume of saline to supernatant, and a cytospin preparation made from 0.05 ml of cell suspension. Differential leukocyte counting was performed after Dif-quick® stain. Conjunctival provocation as described above was repeated once after 24 h. rDer f 2 solution (or saline) was applied to the same eye in the first and second conjunctival challenge.

Lymphocyte Proliferation Assays

The proliferative status of peripheral blood mononuclear cells (PBMC) was evaluated by flow-cytometric analysis of the expression of proliferating cell nuclear antigen (PCNA). PCNA is a processivity factor required by eukaryotic DNA polymerase delta and expressed in the cellular nuclei in S/G2/M phase [16]. It has been shown that the amount of PCNA expression accurately reflects the status of cellular proliferation [17, 18].

PBMC were prepared on a Ficoll-paque® (No. 17-0840-02, Pharmacia, Uppsala, Sweden) density gradient and incubated in serum-free medium, AIM-V (Gibco, N.Y., USA) in the presence or absence of mite allergen (5 μ g/ml of rDer f 2) for 72 h. After incubation, cells were fixed with 5% formalin and treated with 0.1% saponin for membrane permeabilization to enable antibodies to detect the intracellular

antigen. Cells were then treated with FITC-conjugated mouse anti-human PCNA, clone PC-10 (No. 32554A, Pharmingen, Calif., USA) that is known to cross-react with primate PCNA [19], and subjected to flow-cytometric analysis with FACScan (Becton-Dickinson, Calif., USA). Proliferative activities were expressed by the PCNA-positive ratio:

$$\frac{\% \text{ of PCNA-positive cells cultured in allergen-containing medium}}{\% \text{ of PCNA-positive cells cultured in allergen-free medium}}$$

Results

Plasma Antigen-Specific IgE and IgG Levels

Mite extract and rDer f 2-specific IgE levels were determined before and after immunizations by ELISA. Anti-mite extract IgE levels were raised after 8 immunizations with mite extract in 5 (1101, 1102, 1104, 1105, 1106) of the 6 sensitized monkeys as shown in figure 2a. Anti-mite IgE levels determined in the third assay were comparative with the results in the second assay (data not shown). Since the crude mite extract used in the present study contained native Der f 2 antigen, we expected to detect anti-Der f 2 IgE in monkeys in the second assay. However, no Der f 2-specific IgE was detected (fig. 3a). We immunized 5 monkeys positive for anti-mite extract IgE with rDer f 2 5 times. After the immunization, IgE against rDer f 2 was detected in 3 (1101, 1102, 1104) of the 5 monkeys (fig. 3a).

We also determined anti-mite extract and anti-rDer f 2 IgG levels before and after immunizations by ELISA (fig. 2b, 3b). All 6 monkeys showed anti-mite extract IgG production, and 5 monkeys (1101, 1102, 1104, 1105, 1106) showed an anti-rDer f 2 IgG response. The positive and negative distributions for IgG production were dependent on the experience of immunization with mite extract and rDer f 2. Antigen-specific IgG levels did not show a clear correlation with IgE levels.

Fig. 3. Anti-rDer f 2 IgE (**a**) and IgG (**b**) in plasma as determined by an ELISA in the second (□) and third (■) assay, which corresponded to before and after the immunization with rDer f 2.

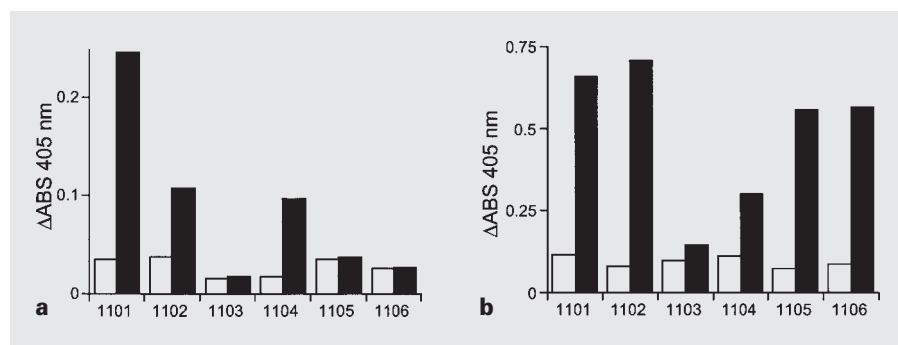


Table 1. Minimum provocative concentration of mite extract and rDer f 2 for cutaneous reaction and histamine release

Provoking antigen	Mon-key	s.c. immunization		Cutaneous reaction μg/ml			Histamine release μg/ml		
		mite	rDer f 2	1st assay	2nd assay	3rd assay	1st assay	2nd assay	3rd assay
Mite ¹	1101	○		>100	1	1	>10	0.01	n.d.
	1102	○		>100	10	10	>10	0.01	n.d.
	1103	○		>100	>100	>100	>10	>10	n.d.
	1104	○		>100	10	10	>10	0.01	n.d.
	1105	○		>100	10	10	>10	0.1	n.d.
	1106	○		>100	10	10	>10	1	n.d.
rDer f 2	1101		○	n.d.	>10	0.1	n.d.	>10	0.01
	1102		○	n.d.	>10	1	n.d.	>10	0.1
	1103			n.d.	>10	>10	n.d.	>10	>10
	1104		○	n.d.	>10	0.1	n.d.	>10	0.1
	1105		○	n.d.	>10	1	n.d.	>10	0.01
	1106		○	n.d.	>10	>10	n.d.	>10	>10

= Done; + = not done; n.d. = not determined.
¹ 'Mite' stands for crude mite extract.

Cutaneous Reactivity Test

An immediate skin reaction to mite extract and rDer f 2 appeared after immunizations as shown in table 1. The positive and negative distributions for the cutaneous reaction in 6 monkeys almost coincided with those of plasma IgE levels both for mite extract and rDer f 2, with the exception of No. 1105/rDer f 2. No late phase cutaneous reaction was observed 24 h after the intradermal injection of antigens, however. Cutaneous reactivity to mite extract and rDer f 2 4 months after the last subcutaneous immunization with rDer f 2 was comparable to that in a third assay (data not shown).

Assay of Histamine Release from Peripheral Blood Leukocytes

Although no histamine release was observed before immunization with antigen in any of the monkeys (data not shown except for No. 1101), a reaction appeared in several monkeys after immunization. The percent histamine release from leukocytes caused by mite extract in the second assay and by rDer f 2 in the third assay is shown in figures 4 and 5, respectively. The positive and negative distribution for the histamine release in 6 monkeys coincided with that of cutaneous reactions to both mite extract and rDer f 2. However, the minimum antigen concentration at which a histamine release was provoked was not correlated with that for the cutaneous reaction (table 1).

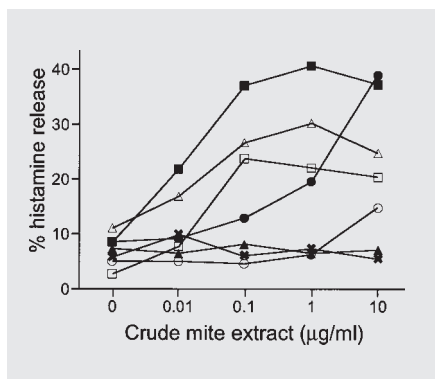


Fig. 4. Histamine release from peripheral blood leukocytes after stimulation with mite extract in the second assay. Reaction with 0 µg/ml of the antigen means the spontaneous release of histamine. ■ = No. 1101; □ = No. 1102; ▲ = No. 1103; △ = No. 1104; ● = No. 1105; ○ = No. 1106. Histamine release in No. 1101 before immunization is shown for comparison (×).

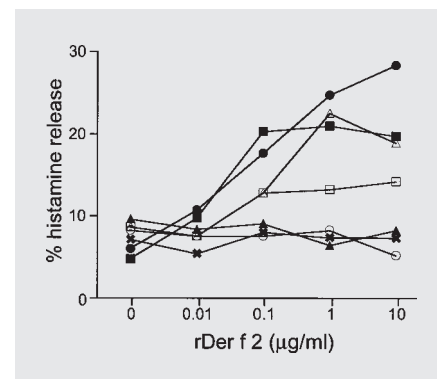


Fig. 5. Histamine release from peripheral blood leukocytes after stimulation with rDer f 2 in the third assay. ■ = No. 1101; □ = No. 1102; ▲ = No. 1103; △ = No. 1104; ● = No. 1105; ○ = No. 1106. Histamine release in No. 1101 before immunization is shown for comparison (×).

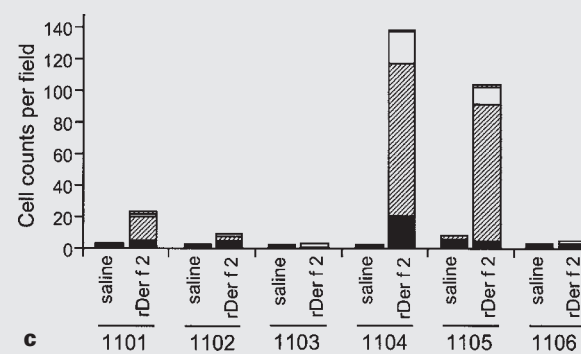
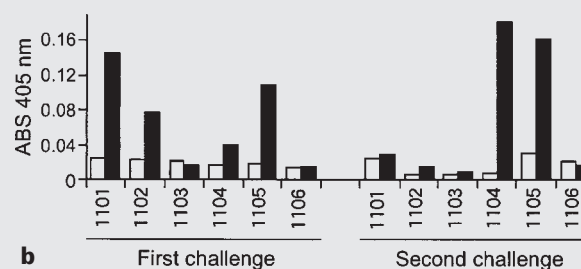
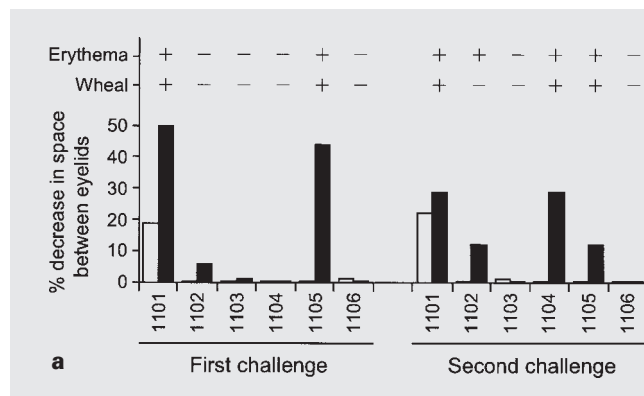


Fig. 6. Evaluation of conjunctivitis after two applications of rDer f 2 to the eyes with a 24-hour interval. **a** Visible symptoms after the first and second challenge. '+' and '-' stand for 'observed' and 'not observed' in the eyes treated with rDer f 2, respectively. Neither erythema nor wheals were observed in the eyes challenged with saline. Values of the eyes treated with saline (□) and rDer f 2 (■). **b** Hexosaminidase activity of the CLF collected 20 min after the challenges. Values of the eyes treated with saline (□) and rDer f 2 (■). **c** Leukocyte counts in the CLF taken from eyes treated with saline or rDer f 2 after the second challenge. ■ = Macrophages; ▨ = eosinophils; □ = neutrophils; ▩ = lymphocyte.

Conjunctival Provocation Test

Since several monkeys began to show allergic symptoms in the course of immunization, we evaluated the degree of the conjunctivitis upon provocation with rDer f 2. Conjunctival challenge was performed twice with a 24-hour interval (first and second challenge). Allergic symptoms scored as

described in Materials and Methods are shown in figure 6a. Hexosaminidase activity and cellular count in the CLF are shown in figure 6b and c, respectively. A positive immediate reaction was observed in No. 1101 and 1104 after the first challenge with rDer f 2, and No. 1101, 1102, 1104 and 1105 after the second challenge with the antigen. No reac-

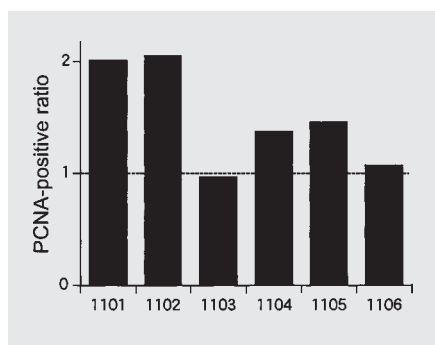


Fig. 7. PBMC proliferation after culture with 5 $\mu\text{g}/\text{ml}$ of rDer f 2 for 72 h, as determined by flow-cytometric analysis of PCNA expression.

tion was observed in eyes challenged with saline except in No. 1101. Although no leukocyte influx was observed after the first challenge (data not shown), a severe infiltration by eosinophils and neutrophils was observed in No. 1104 and 1105, and a moderate reaction in No. 1101, after the second challenge. Hexosaminidase activity was correlated with immediate reaction as scored by the space between eyelids in the first challenge, and correlated with numbers of infiltrating leukocytes on the 2nd day.

Lymphocyte Proliferation

PBMC were cultured in the presence or absence of 5 $\mu\text{g}/\text{ml}$ of rDer f 2 for 72 h, then subjected to flow-cytometric analysis of PCNA expression. PBMC taken from 4 monkeys positive for cutaneous, conjunctival reaction, and histamine release after rDer f 2 challenge showed a proliferative response to rDer f 2 stimulation (fig. 7).

Discussion

For the study on allergic diseases, several experimental models showing type I reaction or eosinophilic inflammation have been developed in mice, rats, and guinea pigs by using *Ascaris*, ovalbumin, and other allergens [20]. In addition, several studies showed that active immunization of monkeys with pollen allergens including recombinant Bet v 1 and Bet v 2 induced antigen-specific IgE production and some allergic symptoms [8, 9]. Concerning a mite allergic model, Hsiue et al. [4] recently reported the induction of allergic airway inflammation in guinea pigs that showed severe eosinophilia of the airway and immediate bronchoconstriction after intravenous injection of crude mite extract [4]. We have also developed murine allergic models exhib-

iting immediate airway constriction or neutrophil influx into the airway after antigen inhalation by using rDer f 2 as a sensitizing and provoking antigen [21, 22]. However, no reports on the induction of anti-mite IgE in primates have been published to our knowledge. In the present study, we attempted to induce sensitization in rhesus monkeys with mite allergens. We used crude body extract and rDer f 2 as sensitizing antigen. rDer f 2 has recently been developed, and shown to exhibit allergenic activity comparable with native Der f 2 prepared from crude mite extract in humans and mice [9, 10]. Because of the high allergenic activity of rDer f 2 and the fact that over 80% of patients with house dust mite allergy are sensitized to Der f 2 allergens as shown in several studies [23, 24], rDer f 2 may be a promising allergen for the diagnosis and immunotherapy of house dust mite allergy, and for basic research into allergies and immunology.

After 4 months of subcutaneous immunization with crude mite extract, 5 of 6 monkeys became sensitized to mite extract, as determined by three assays, ELISA for plasma IgE, cutaneous reactivity, and histamine release from leukocytes. Five monkeys were positive for mite extract in all 3 assays, while 1 was negative in all assays. Although several studies in humans have shown a correlation between plasma IgE levels, cutaneous reactivity, and histamine release [25, 26], this was not clear in the sensitized monkeys in the present study. To clarify the relationships, more monkeys must be included in the experiment. Since mite extract contains a little native Der f 2, we expected an IgE response to Der f 2 after the immunization with crude mite extract. However, no IgE production to Der f 2 was observed in any of the monkeys in the second assay. The amount of Der f 2 (about 0.03 $\mu\text{g}/\text{immunization}$) seemed to be too small to elicit an IgE response. To enable immunological analysis of the monkeys with rDer f 2, and to examine the sensitizing activity of rDer f 2 in monkeys, we immunized the 5 monkeys positive for mite extract with rDer f 2. After 4 months of immunization, an IgE response to rDer f 2 was detected in 4 monkeys. Experimental evidence of the capacity of rDer f 2 to induce IgE response was obtained. From the results of assays using crude mite extract and rDer f 2, IgE production in response to rDer f 2 was comparable with that to mite extract which contained many minor antigens. Since many mite-allergic patients react to Der f 2 allergens, the 4 monkeys positive for both rDer f 2 and mite extract should be a good model of house dust mite allergy in humans. Although sensitivity and reactivity to the sensitizing antigen in our monkeys were comparable with that of pollen-sensitized animals in several studies [8, 27], monkeys showing a larger histamine release from basophils (60–80%) and a

lower provocative concentration for skin reaction ($<0.1 \mu\text{g/ml}$ of antigen) in response to *Ascaris suum* or Japanese cedar allergens were reported [28, 29]. Additional immunization of our monkeys may achieve a stronger sensitization to mite antigens. All monkeys immunized with either mite extract alone or mite extract + rDer f 2 showed an IgG response to the immunizing antigen irrespective of the IgE response. In humans, it has been reported that many healthy subjects as well as most of the mite-allergic patients showed an IgG production to mite antigens. Anti-mite IgG4 levels in patients seem to be higher than that in normal people, while differences of other IgG subclasses specific for mite antigens between normal and allergic people are still unclear [30–34]. We are planning to study anti-mite IgG subclasses in sensitized monkeys to clarify the differences between IgE-positive and IgE-negative animals.

A type I allergic reaction is triggered by the release of chemical mediators from basophils, connective tissue-type mast cells, and mucosal mast cells [35, 36]. On the basis of cutaneous reaction and histamine release assay of peripheral blood leukocytes, we were able to examine the activity of basophils and skin mast cells most of which are of the connective tissue-type. Bronchial asthma, a major symptom of house dust mite allergy, is a reaction of the airway where mucosal mast cells are more dominant than the connective tissue-type ones [37, 38]. However, bronchial provocation tests in monkeys require a large amount of mite allergens which are expensive, and there are some difficulties in preparing biopsy specimens and collecting infiltrating leukocytes from the airway of the monkeys. To study the reaction caused by mucosal mast cells, we chose the conjunctiva as the sensitizing and provoking sites [39, 40]. At the beginning of the conjunctival immunization, monkeys showed few allergic symptoms after application of mite extract and rDer f 2 to the eyes, in spite of the systemic production of antigen-specific IgE. However, after two sets of conjunctival immunization, 4 monkeys reacted clearly to the conjunctival challenge with rDer f 2. Local application induced local hyperreactivity in monkeys as well as in rodents sensitized sequentially by intranasal administration or inhalation of antigens [41, 42]. Repeated antigen challenges might induce local IgE synthesis, destruction of the mucosal barrier, or other immunological changes which promote conjunctivitis [43–45]. Three of the 4 sensitized monkeys also showed eosinophilia 24 h after antigen challenge to conjunctiva, which is frequently observed in patients with ocular allergy [46, 47]. In the present study, we initially immunized monkeys by subcutaneous injection with mite antigens adsorbed in aluminum hydroxide according to several studies in primates. However, reports on naturally occurring

Japanese cedar pollinosis in Japanese monkeys as well as studies using rodents suggest the possibility that local immunization without adjuvant may induce systemic IgE production in monkeys [29, 42, 48]. Repeated conjunctival and intranasal immunization of naive monkeys with mite antigen alone, which resembles the natural way of sensitization to aeroantigen in humans, remains to be done. Results of the lymphocyte proliferation assay suggested that the monkeys were sensitized at the T cell level. Cytokine production from PBMC after antigen stimulation is necessary to clear the Th1/Th2 bias that is a major feature of type I allergy in humans [49, 50].

It has been reported that several antiallergic drugs which were effective in rodent models of allergy were not fully effective in human patients [51, 52]. The development of models that utilize primates, which are genetically close to humans, is therefore important not only for the establishment of medical treatments of allergy but also for the study of the mechanism of the occurrence. In the present study, we developed a monkey model for the house dust mite allergy, which showed a cutaneous reaction, histamine release from basophils, and conjunctivitis. We are planning to investigate the T cell function in sensitized monkeys.

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