

Experimental Cedar Pollinosis in Rhesus Monkeys

23513

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Abstract. In order to develop an experimental model for human cedar pollinosis, 4 rhesus monkeys (*Macaca mulatta*) were subcutaneously immunized with crude cedar pollen antigen, which contained 1.0 µg or 10 µg protein with aluminium hydroxide gel, six times every month. All 4 animals showed immediate-type cutaneous reactivity. Serum IgE antibody responses against the pollen antigen, as quantitated by the Prausnitz-Küstner reaction, were seen in 3 of 4 monkeys. Radioallergosorbent test scores increased in 2 monkeys and remained at higher levels throughout the study. Histamine release from leukocytes was positive in all animals. In conjunctival provocation tests, they showed clinical signs such as eye-scratching after the antigen was dropped into their eyes. This reaction is similar to that observed in humans and suggests this animal model might be useful in studying human cedar pollinosis.

Introduction

Human allergy to Japanese cedar (*Cryptomeria japonica*) pollen was first reported in 1964 [1]. In Japan, Japanese cedar contributes the most important group of pollens causing allergies because of its abundant pollination and widespread distribution, and recently the number of patients suffering from allergies is remarkably increasing. The symptoms of pollinosis are snivelling, sneezing, eye mucus, and/or tears resulting from inflammation of mucous membranes of nostrils and/or eyes.

Approach to the experimental study of human pollinosis in order to establish medical treatment has led to a continuing search for an animal model, such as mouse or guinea pig [2, 3]. 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 are not similar to those of humans.

In 1987, Yokota et al. [4] reported naturally occurring pollinosis in wild Japanese monkeys that had symptoms similar to human pollinosis. Generally, monkeys have been used for cellular, pharmacologic, and immunologic studies to minimize concerns about

interspecies variability. The use of primates for a model of immediate hypersensitivity in man has been reported for a long time, e.g., in the transfer of human reaginic sera to monkeys [5, 6] and experimental asthma by *Ascaris* infection [7]. However, there are few reports about experimental pollinosis of primates [8].

The present paper demonstrates experimentally induced cedar pollinosis in monkeys.

Materials and Method

Animals

Two male *Macaca mulatta* monkeys weighing 5.7 and 6.1 kg and 2 female monkeys weighing 4.0 and 4.0 kg were purchased from Japan Clea. These monkeys were individually kept in stainless steel cages (58 × 66 × 65 cm) placed within an air-conditioned room at room temperature (24 ± 2°C), 55 ± 5% relative humidity, controlled illumination (light on from 05.00 to 19.00 h), and fed a commercial diet (PS; Oriental Yeast) and tap water ad libitum.

Antigen

Preparation of antigen was performed according to the methods of Yasueda et al. [9]. Briefly, the pollen was defatted with ether and extracted in 20 vol of 0.125 M ammonium bicarbonate (pH 8.0) at 20°C for 48 h. After extraction, the supernatant was obtained by centrifugation. Solid ammonium sulfate was added to the superna-

tant to 80% saturation. After stirring at 4°C overnight, the precipitate was obtained by centrifugation, dialyzed in 0.05 M Tris-HCl (pH 7.8), and passed through a DEAE-cellulose column. The fraction which contained protein (3%) and other materials, mainly polysaccharides, was used in this experiment.

Immunization

The antigens containing 1 or 10 µg protein were mixed with 50 mg aluminium hydroxide gel. The animals were anesthetized with ketamine chloride (Sankyo; 10 mg/kg i.m.). One male (No. 16) and 1 female (No. 19) monkey were subcutaneously immunized with the antigen at 1 µg protein/body in the dorsal part of the body, and another male (No. 17) and a female (No. 20) monkey at 10 µg protein/body. These animals were boosted every month and received a total of six injections. Blood was collected from the saphenous vein every 7 days following each booster injection, and separated serum was stored at -20°C until used.

Cutaneous Reactivity

The animals were anesthetized by intramuscular injection of ketamine chloride (8-15 mg/kg body weight) and intradermally injected with 0.02 ml of antigen into sites in the arm. After 15 min, the diameters of erythema wheal at the sensitized sites were measured.

Prausnitz-Küstner Reactions

Sera were collected from the saphenous veins of immunized monkeys. The tests were carried out on normal monkey which had negative skin reactions to cedar pollen antigen. The normal monkey was anesthetized by an intramuscular injection of ketamine chloride (20 mg/kg body weight). 0.1 ml of serial twofold dilutions of various sera was injected intradermally into sites in the arms. Forty-eight hours later, the monkeys were intradermally injected with antigen into the sensitized sites. Diameters at the sites were measured after 15 min, and a positive reaction was characterized by an edema >9×9 mm. For determination of heat lability, each serum was heated to 56°C for 4 h in a water bath and tested similarly.

Serum IgE Determination

The total serum IgE concentration was measured by radioimmunosorbent test [10], and IgE antibodies specific to cedar pollen were assayed by radioallergosorbent test (RAST) [11].

Preparation of Mixed Leukocytes

Heparinized blood obtained from the saphenous veins of the monkeys was mixed with an equal volume of Haemacel (Hoechst Japan) and left for 60 min at room temperature. After sedimentation, the leukocyte-rich supernatant was separated, followed by centrifugation (250 g for 5 min at 4°C) and hypotonic shock for lysis of erythrocytes, then washed twice with Tris-ethylenediaminetetraacetic acid (EDTA) buffer [31 mM Tris(hydroxymethyl) aminomethane, 120 mM NaCl, 5 mM KCl, 10 mM EDTA-2 Na, 0.03% human serum albumin, pH 7.6], followed by final washing with Tris-ACM buffer [31 mM Tris(hydroxymethyl) aminomethane, 120 mM NaCl, 5 mM KCl, 0.6 mM CaCl₂·2H₂O, 0.1 mM MgCl₂·7H₂O, 0.03% human serum albumin, pH 7.6]. The viability of the cells, as estimated by the trypan blue exclusion test, was >95%, and the cell number was adjusted to 1.0 × 10⁶ cells/0.45 ml in Tris-ACM buffer.

Histamine Release from Mixed Leukocytes

The mixed leukocytes (1.0 × 10⁶ cells) in 0.45 ml were transferred into a polypropylene tube. After incubation for 10 min at 37°C, stimulation was performed by adding 0.05 ml of antigen (final concentration 1 µg/ml) at 37°C for 45 min. The release process was stopped by placing the tubes in an ice bath. After centrifugation (800 g at 4°C for 10 min), the supernatant was collected and stored at -20°C until assay.

Measurement of Histamine

Histamine in the supernatant was measured with a commercial radioimmunoassay kit (Immunotech). The total histamine content was determined by lysing cells with perchloric acid (final concentration 1 µg/ml). The results are expressed as the percentage of the total histamine release with correction for spontaneous release.

Measurement of Antipollen IgG Antibody

IgG antibodies against pollen were measured by enzyme-linked immunosorbent assay (ELISA) according to methods previously described [12]. The sequence of the addition of reagents to the wells was as follows: pollen antigen (1 µg/well), test serum (dilution 1:200), enzyme-linked goat antimouse IgG antibody, and substrate. The antipollen IgG antibody activity was recorded as the absorbance values and expressed as units converted with pooled standard serum obtained from monkey No. 19, arbitrary value 20 U/ml.

Conjunctival Provocation Test

The monkeys were restrained in monkey chairs. Antigen solution or saline was dropped into their eyes, and immediately they were returned to their cages. Clinical signs were recorded for 30 min by videotape recorder to avoid stress. Counts of eye-scratching were performed 5-30 min after provocation, excluding nonspecific counts during the first 5 min.

Blood Examination

Blood examination was performed after the 4th immunization, and the number of erythrocytes and leukocytes was counted by Sysmex (model CC-180A; TOA Medical Electronics). Furthermore, differential count of leukocytes was also performed.

Results

Cutaneous Reactivity

Immediate-type skin reactivity to pollen antigen appeared after the 4th immunization (table 1). In 3 of 4 monkeys (Nos. 16, 17, and 20) a significant reactivity remained 3 months after the last immunization, but 1 monkey (No. 19) had a weak reactivity throughout the study.

Prausnitz-Küstner Reactions

Serial dilutions of the sera collected after the 4th immunization were injected intradermally into monkeys (table 1). Positive reactions were observed in 3 of 4 monkey sera (1:1,280, 1:320, and 1:320), but 1

Table 1. Results of the skin test and Prausnitz-Küstner titers in monkeys after the 4th immunization

Immuno- antigen μg	Monkey		Allergen		Prausnitz- Küstner titer
	No.	sex	saline	pollen antigen	
1	16	♂	5.0 × 4.0 ^a	9.0 × 9.0	1,280 ^b
	19	♀	5.5 × 4.0	8.0 × 7.0	< 20
10	17	♂	7.0 × 5.0	12.0 × 10.0	320
	20	♀	5.0 × 4.5	10.0 × 9.5	320

^a Maximum diameter × minimum diameter (mm).^b Maximum dilution of the samples required to give positive reactions.

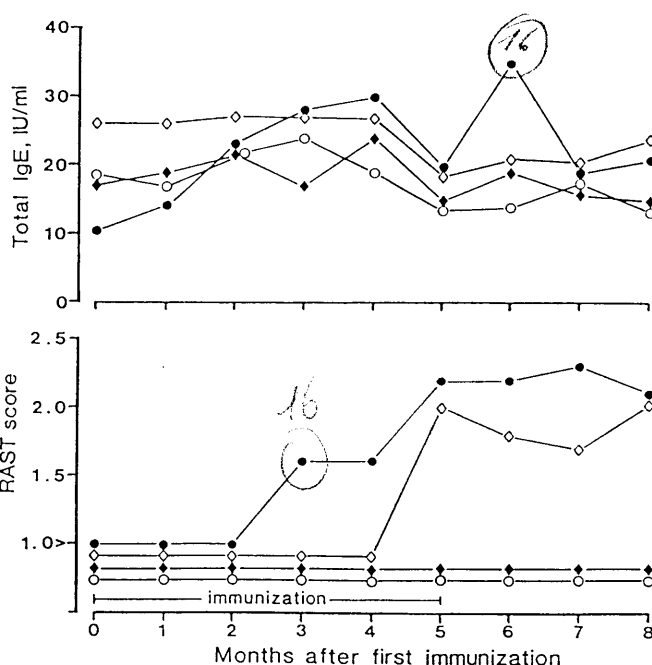
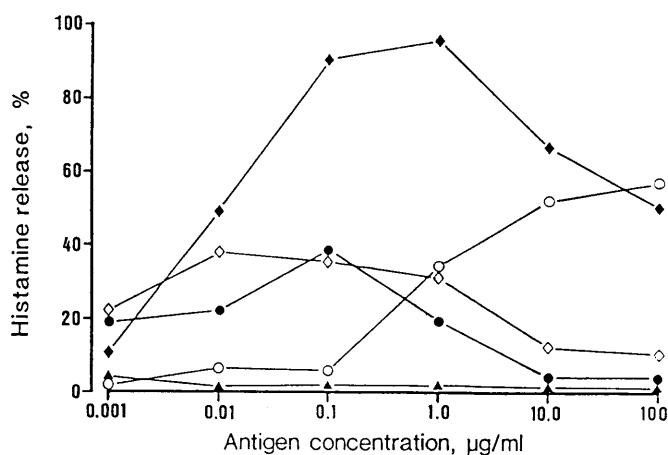
monkey serum (No. 19) showed a negative reaction (<1:20). Heating of the serum to 56°C for 4 h reduced the skin-sensitizing activity.

Serum IgE Determination

Changes of total serum IgE levels and RAST scores were followed for up to 3 months after the last immunization (fig. 1). Changes of total IgE levels were not observed during the experiment. RAST scores increased after the 3rd immunization in 1 monkey (No. 16) and after the 5th immunization in 1 (No. 20), and these monkeys showed a persistence at higher levels. Two monkeys (Nos. 17 and 19) were considered to be RAST negative. A relationship between total IgE and RAST scores was not observed.

Histamine Release

The percent histamine release from leukocytes caused by antigen is shown in figure 2. The results show that in experiments with any immunized animals, the release of histamine occurred as a result of exposure to antigen. In 3 of 4 monkeys (Nos. 16, 17, and 20), bell-shaped concentration-dependent histamine release curves at 0.01, 0.1, or 1.0 μg/ml of antigen were induced. In 1 monkey (No. 19), histamine release began at 1.0 μg/ml of antigen and increased to peak at 100 μg/ml of antigen. The leukocytes from unimmunized monkeys did not release histamine after addition of antigen up to 100 μg/ml.

**Fig. 1.** Changes of total IgE levels and RAST scores. ● = Monkey No. 16; ♦ = No. 17; ○ = No. 19; ◇ = animal No. 20.**Fig. 2.** Relationship between pollen antigen concentration and percent histamine release in leukocytes from monkeys after the 4th immunization. ▲ = Control (unimmunized); ● = monkey No. 16; ♦ = No. 17; ○ = No. 19; ◇ = animal No. 20.

Measurement of IgG Antibodies

Antipollen IgG antibodies measured by ELISA are shown in figure 3. In 3 of 4 monkeys, IgG antibodies appeared slowly and remained at a low level. However, in 1 monkey (No. 19) IgG antibodies increased after the 3rd immunization and showed about a two to three times higher level than the others.

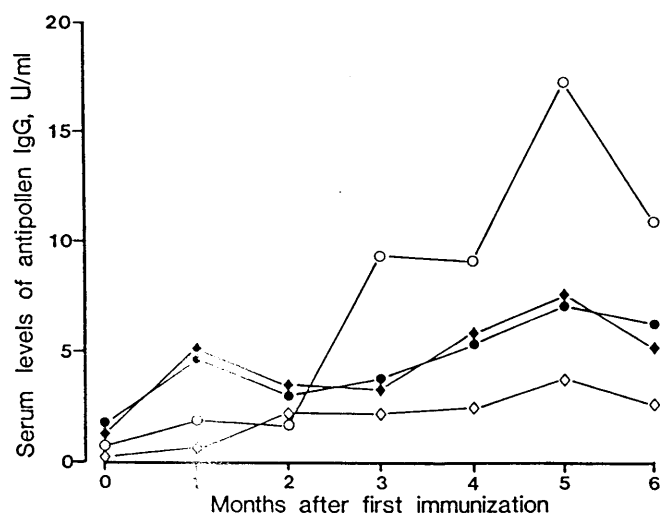


Fig. 3. Changes in serum levels of IgG antibodies against cedar pollen antigen. ● = Monkey No. 16; ◆ = No. 17; ○ = No. 19; ◇ = animal No. 20.

Table 2. Results of the conjunctival provocation test

Immuno-antigen μg	Monkey		Scratching ^a	
	No.	sex	saline	pollen antigen
Control	15	♂	0.0 ^b	2.0
	16	♂	3.5	11.3
	19	♀	4.0	13.7
10	17	♂	not done	not done
	20	♀	0.5	7.3

^a Scratching was counted from 5 to 30 min after provocation.

^b The tests were performed two times with saline and three times with pollen antigen. The results are expressed as average counts.

Table 3. Blood values of monkeys after the 4th immunization

Immuno-antigen μg	Monkey		Red blood cells × 10 ⁴ /mm ³	White blood cells × 10 ² /mm ³	Differential white blood cell count, %				
	No.	sex			lymphocytes	neutrophils	eosinophils	basophils	others
Control	18	♂	594	81	74.5	23.5	0.0	0.5	1.5
	16	♂	635	79	67.0	30.0	0.5	0.5	2.0
	19	♀	574	43	88.0	11.0	0.0	0.0	1.0
10	17	♂	580	56	76.5	20.5	1.0	1.0	1.0
	20	♀	538	62	66.5	30.0	3.0	0.5	0.0

Conjunctival Provocation Test

Count of eye-scratching in 3 of the 4 immunized monkeys were 7.3–13.7 and 2.0 in the normal monkey after dropping of the cedar pollen antigen (table 2). The counts for immunized monkeys were higher than that for the normal monkey. When the monkeys were sensitized with saline, the counts for immunized monkeys ranged from 0.5 to 4.0. Scratching for monkey No. 17 could not be counted because this animal moved fairly frequently.

Blood Examination

Blood examination was performed after the 5th immunization (table 3). The numbers of erythrocytes and leukocytes in the immunized monkeys were in the normal range reported previously [13]. Differential counts of leukocytes were also similar to the normal monkey.

Discussion

An experimental model of cedar pollinosis was developed in rhesus monkeys by subcutaneous injection of cedar pollen antigen.

Antigen concentrations which caused maximal histamine release from leukocytes were 0.01 μg/ml in monkey No. 16 and 0.1 μg/ml in animal No. 20. Their RAST scores were ≥ 2.0, and Prausnitz-Küstner titers were 1:1,280 and 1:320, respectively. In 1 monkey (No. 19) the skin test reaction was weak, whereas RAST score and Prausnitz-Küstner titer were negative, and histamine release began at 1.0 μg/ml of antigen and increased to peak at 100 μg/ml. The anti-pollen IgG antibody concentration of this monkey was also two to three times higher than that of the others. In humans, the symptom index has been significantly correlated with the level of positive skin test,

RAST, and leukocyte histamine release [14]. In addition, there has been a significant correlation between asthmatic severity and allergen concentrations of 50% histamine release from leukocytes [15]. Significantly, leukocytes in mild asthmatics require more antigen to cause maximal histamine release than those in severe asthmatics. Therefore, it was hypothesized that monkey No. 19 was only slightly sensitized. In the Prausnitz-Küstner test, the skin-sensitizing activity could not be observed in all sera heated to 56°C for 4 h, suggesting that IgE antibodies were produced in monkeys by immunization with cedar pollen. However, despite the fact that the Prausnitz-Küstner titer of monkey No. 17 was 1:320 and the antigen concentration of maximal histamine release 1.0 µg/ml, the RAST score of the animal was negative. The reasons why the monkey was negative on RAST are because RAST cannot detect IgE antibodies specific for non-protein antigens such as polysaccharides, and the RAST score was obtained by using antihuman IgE antibody. Since we used crude antigen containing only 3% protein, monkey No. 17 might produce IgE antibody against other materials, but not protein. Indeed, there are reports on positive skin test but RAST-negative children who have asthma from Japanese cedar pollen [16]. Tamura et al. [2] have reported that allergen components other than the protein of Japanese cedar pollen were involved in IgE antibody response of pollen-sensitized mice. Furthermore, monkey serum containing IgE antibody similar in antigenic structure and physicochemical properties to human IgE antibody [17], but approximately ten times the concentration of human reaginic serum, was required to sensitize monkey skin in the same manner as required to sensitize human skin [18].

Levine et al. [19] have reported a low-dose immunization schedule of preferentially induced reagin formation in the guinea pig. The monkeys were immunized with two doses (1.0 or 10 µg) of antigen in this experiment, but differences of reaginic response between the two groups were not clearly observed because of the small number of animals used. We could not determine the correlation between antigen dose used for immunization and reaginic response.

Clinical signs such as snivelling, sneezing, eye mucus, and mucous membrane inflammation of the nostrils and/or eyes are obtained after a nasal or conjunctival provocation test in humans and wild monkeys with pollinosis. Marsh et al. [20] have reported that humans experimentally sensitized with ryegrass

pollen developed skin reactivity and leukocyte histamine release characteristic of immediate hypersensitivity, but there were no signs of clinical allergy in any of these people during natural exposure to the pollen. We obtained similar results in that the sensitized monkey showed only a particular clinical sign, such as eye-scratching after the antigen was dropped into their eyes. The reason for this finding was not immediately evident, but there might be some differences in the characteristics of IgE antibodies due to both naturally induced and experimentally induced pollinosis. Furthermore, Otsuka and Okuda [21] have reported that the results of nasal provocation tests during the pollen season differ from those out of season. During pollen season mast cells and basophils migrate to the mucous membranes of the nostrils and/or eyes, so antigen-specific reactions readily occur at these sites. If monkeys were continuously sensitized with pollen, they might well show more apparent clinical signs.

It has been reported that several antiallergic drugs which were effective in rodent models of allergy, such as the rat and/or guinea pig, were not fully effective in human patients [22, 23]. It is important to develop more suitable models to establish a means for medical treatment of pollinosis. Although naturally occurring pollinosis was found in wild Japanese monkeys, it was almost impossible to use them as experimental animals. These monkeys are protected animals in Japan, and they might have been sensitized with several unknown allergens in nature. Therefore, rhesus monkeys which were bred in a breeding colony for experimental animals and which belonged to the same genus as the Japanese monkeys were immunized with cedar pollen. We conclude that rhesus monkeys immunized with cedar pollen could be a useful model in the investigation of human cedar pollinosis. Further studies to characterize allergens and to induce symptoms other than eye-scratching alone remain to be performed.

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Received: March 6, 1990

Accepted after revision: September 14, 1990

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