

An experimental allergic conjunctivitis induced by topical and repetitive applications of Japanese cedar pollens in guinea pigs

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Abstract. *Objective and Design:* To develop a model of experimental allergic conjunctivitis, guinea pigs were given repetitive and topical applications of Japanese cedar pollen as an antigen, and the resulting allergic reaction was characterised.

Subjects: Male Hartley guinea pigs.

Treatment: Guinea pigs were sensitised by insertion of small gelatin sponge pieces (3 pieces/eye, both eyes) containing the pollen extracts + Al(OH)₃ into the palpebra superior/inferior sulci of both eyes for 8 h/day for 6 days. Then the animals were challenged by dropping pollen suspension in each eye once every week.

Methods: Time-course changes of conjunctivitis intensity score (CIS), which represents oedema and redness, and scratching frequency, and γ_1 and IgE levels in sera were assessed following the respective 1st–17th challenges. Ophthalmic lavage was performed to assay for albumin leakage and migrated leukocytes at each of the odd-numbered challenges.

Results: At relatively early stages of the repeated challenge, only a gradual increase of CIS was observed. However, thereafter, considerably higher CIS with the maximum at 30 min after the challenge was provoked at the 12th–17th challenges. Quite interestingly, there were differences in the CIS between the right and left eyes, depending on the individuals. Scratching, and albumin leakage and neutrophil influx into the lavage fluid were also developed during the challenges. Although the anaphylactic antibodies against the antigen were detected in the sera from half of the animals, the levels were not correlated to severity of the symptoms.

Conclusions: The present sensitisation/challenge procedures are unique in terms of topical application of antigen. The lack of any correlation between the levels of the anaphylactic antibodies and severity of anaphylactic symptoms, and the difference of CIS between the left and right eyes of an individual strongly suggest that the anaphylactic antibodies are formed in locally limited tissue surrounding an eye. The pre-

sent method should be useful for analysing the mechanisms of allergic conjunctivitis.

Key words: Allergic conjunctivitis – Itching – Pollenosis – IgE – Allergic model

Introduction

Allergic conjunctivitis is one of the typical atopic diseases, which is classified into the seasonal type caused by air-borne allergens such as ragweed [1] and cedar pollens [2] and the perennial type caused by house dust mites [3], cat and dog dander [4], various fruits, vegetables and moulds [5]. Recently, a number of patients in Japan have been suffering from Japanese cedar pollenosis that include the symptoms of allergic rhinitis and conjunctivitis. The prevalence of allergic conjunctivitis is estimated to be approximately 20% of the general Japanese population. The ocular allergy is characterised by severe itching, hyperaemia and oedema, the medical therapy for which consists of topical applications of anti-histamines with or without vasoconstrictors, mast cell stabilisers, nonsteroidal anti-inflammatory drugs and glucocorticoids [6]. However, since these drugs do not relieve the symptoms completely or cause severe side effects [6], other effective and safe drugs must be developed.

Experimental animal models are indispensable for assessing new therapeutic drugs and for investigating the pathophysiology of allergic conjunctivitis. Most experimental animal models of ocular allergy have been developed by actively sensitising guinea pigs with an intraperitoneal injection of a non air-borne antigen such as ovalbumin (OA) [7] and dinitrophenylated lysine [8] mixed with an adjuvant or by passively sensitising animals with antisera [9, 10], and subsequent single conjunctival challenge with a solution of the specific antigen. These models are considerably different from the clinical situation because the sensitised state was not achieved locally at the conjunctival tissue but induced

systemically and the antigen challenge was not performed chronically.

The present study was undertaken to develop an experimental animal model of allergic conjunctivitis in which the sensitised condition is achieved at the local conjunctival tissue site: Guinea pigs were sensitised by conjunctival applications of extracts of Japanese cedar pollens adsorbed on $\text{Al}(\text{OH})_3$ and then challenged with repeated dropping of pollen suspension in both eyes over a long term.

Materials and methods

Animals

Male, 3-week-old, Hartley guinea pigs weighing 250–300 g were purchased from Japan SLC, Hamamatsu, Japan. The animals were housed in an air-conditioned room at a temperature of $23 \pm 1^\circ\text{C}$ and $60 \pm 10\%$ humidity with lighting time at 8:00 a.m.–8:00 p.m., fed a standard laboratory diet and given water ad libitum. The first sensitisation was started 1–2 weeks after the purchase.

This animal study was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

Materials

The following reagents were obtained from the indicated commercial sources: sodium pentobarbital (Abbott Lab., North Chicago, IL, USA); guinea pig albumin and anti-guinea pig albumin antibody rabbit serum (Organon Teknica, West Chester, PA, USA); biotin-*N*-hydroxysuccinimide and streptavidin-horseradish peroxidase conjugate (Gibco BRL, Gaithersburg, MD, USA); ovalbumin (OA) and *o*-phenylenediamine (Wako Pure Chem., Osaka, Japan); gelatin sponge (Spongel®, Yamanouchi Pharm. Co., Ltd., Tokyo, Japan) and Evans blue (Merck,

Darmstadt, Germany). Japanese cedar pollens were donated from the laboratory of Torii Pharm. Co. Ltd. (Chiba, Japan). The other reagents used were the highest grade of commercial products available.

$\text{Al}(\text{OH})_3$ gels were prepared with 0.5 N NaOH and 0.5 N $\text{Al}_2(\text{SO}_4)_3$ as previously described [11], which was a modification of the method of Levine and Vaz [12].

Anti-OA guinea pig serum, which was used as a standard for quantification of γ_1 and IgE antibody levels in sensitised guinea pig sera by passive cutaneous anaphylaxis (PCA), was obtained according to the method of Levine et al. [13]. In brief, guinea pigs were sensitised by i.p. injection of 10 μg OA/mg $\text{Al}(\text{OH})_3$ /animal/time, once every 2 weeks, totally 10 times. The 4-h and 7-day homologous PCA titres of the serum were both 1:500.

Preparation of Japanese cedar pollen extracts

The cedar pollen extracts used for the sensitisation were prepared according to the previously described method [14]: In brief, the pollens were suspended in physiologic saline at 50 mg/ml and allowed to stand at 4°C for 18 h under mild stirring. After centrifugation at $1,700 \times g$ for 15 min at 4°C , the resultant supernatant was used as the sensitisation antigen.

Sensitisation and challenge

Sensitisation with cedar pollen extracts was conducted by the two methods, Experiment 1 and 2, shown in Chart 1.

Experiment 1: Guinea pigs were sensitised locally by dropping cedar pollen extracts adsorbed on $\text{Al}(\text{OH})_3$ in both eyes at a dose of 0.3 μg pollen protein/0.6 mg $\text{Al}(\text{OH})_3$ /10 μl /eye/time, four times a day, for successive six days (Group 1a). As a positive control, the other guinea pigs were sensitised systemically by intraperitoneal injection with the extracts + $\text{Al}(\text{OH})_3$ twice within a week at a dose of 10 μg pollen protein/20 mg $\text{Al}(\text{OH})_3$ /ml/animal/time (Group 1b).

Experiment 2: Guinea pigs were sensitised locally by insertion of 3 pieces ($2 \times 3 \times 0.5$ mm in size)/eye of a gelatin sponge soaked with

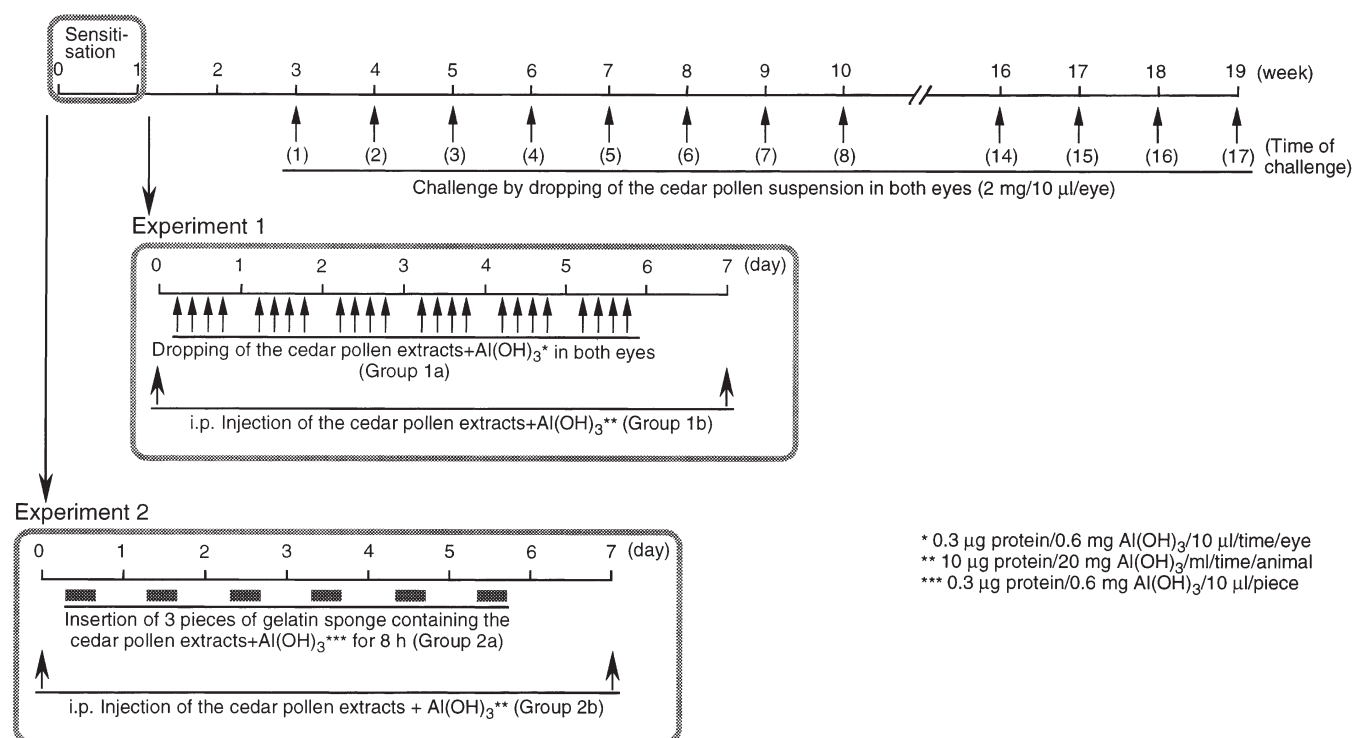


Chart 1. Schedules for sensitisation and challenge with Japanese cedar pollen in the guinea pig.

0.3 µg pollen protein/0.6 mg Al(OH)₃/10 µl/piece of the extracts + Al(OH)₃ into the palpebral superior (1 piece) and inferior sulci (2 pieces) of each of their eyes under pentobarbital anaesthesia (40 mg/kg, i.p.). The same antigen suspension (10 µl/eye) was additionally dropped in both eyes, and then the sponge pieces were removed 8 h after their insertion. This procedure was conducted for successive six days (Group 2a). As a positive control, the other guinea pigs were sensitised systemically in the same manner as Group 1b (Group 2b). Non-sensitised-non-challenged guinea pigs were also prepared for a negative control as Group 2c.

On the 21st day after the first sensitisation, the respective groups, except for Group 2c, were challenged by dropping of the pollen suspension without Al(OH)₃ in each of their eyes at a dose of 2 mg pollens/10 µl/eye, followed by repeated challenges with the same dose of antigen once every week.

Quantification of antigen remaining on the surface of the conjunctiva

Residual rates of antigen on the surface of the conjunctiva following the application at the sensitisations, which were executed according to the two sensitisation methods of Groups 1a and 2a, were quantitated using Evans blue in place of the antigen extract: Evans blue + Al(OH)₃ (50 µg Evans blue/0.6 mg Al(OH)₃/10 µl/eye with and without the gelatin sponge pieces) was applied to the both eyes. Thirty minutes after the application, the guinea pig was killed by exsanguination, followed by isolation of the conjunctiva with the bulbus oculi (and the gelatin sponge pieces). The amount of Evans blue remaining on the conjunctiva (and the gelatin pieces) was quantitated according to the method of Katayama et al. [15].

Evaluation of conjunctivitis

Time-course changes of conjunctival oedema and redness 0.5–10 h after the respective antigen challenges were judged macroscopically. Conjunctivitis intensity score (CIS) was evaluated numerically accord-

ing to an arbitrary 5-point graded scale, from 0 to 4, increasing with severity (0, no symptoms; 1, light; 2, mild; 3, moderate; 4, severe) according to the method of Ballas et al. [16]. Additionally, the symptom falling between score 0 and 1 was judged as score 0.5.

Measurement of scratching frequency

Scratching frequencies at 0–30 min, 0.5–1 h, 1–2 h and 2–3 h after the respective pollen challenges were counted. The scratch response was defined as an uninterrupted cluster of rapid hind limb movements that were precisely directed to the ocular surface.

Ophthalmic lavage

The collection of ophthalmic lavage fluid was carried out as follows: Ten-µl of sterile physiologic saline was applied to the eye using a micropipette (Eppendorf, Hamburg, Germany), without touching the eye. After two or three forced blinks, the lavage fluid was collected. The lavage was repeated 5 times in each eye, and the lavage fluids obtained from both the eyes were combined. The ophthalmic lavage was conducted every hour from 1 h before to 10 h after the respective odd-numbered challenges.

Measurement of albumin in the ophthalmic lavage fluid

Following the centrifugation (4 °C, 120 × g, 5 min) of the fluid, albumin in the supernatant was measured by enzyme-linked immunosorbent assay according to the method of Gawin et al. [17].

Measurement of total leukocytes and their classification in the ophthalmic lavage fluid

The total and the number of classified leukocytes in the fluid were microscopically counted: After the centrifugation of the fluid, the

Group	PCA	Time of challenge	No. of tested sera	No. of sera showing the indicated PCA titre					
				0	1–8	16	32	64	128 ≤
Group 1a	4-h PCA	1	4	4	0	0	0	0	0
		5	4	4	0	0	0	0	0
		9	4	4	0	0	0	0	0
		13	4	4	0	0	0	0	0
		15	4	4	0	0	0	0	0
	7-day PCA	1	4	4	0	0	0	0	0
		5	4	4	0	0	0	0	0
		9	4	4	0	0	0	0	0
		13	4	4	0	0	0	0	0
		15	4	4	0	0	0	0	0
Group 1b	4-h PCA	1	4	0	2	1	1	0	0
		5	4	0	0	0	0	0	4
		9	4	0	0	0	0	0	4
		13	4	0	0	0	0	0	4
		15	4	0	0	0	0	1	3
	7-day PCA	1	4	0	0	0	3	0	1
		5	4	0	0	0	0	0	4
		9	4	0	0	0	0	0	4
		13	4	0	0	0	0	0	4
		15	4	0	0	0	0	1	3

The serum was obtained on the day before the time of the indicated challenge.

Table 1. Four-h and 7-day passive cutaneous anaphylaxis (PCA) titres of sera from the guinea pigs of Groups 1a and 1b.

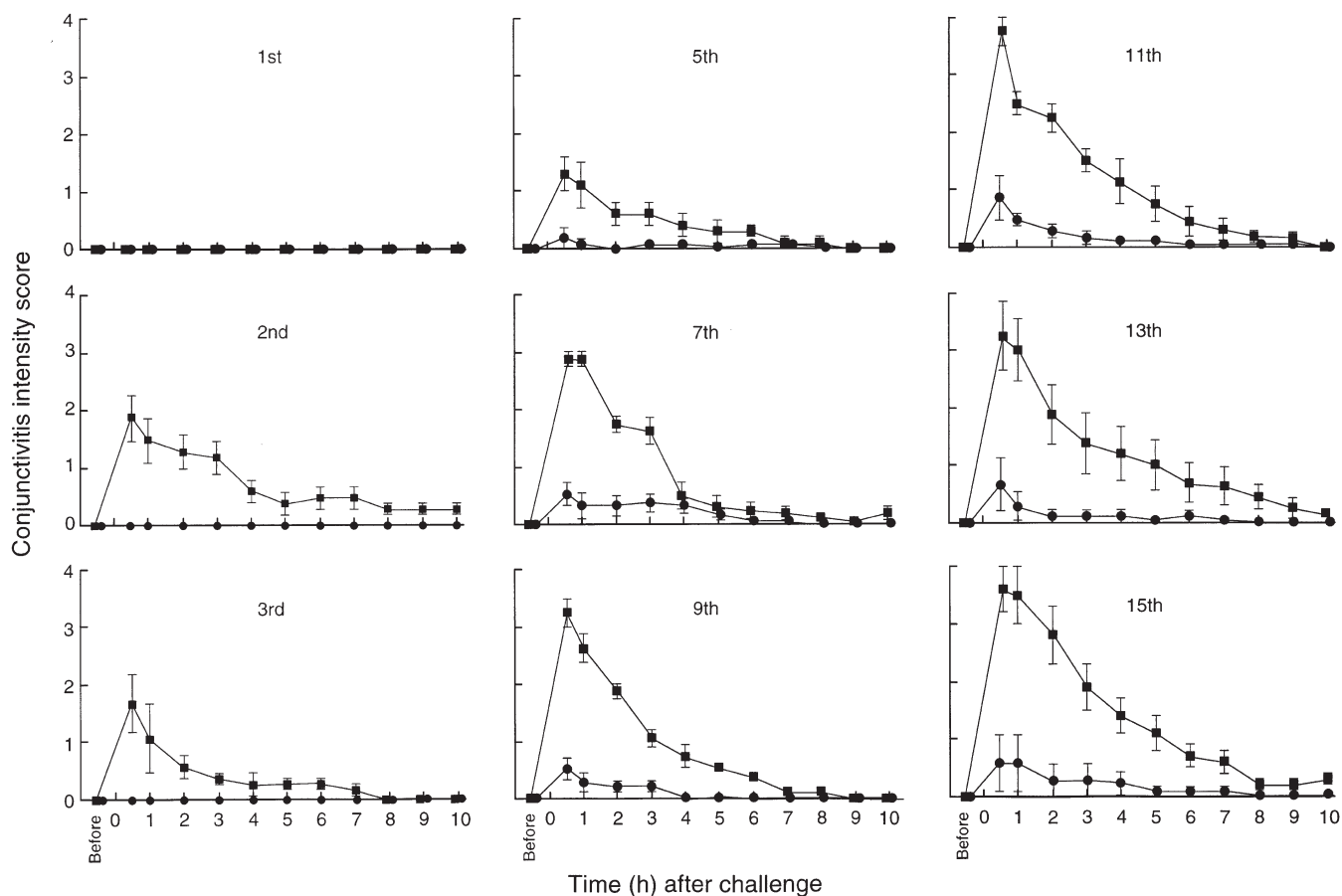


Fig. 1. Time-course changes of conjunctivitis intensity score (CIS) following the 1st, 2nd, 3rd, 5th, 7th, 9th, 11th, 13th and 15th challenges by dropping of Japanese cedar pollen suspension in both eyes of sensitised guinea pig of experiment 1. CIS was evaluated as follows: 0, no symptoms; 1, light; 2, mild; 3, moderate; 4, severe. Closed circle: Group 1a, closed square: Group 1b. Each point represents the mean $\pm$ SEM of 5 (Group 1a) and 4 or 5 (Group 1b) animals.

resultant cell pellet was suspended with a defined volume (50–500 μ l/sample) of physiologic saline. For determining the number of total leukocytes, a portion of the cell suspension was stained with Turk's solution (Nacalai Tesque, Kyoto, Japan). For the classification of leukocytes, an aliquot of the cell suspension was centrifuged on the Settling chamber (Neuro Probe, Cabin John, MD, USA) at $50 \times g$ for 30 s at 4°C and staining with Diff-Quik® solution (International Reagent, Kobe, Japan). Four hundred cells were counted in total.

PCA

For the estimation of specific γ_1 and IgE antibody titres of the serum in the sensitised guinea pig during the period of the challenges, 4-h and 7-day PCAs were performed according to the method of Ovary [18] and Levine et al. [13], respectively. The serum for the PCAs was obtained on the day before the respective odd-numbered challenges.

Results

Time-course changes of allergic conjunctivitis in experiment 1

Figure 1 shows time-course changes of CIS following the respective antigen challenges in experiment 1. Each individual score was the mean of the CISs of the right and left eyes. Up to the 3rd challenge, no increases of CIS were

observed in Group 1a. Although a modest increase of CIS was seen at the 5th challenge, the symptom of conjunctivitis did not become any more severe with subsequent challenges. Scratching, and leakage of albumin and increase of leukocyte number in the ophthalmic lavage fluid were hardly observed throughout the 1st to 15th challenges in Group 1a (data not shown).

On the other hand, guinea pigs of Group 1b showed apparent elevation of CIS at the 2nd challenge with its peak at the 30th min. After that, with further repeated pollen challenges, CIS continued to increase until the 11th challenge, with higher peak levels (Fig. 1). In addition, the intraperitoneally sensitised animal exhibited more than 10 scratching episodes within 30 min after the respective 2nd–15th challenges (data not shown). An immediate increase of albumin amount and relatively sustained elevations of numbers of eosinophils and neutrophils in the ophthalmic lavage fluid were recognised at the 7th–15th, 3rd–15th and 7th–15th challenges, respectively (data not shown).

γ_1 and IgE antibody levels in the serum in experiment 1

The levels of γ_1 and IgE antibodies in the serum obtained on the day before the respective challenges in the sensitised

guinea pig were quantitated by measuring their titres of 4-h and 7-day PCA, respectively. These results are shown in Table 1. Sera from all the animals of Group 1a were negative for both PCAs throughout the course of the repeated challenges. On the other hand, all sera obtained from Group 1b were already positive for both PCAs at the 1st challenge followed by sustaining their titres reaching more than $64 \times$ at the 5th–15th challenges.

Residual rates of antigen on the surface of the conjunctiva at sensitisations

When the Evans blue (for antigen) + $\text{Al}(\text{OH})_3$ suspension was dropped in the eyes according to the method of Group 1a, merely 6% of the applied Evans blue remained on conjunctiva 30 min after the application (Table 2). On the other hand, when the gelatin sponge pieces containing the suspension were inserted under the eyelids, the method of Group 2a, more than 90% of the dye was detected in the gelatin sponge pieces and on the conjunctival tissue 30 min after the insertion (Table 2).

Table 2. Amount of residual Evans blue on conjunctiva 30 min after dropping of Evans blue + $\text{Al}(\text{OH})_3$ suspension in both eyes or insertion of gelatin sponge pieces containing the suspension into the palpebral superior and inferior sulci of the eyes in the guinea pig.

Application method	Amount of residual Evans blue (% of the applied amount)
Ophthalmic drop	6.0 ± 1.9
Gelatin sponge insertion	90.4 ± 3.5

Each value represents the mean $\pm$ SEM of 4 experiments.

Time-course changes of CIS in experiment 2

The respective time-course changes of CIS at the 1st to 17th antigen challenges in experiment 2 are shown in Figs. 2A and 2B. The individual score was the mean of the CISs of the right and left eyes. Although, in Group 2a, no symptoms were observed at the 1st to 3rd challenges, modest increases of CIS were produced by the 4th–11th challenges, which had scores of less than 1. Furthermore, subsequent challenges

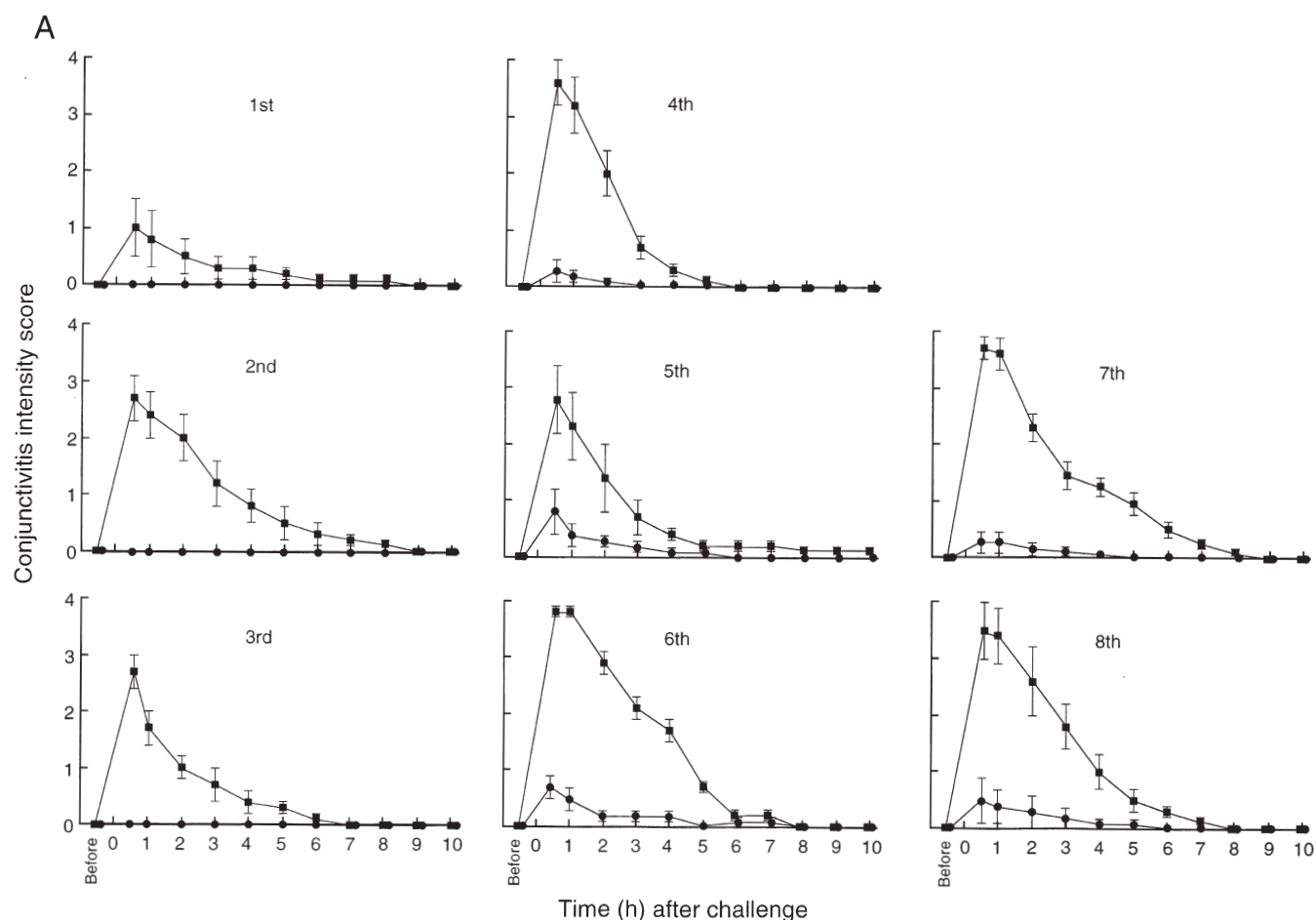


Fig. 2. Time-course changes of conjunctivitis intensity score (CIS) following the 1st–8th (A) and 9th–17th (B) challenges by dropping of Japanese cedar pollen suspension in both eyes of sensitised guinea pig of experiment 2. CIS was evaluated as follows: 0, no symptoms; 1, light; 2, mild; 3, moderate; 4, severe. Closed circle: Group 2a, closed square: Group 2b. Each point represents the mean $\pm$ SEM of 5 (Group 2a) and 5 or 6 (Group 2b) animals.

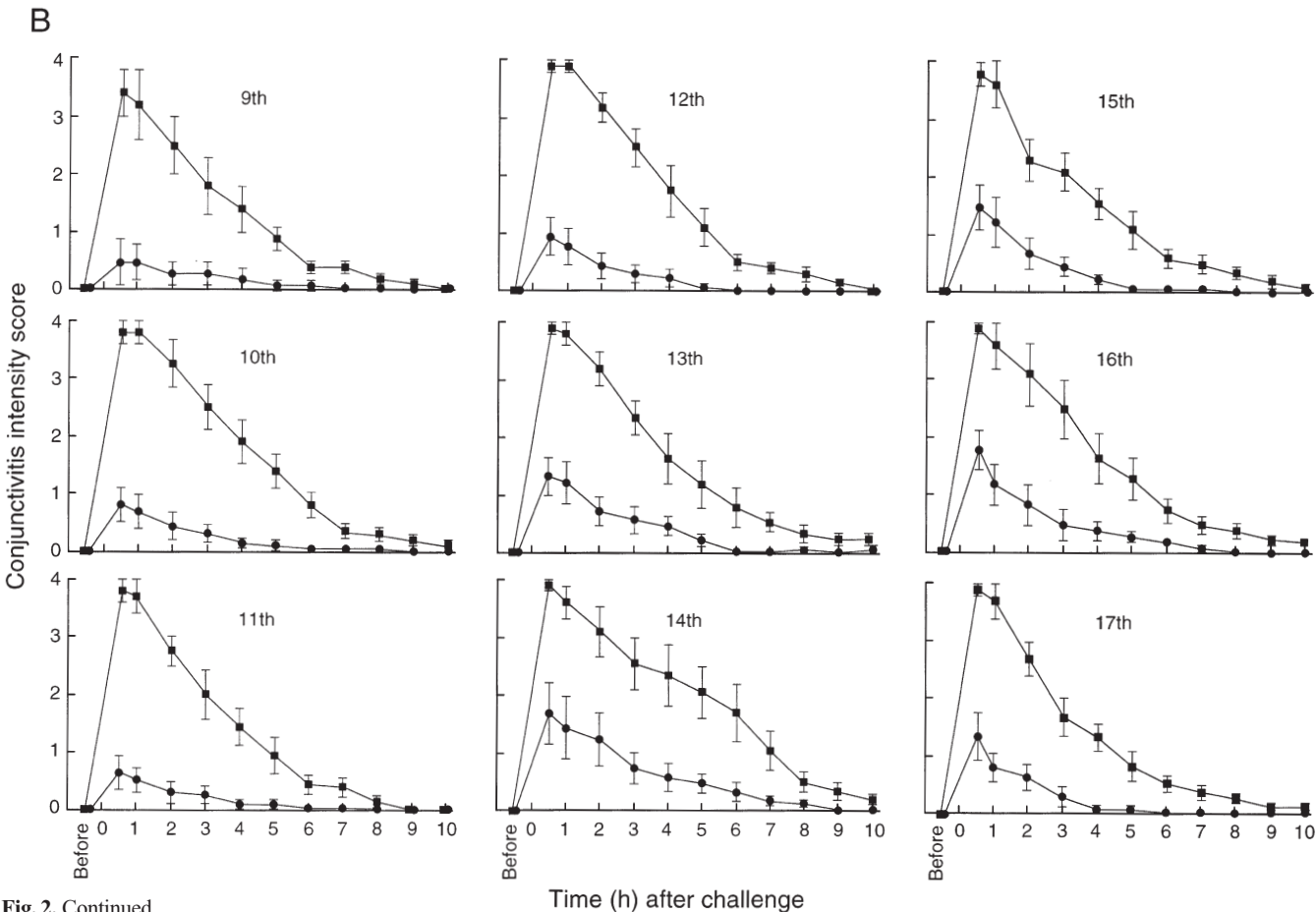


Fig. 2. Continued.

induced relatively intensive symptoms: at the 12th–17th challenges, CISs 1–2 were observed in Group 2a. On the other hand, guinea pigs of the positive control (Group 2b) show increases of CIS that were almost the same as those of Group 1b in Experiment 1. The negative control animals (Group 2c) showed no symptoms.

Table 3 shows the scores of the respective right and left eyes in the individual animals, which were examined 30 min after the 1st, 5th, 10th and 15th challenges in Groups 2a and 2b. In Group 2a, there were no symptoms in any animals at the first challenge as shown in Fig. 2A. However, quite interestingly, the score of the left eye differed from that of the right eye in each animal at the 5th to 15th challenges. A typical example is represented by animal No. 303, where the left eye had the highest score, while the right eye developed no symptoms at the 10th and 15th challenge. In contrast, in systemic sensitisation (Group 2b), almost animals exhibited scores of the same degree at the 10th and 15th challenges.

Time-course changes of scratching frequency in experiment 2

Figures 3A and 3B show the time-courses of scratching frequency after the respective 1st to 17th challenges in Groups 2a, 2b and 2c. In Group 2a, the number was less than 10 times within 30 min following the respective 1st to 9th challenges. However, at the 10th to 15th challenges, the scratch-

ing behaviour, almost of which was induced within 30 min after the challenge, tended to become more frequent. On the other hand, the scratching frequency in Group 2b increased with challenges until the 6th provocation. Thereafter, the

Table 3. Conjunctivitis intensity score (CIS) in the right and left eyes induced by the 1st, 5th, 10th and 15th challenges by dropping of Japanese cedar pollen suspension in both eyes of sensitised guinea pigs of Groups 2a and 2b.

Group	Animal No.	CIS Time of challenge							
		1st		5th		10th		15th	
		R	L	R	L	R	L	R	L
Group 2a	1	0	0	0	1	1	0.5	2	0.5
	2	0	0	0	0.5	2	0.5	4	1
	3	0	0	0	2	0	4	0	4
	4	0	0	3	2	1	0.5	1	2
	5	0	0	0.5	1	0	0	0.5	0.5
Group 2b	1	0	0	0	1	4	2	3	3
	2	0	0	4	3	4	4	4	4
	3	3	3	3	4	4	4	4	4
	4	0	0	3	2	4	4	4	4
	5	2	1	4	4	4	4	4	4

Symptoms were observed 30 min after each challenge. R: right eye, L: left eye.

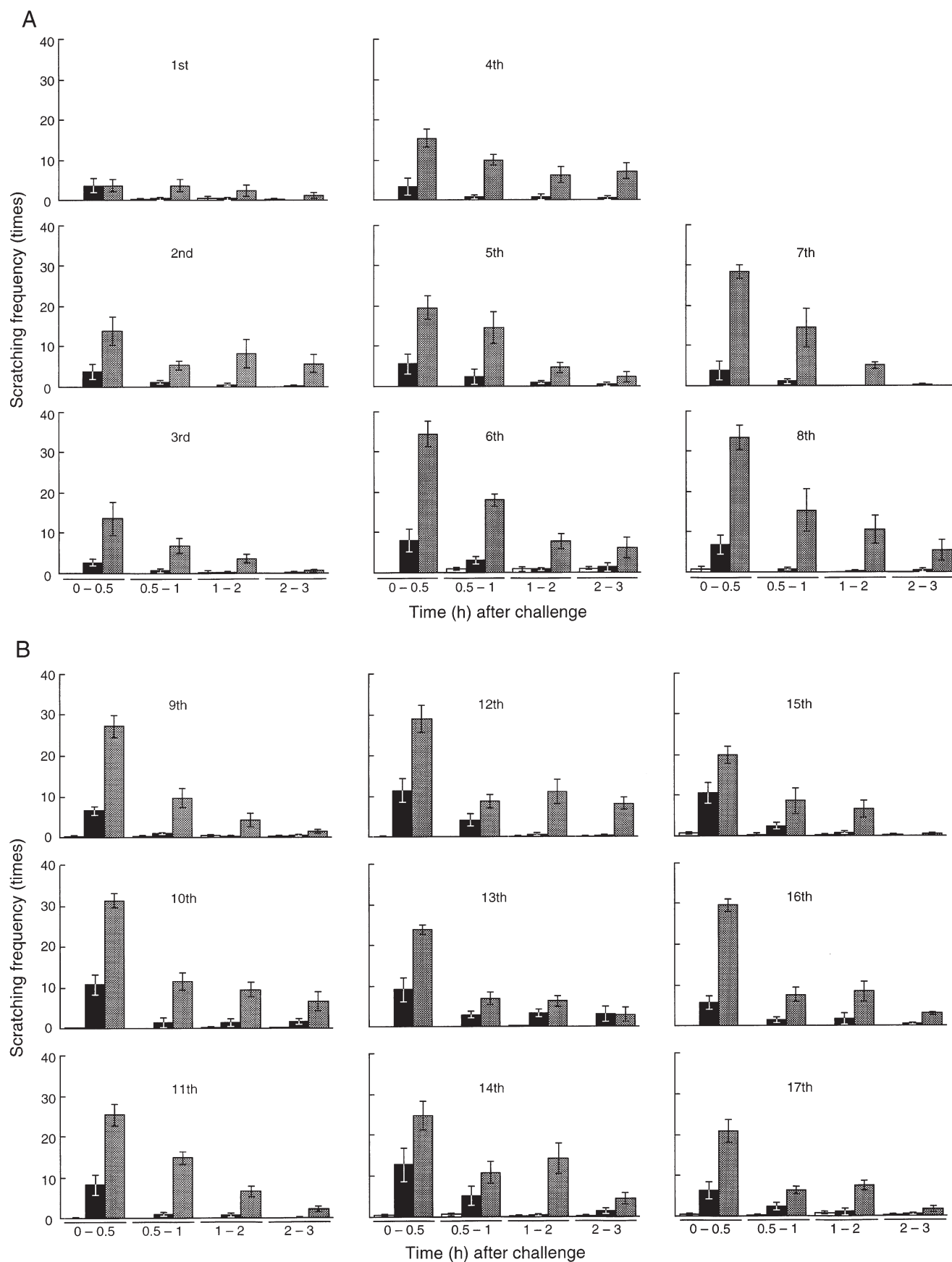


Fig. 3. Time-course changes of scratching frequency following the 1st–8th (A) and 9th–17th (B) challenges by dropping of Japanese cedar pollen suspension in both eyes of sensitised guinea pig of experiment 2. Closed column: Group 2a, dotted column: Group 2b, open column: Group 2c. Each column represents the mean $\pm$ SEM of 5 (Group 2a), 5 or 6 (Group 2b) and 6 (Group 2c) animals.

frequency was almost constant with approximately 20–30 times scratching within the first 30 min after the challenges and then decreased gradually with time. Scratching was hardly seen in the normal guinea pigs (Group 2c).

Time-course changes of amount of albumin in the ophthalmic lavage fluid in experiment 2

Time-related changes of amounts of albumin in the ophthalmic lavage fluid from Groups 2a, 2b and 2c are shown in Fig. 4. In Group 2a, little albumin was found from the 1st to 11th challenges. However, at the 13th to 17th challenges, modest increases of albumin level were recognised, each peaking at 1 h after the challenges. The fluid collected from Group 2b contained apparent levels of albumin already at the 1st challenge. No increases of albumin amount by repeated washing were observed (Group 2c).

Time-course changes of number of leukocytes in the ophthalmic lavage fluid in experiment 2

Figure 5 shows time-course changes of the total number of leukocytes and the respective numbers of mononuclear cells, eosinophils and neutrophils in the ophthalmic lavage fluid

recovered from the conjunctiva following the 1st, 9th and 17th challenges. At the 1st and 9th challenges, no increases of any leukocytes were observed in Group 2a. However, at the 17th challenge, increases of the total number, mainly due to increases in neutrophils, were observed. On the other hand, in Group 2b, marked elevations of these leukocytes were seen at all challenging times, yet these infiltrations were not consistent in their degrees and time courses. No apparent increases of leukocytes were seen in Group 2c.

γ_1 and IgE antibody levels in the serum in experiment 2

The levels of γ_1 and IgE antibodies in the sera obtained from Groups 2a and 2b on the day before the repetitive challenges were estimated as the respective 4-h and 7-day PCA titres (Table 4). Although all sera from Group 2a were negative for both PCAs at the 1st challenge, 4 and 1 out of 10 animals were weakly positive ($1-8 \times$) for the respective 4-h and 7-day PCAs at the 5th challenge. By subsequent challenges, both PCA titres modestly increased. At the 17th challenge, the sera from 5 out of 10 animals were positive for both PCAs. The course of these PCA titres in Group 2b progressed similarly to that of Group 1b in experiment 1.

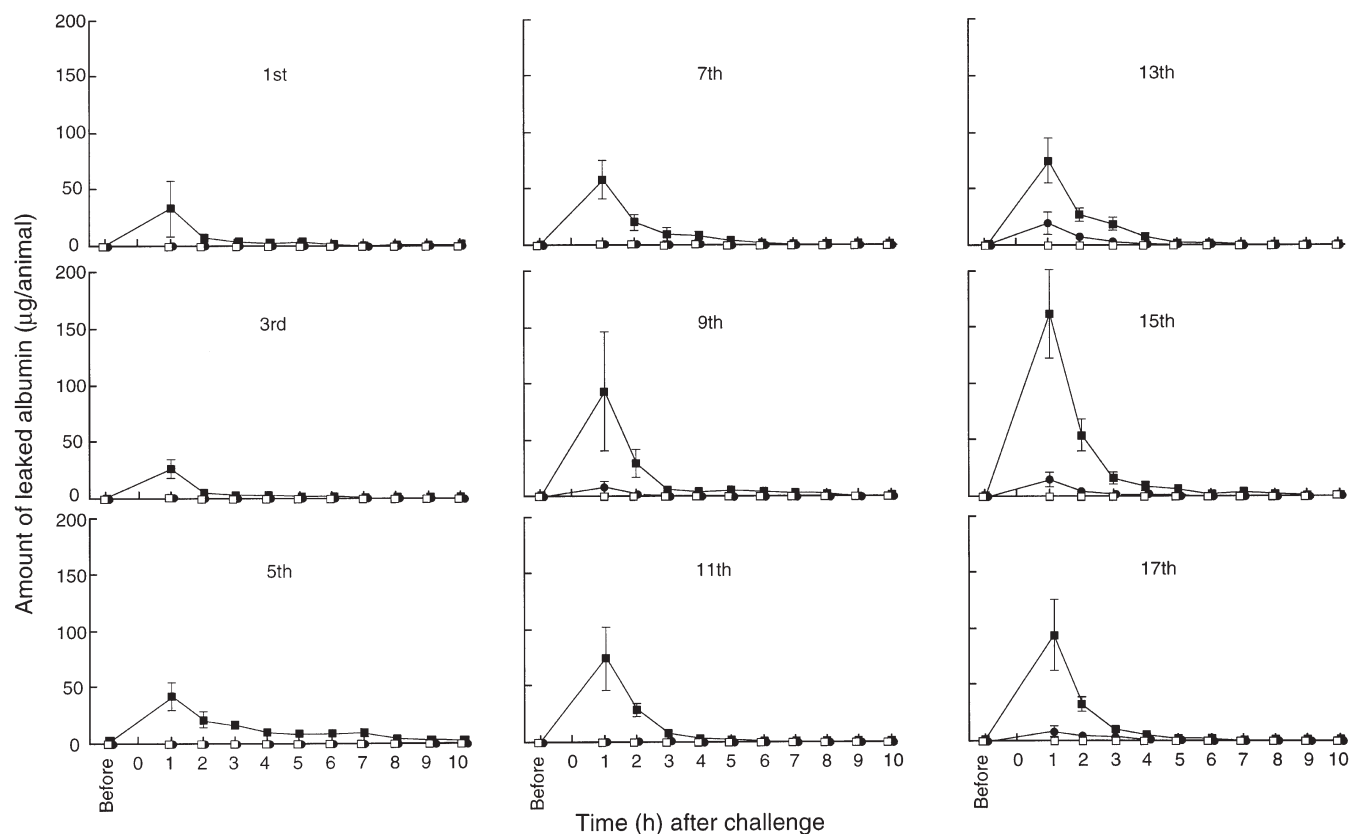


Fig. 4. Time-course changes of amount of albumin in the ophthalmic lavage fluid following the 1st, 3rd, 5th, 7th, 9th, 11th, 13th, 15th and 17th challenges by dropping of Japanese cedar pollen suspension in both eyes of sensitised guinea pig of experiment 2. Closed circle: Group 2a, closed square: Group 2b, open square: Group 2c. Each point represents the mean $\pm$ SEM of 5 or 6 (Groups 2a and 2b) and 5 (Group 2c) animals.

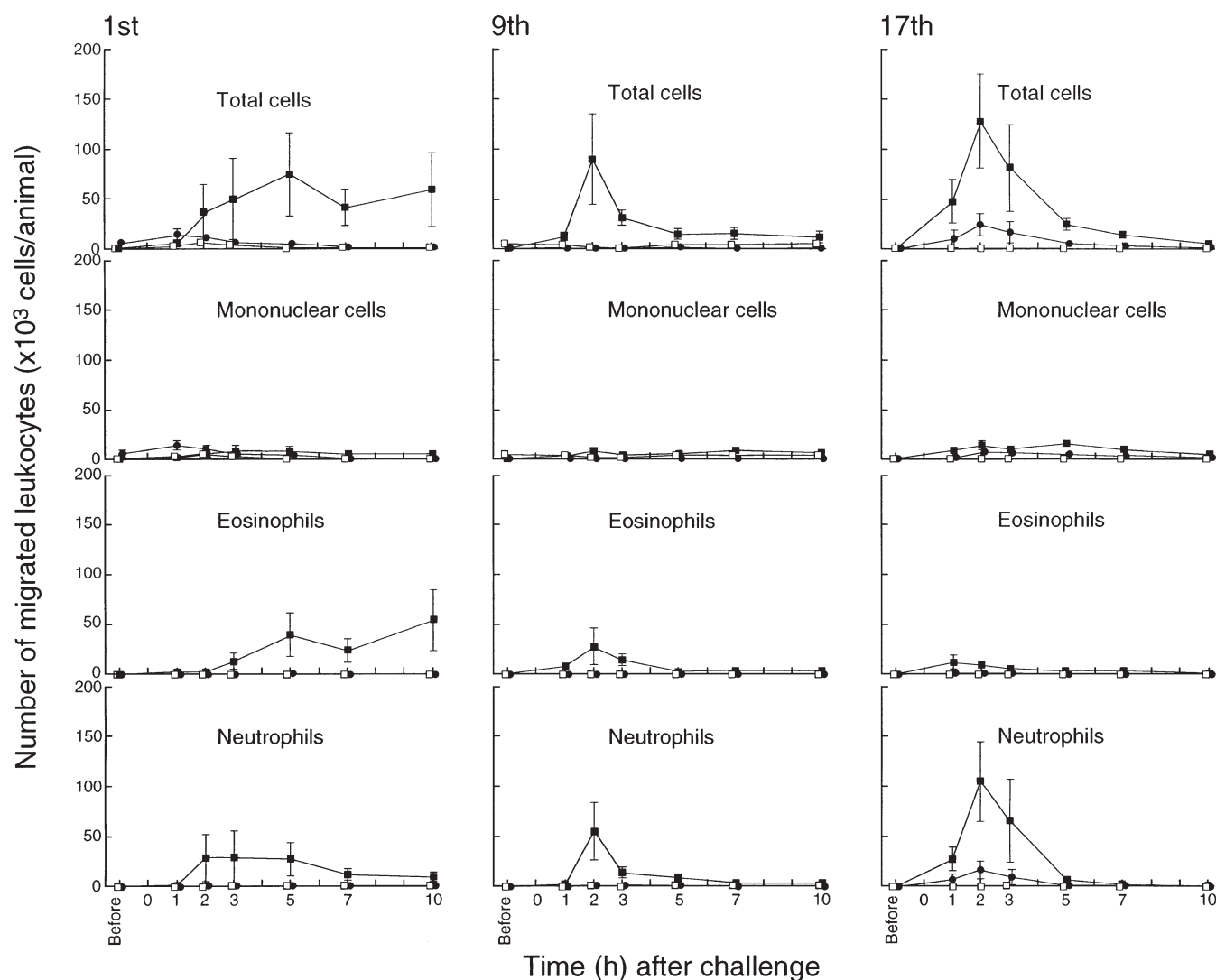


Fig. 5. Time-course changes of the number of leukocytes in the ophthalmic lavage fluid following the respective 1st, 9th and 17th challenges by dropping of Japanese cedar pollen suspension in both eyes of sensitised guinea pig of experiment 2. Closed circle: Group 2a, closed square: Group 2b, open square: Group 2c. Each point represents the mean $\pm$ SEM of 5 or 6 (Groups 2a and 2b) and 5 (Group 2c) animals.

Discussion

We undertook the development of an experimental animal model of allergic conjunctivitis by repetitive and long-term topical applications of cedar pollen to the eyes of locally or systemically sensitised guinea pigs. Firstly, active sensitisation was performed by dropping the pollen extracts + $\text{Al}(\text{OH})_3$ in both eyes (Group 1a). This method produced neither obvious development of symptoms of allergic conjunctivitis nor detectable levels of anaphylactic antibodies in the sera even by repeated challenges with the pollen. In contrast, intraperitoneally sensitised guinea pigs (Group 1b) showed severe symptoms as well as elevations of circulating anaphylactic antibody levels.

The aim of our present study is primarily to establish an allergic conjunctivitis model in which the sensitisation is attained locally. We considered that the failed sensitisation of the experiment in Group 1a might be caused by rapid

drainage of the antigen + $\text{Al}(\text{OH})_3$ into the nasopharyngeal cavity through the nasolacrimal duct. In fact, only 6% of the $\text{Al}(\text{OH})_3$ remained on the conjunctiva at 30 min after the application. Therefore, we devised a new sensitisation method in which the conjunctiva remains effectively in contact with the antigen extracts + $\text{Al}(\text{OH})_3$ for a long time. When the gelatin sponge pieces soaked with the antigen suspension were inserted into the palpebra sulcus, a much higher amount of the antigen was sustained there, presuming that the antigen + $\text{Al}(\text{OH})_3$ is continuously released from the gelatin sponge. Furthermore, no injury of the conjunctiva was macroscopically induced by the inserted gelatin sponges during sensitisation.

When guinea pigs sensitised by the second method were challenged with the cedar pollen repeatedly, relatively apparent increases of CIS were seen at the 12th and subsequent challenges compared to the prior provocations. More interestingly, different from the intraperitoneally sensitised

Group	PCA	Time of challenge	No. of tested sera	No. of sera showing the indicated PCA titre					
				0	1–8	16	32	64	128 ≤
Group 2a	4-h PCA	1	10	10	0	0	0	0	0
		5	10	6	4	0	0	0	0
		9	10	6	3	1	0	0	0
		13	10	6	4	0	0	0	0
		17	10	5	4	0	1	0	0
	7-day PCA	1	10	10	0	0	0	0	0
		5	10	9	1	0	0	0	0
		9	10	8	2	0	0	0	0
		13	10	8	2	0	0	0	0
		17	10	5	5	0	0	0	0
Group 2b	4-h PCA	1	10	1	4	1	1	1	2
		5	10	0	0	0	0	0	10
		9	10	0	0	0	0	0	10
		13	10	0	0	0	2	3	5
		17	10	0	0	0	2	0	8
	7-day PCA	1	10	1	1	1	3	1	3
		5	10	0	0	0	1	0	9
		9	10	0	0	0	0	0	10
		13	10	0	0	0	0	1	9
		17	10	0	0	0	0	1	9

The serum was obtained on the day before the time of the indicated challenge.

Table 4. Four-h and 7-day passive cutaneous anaphylaxis (PCA) titres of sera from the guinea pigs of Groups 2a and 2b.

guinea pigs, all animals showed different CISs between the right and the left eyes, although the degree of the difference individually varied, and it was not observed that CIS in either the right or left eye is consistently higher than the other among the animals. This phenomenon tended to become marked as the challenges were repeated. In addition, scratching, neutrophil infiltration and the albumin leakage from the conjunctiva were recognised. These results are closely similar to the clinical observation that the degrees of symptoms of seasonal allergic conjunctivitis in the left and the right eyes are not all symmetric [19] and strongly indicate the probability that sensitisations of each eye are initiated locally and separately. In addition, the unsymmetry is also recognised in vernal catarrh [19], a chronic state of allergic conjunctivitis [20].

Circulating γ_1 and IgE antibody levels of a few animals of Group 2a became positive at the 5th challenge, and 5 out of 10 animal sera were positive for both PCAs at the 17th challenge. However, there was no correlation between the levels and severity of the symptoms (data not shown). In contrast to the observations in Group 2a, γ_1 and IgE antibody titres in sera from the intraperitoneally sensitised groups (Group 1b and Group 2b) were already high at the 1st challenge. Furthermore, relatively good correlation was recognised between the circulating antibody levels and severity of conjunctivitis in Group 1b and Group 2b. In the systemically sensitised groups, CISs in the left and the right eyes were almost the same degree of intensity. However, it has been reported that symptoms of patients suffering from allergic conjunctivitis are significantly related to the IgE levels in tears rather than to those in the blood [21]. Needless to say, in the clinical situation of allergic conjunctivitis, triggering of pro-

duction of the anaphylactic antibodies is caused by topically invaded antigens. In the intraperitoneally sensitised animals, it could be considered that the antigen-specific antibodies were firstly produced systemically, and then they were spread to the local conjunctival site. Yet, in the topically sensitised group, these antibodies may be produced locally after activation of immune-component cells in the conjunctiva-associated lymphoid tissues followed by spreading over the whole body. From these considerations, the sensitisation process of Group 2a is largely different from that of the intraperitoneally sensitised groups and considerably similar to that of humans, indicating that local sensitisation is more meaningful than systemic sensitisation as a favourable model for research on the disease.

On the other hand, Merayo-Llodes et al. [22] established an experimental allergic conjunctivitis by exposing guinea pigs to ragweed pollen through topical contact on both nasal and conjunctival mucosa, in which it was shown that ragweed pollen powders without adjuvant could achieve a sensitised state and that a single challenge with the pollen induced apparent symptoms of conjunctivitis. In our previous studies [14, 23], when Japanese cedar pollen extracts adsorbed on $Al(OH)_3$ were repeatedly applied to nasal mucosa of guinea pigs for sensitisation and then the animals were challenged by forced inhalation with the pollen powders, symptoms of allergic rhinitis including nasal blockage could be clearly observed. However, intranasal applications with the cedar pollen without the adjuvant produced no elevations of anaphylactic antibodies in the sera and minimal nasal obstructive responses. The reason for the difference between our previous studies [14, 23] and their report [22] is not clear. Although it might be due to the different kind of pollens used,

our experience strongly suggests that an adjuvant is indispensable for local sensitisation by Japanese cedar pollens, like in the present experiment.

Itching is one of the most characterised features of allergic conjunctivitis [24]. It can be easily considered that scratching makes the disease worse and protracted. Suppression of itching is important for improving the quality of life of the patients. The present model will contribute to understanding the pathophysiology of the occurrence of itching and the development of new-type drugs that suppress it.

It has been reported that marked infiltrations of eosinophils and neutrophils into conjunctival tissue were seen in patients with allergic conjunctivitis [25–28]. However, different from the intraperitoneally sensitised guinea pigs, neutrophils but not eosinophils migrated outside the conjunctiva of the topically sensitised animals. The reason for this difference is not clear. Recent findings show that mite and pollen extracts were able to directly induce the production of interleukin-8, which possesses a potent chemotactic activity for neutrophils, in both cultured human epithelial cell lines and nasal epithelial cells collected from non-allergic individuals in vitro [29, 30]. Although neutrophil infiltration was not induced at the 1st challenge in the topically sensitised group (Group 2a), the conjunctival epithelial cells might become responsive to the pollen extracts to produce some neutrophil chemotactic factors by repeated contacts with the antigen.

In conclusion, when guinea pigs were sensitised by insertion of gelatin sponge pieces containing the suspension of the pollen extracts + Al(OH)₃ into the palpebra sulci and repeatedly challenged with the pollen over a long term, allergic conjunctivitis accompanied by different severity of the right and left eyes developed without any correlation with circulating anaphylactic antibody levels. These results strongly suggest that the application of antigen to conjunctival tissue initiates sensitisation at the local site. In a separate experiment, we could obtain almost reproducible results showing that apparent symptoms of conjunctivitis were induced by more than 10-time challenges in topically sensitised guinea pigs. The present sensitisation/challenge procedure and resulting symptoms may be similar to the clinical situation, suggesting that this model is valuable for analysing the pathophysiological mechanism of the disease and should aid in the development of new therapeutic drugs.

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