

Intranasal Sensitization of Japanese Cedar Pollen by the Co-Administration of Low Doses of Cholera Toxin but Not Its Recombinant B Subunit to Mice

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Abstract: We evaluated the effects of cholera toxin (CT) and the B subunit of cholera toxin (CTB) on the intranasal sensitization of Japanese cedar pollen (JCP) in mice. JCP suspended in phosphate-buffered saline was administered into the nostrils of mice in combination with varying doses of CT or recombinant CTB(r-CTB) once a week for 5 weeks. Antibody responses specific to sugi basic protein (SBP) were monitored by ELISA for seven weeks. The sensitization of JCP alone did not induce IgG1, IgG2b, IgG2a, IgE or IgA. In contrast, sensitization of JCP in combination with CT (JCP/CT) elicited the prominent production of SBP-specific IgG1 and low levels of IgG2b and IgG2a on Day 49. IgE production was detected only in the serum of mice which were treated with JCP/CT, and not under any other protocol. Using spleen cells from these mice, cytokine production was examined by ELISA in culture supernatants after they had been stimulated *in vitro* with major cedar pollen allergens, Cry j 1, Cry j 2 or SBP. Notable responses were an increase of IFN- γ as well as IL-4 in JCP/CT-sensitized cells stimulated with Cry j 2, but not in those stimulated with Cry j 1. No significant differences were detected in IL-5 production among the experimental groups. Histopathological examination, however, showed that eosinophil infiltration was evident in the nasal mucosa of the JCP/CT-sensitized mice following challenge with JCP/CT, but weak with BSA/CT or CT alone. Thus, the immunological and histological analyses indicated that the co-administration of a low dose of CT in combination with JCP allows the induction of pollen-allergic states in mice.

Key words: Intranasal sensitization, Murine model, Japanese cedar pollen, Cholera toxin

Japanese cedar pollen contains two major allergens, Cry j 1 and Cry j 2. Cry j 1 is a polypeptide of 41–45 kDa with pectate lyase activity and is localized on the exine (exterior) layer. Cry j 2 is a 42 kDa polypeptide bearing amino acid sequence homology with polygalacturonase and is contained in the amyloplast (interior) of the pollen (8, 16).

Murine models have proved useful in various studies investigating allergy immunotherapy. Freund's incomplete and complete adjuvant barely induce IgE response to protein antigen in mice. It is well-known that helminths and aluminium-gel-precipitated protein antigens are good stimulators for the induction of IgE antibodies. However, these sensitization protocols require intraperitoneal or intradermal administration (17) at a dif-

ferent site from that involved in the natural sensitization of pollen. Furthermore, mice do not show allergic reactions such as asthma or atopic dermatitis, although a rise in body temperature can be an indication of an allergic reaction (5).

Recent studies have reported that cholera toxin shows a powerful adjuvant activity in mucosal immunization (4, 10–12). Furthermore, Glenn et al reported that applying a vaccine solution containing cholera toxin (CT) to the surface of the skin elicited antigen-specific immune responses in mice (6, 7). Cholera toxin is an exotoxin produced by *Vibrio cholerae* with a hexameric A-B structure similar to Pertussis toxin and *Escherichia coli*-

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Abbreviations: BSA, bovine serum albumin; Cry j 1, *Cryptomeria japonica* 1; Cry j 2, *Cryptomeria japonica* 2; CT, cholera toxin; CTB, B subunit of cholera toxin; ELISA, enzyme-linked immunosorbent assay; IFN- γ , gamma interferon; Ig, immunoglobulin; IL, interleukin; JCP, Japanese cedar pollen; PBS, phosphate-buffered saline; PCA, passive cutaneous anaphylaxis; r-CTB, recombinant CTB; SBP, sugi basic protein.

heat labile toxin. The active A subunit of the toxin (33 kDa) is composed of a single component with adenyl cyclase activity capable of interfering with signal transduction in mammalian cells. The B oligomer component of the toxin (88 kDa) has a pentameric structure which mediates the binding of the toxin to glycoprotein receptors on the surface of eukaryotic cells and acts as a powerful adjuvant for immunization (13).

Thus, we attempted here to administer cedar pollen intranasally to mice in as natural a manner as possible and examined the resultant immunological profiles. We showed that CT/JCP sensitization induced the appearance of SBP-specific serum IgE in association with the prominent IgG1, but not IgG2b or IgG2a antibody response. The sensitized mice exhibited histopathological infiltration of eosinophil in the nasal mucosa, and the splenic T cells derived from these mice showed a significant increase in the production of IFN- γ and IL-4 but not IL-5 *in vitro*. Conversely, r-CTB was unable to reproduce the activity of CT. Thus, this protocol may be beneficial in the development of allergic murine models for various pollenosis.

Materials and Methods

Animals. Female BALB/c CrSlc mice (6 weeks, 16–20 g) were purchased from SLC Japan, Inc., Shizuoka. Male Sprague-Dawley (SD) rats were purchased from Charles River Japan Inc., Kanagawa. The animals were housed in stainless steel cages and provided with food (CE-2, CLEA Japan Inc.) and water *ad libitum*.

Allergens and other reagents. Japanese cedar pollen (JCP) was stored at -20°C until use. The JCP was ground using an agate mortar and pestle, and suspended in phosphate-buffered saline (PBS, pH 7.3) just prior to sensitization. The sugi basic protein (SBP) was prepared according to the procedure of Yasueda et al (20), with a slight modification for extracting the JCP with a 0.125 M sodium bicarbonate solution. The yield of SBP was 160 ml per 10 g of JCP. SBP was stored frozen at -20°C until use. Cry j 1 and Cry j 2 were purified from JCP by affinity chromatography using monoclonal antibodies specific for either Cry j 1 (mAb 065) or Cry j 2 (mAb 026), as previously described. The contents of Cry j 1 and Cry j 2 were measured by ELISA as described (14, 15). Six-hundred micrograms of JCP contained 289 ng of Cry j 1 and 58 ng of Cry j 2. The cholera toxin (CT) was purchased from Sigma (St. Louis, Mo., U.S.A.). The r-CTB was a gift from Dr. Jan Holmgren of the University of Göteborg, Göteborg, Sweden. These materials were suspended in PBS before use.

Intranasal sensitization. The PBS suspension mixture of finely ground Japanese cedar pollen powder was

administered into the nostrils of BALB/c mice by pipette and plastic tip. Mice were sensitized with 600 μg of JCP mixed with 1 μg CT (JCP/CT:1), 0.3 μg CT (JCP/CT:0.3) and 1 μg r-CTB (JCP/r-CTB:1) or 0.3 μg r-CTB (JCP/r-CTB:0.3), or 600 μg JCP alone (JCP) in a total volume of 6 μl once a week for 5 weeks (9, 11). Four weeks later, these mice were boosted in the same way twice every 10 days. Mouse blood was collected from the jugular vein with a disposable syringe and needle. Blood was centrifuged at 3,000 r.p.m. for 15 min. Sampled sera were stored at -20°C until use.

ELISA. Quantitation of serum antibodies: In brief (3), microtiter plates (MS-8596, Sumitomo Bakelite Co., Tokyo) were coated with SBP (50 μl /well), Cry j 1 (50 ng/well) or Cry j 2 (50 ng/well) for 1 hr at room temperature and the remaining binding sites were blocked by the addition of 420 μl of PBS containing 0.5% BSA. After incubation for 1 hr at room temperature, the plates were washed 4 times with a wash buffer (PBS containing 0.5% tween-20). The sera were diluted with PBS containing 0.5% tween-20 and added to the wells. After 1 hr at room temperature, the plates were washed 4 times with the wash buffer. Alkaline-phosphatase (AP)-conjugated rat anti-mouse IgM, IgG1, IgG2b, IgG2a, IgE or IgA antibody (50 μl /well, MBL, Nagoya, Japan) was added at a dilution of 1:1,000 or 1:2,000 (IgM) and incubated for 1 hr at room temperature. After washing the plates 4 times, the wells were incubated with 100 μl of substrate containing 10% *p*-nitrophenyl phosphate and 10% diethanolamine (Wako Co., Osaka, Japan). Absorbance was read at 405 nm by an Auto Well Reader (SME3400, Iwaki Glass Co., Ltd., Tokyo).

Passive cutaneous anaphylaxis (PCA) response. PCA was performed as described. In brief, the sensitized mice serum (50 μl /site), diluted at varying ratios, was injected intracutaneously into the shaved backs of SD male rats. Forty-eight hours later, a mixture of SBP (1 ml) and 1% Evans blue solution (1 ml) was injected into the tail veins of these rats. Blue spots on the back side of the skin were measured 30 min later.

Cell culture. T-cell proliferation: Cell cultures were carried out as described. In brief, spleen cells (1×10^5 /0.2 ml/well) were either stimulated with Cry j 1, Cry j 2 (0.19, 0.56, 1.67, 5 μg /ml) or Concanavalin A (2 μg /ml), or remained unstimulated in round-bottomed 96-well plates (Iwaki Glass). Seventy-two hours later, T-cell proliferation was assessed by pulsing the cells with 0.5 μCi ^3H -thymidine (Amersham, St. Louis, Mo., U.S.A.) for the final 18 hr.

Cytokine production. Spleen cells (1.5×10^6 /0.5 ml/well) were stimulated with Cry j 1, Cry j 2 (5 μg /ml), SBP (5%) or Concanavalin A (2 μg /ml), or remained unstimulated in round-bottomed 24-well plates (Iwaki

Glass). Seventy-two hours later, cell culture supernatants were harvested by centrifugation and stored at -80°C until use. IFN- γ and IL-4 were measured by ELISA kits (Quantikine™ Mouse Immunoassay, R&D Systems), as was IL-5 (TiterZyme® Mouse IL-5 Enzyme Immunoassay, PerSeptive Diagnostics, Inc.).

Histopathological analysis. Mice sensitized with JCP/CT:1 were challenged by 600 μg JCP and 1 μg CT (JCP/CT:1), 600 μg BSA and 1 μg CT (BSA/CT:1), or 1 μg CT (CT:1). Naive mice were also challenged by JCP/CT:1. Five hours later, the animals were euthanized by exsanguination under anesthesia by an intraperitoneal injection of sodium pentobarbital solution. The noses of all mice were taken as samples, fixed in 10% neutral buffered formalin, and then decalcified in a mixture of formic acid and formalin. Fixed and decalcified specimens were embedded in paraffin, sectioned, and stained with Hematoxylin-Eosin solution. Slides of tissue specimens were observed by light microscopy.

Statistics. Results are expressed as means $\pm$ standard deviation (S.D.) and analyzed by Student's *t*-test.

Results

Antibody Response to SBP

Mice were intranasally sensitized with 600 μg of JCP with or without 1 μg CT (JCP/CT:1), 0.3 μg CT (JCP/CT:0.3), 1 μg r-CTB (JCP/rCTB:1), or 0.3 μg r-CTB (JCP/rCTB:0.3) once a week for 5 weeks. To estimate the antibody responses to JCP, SBP-coated plates were used for ELISA. The subclasses examined were serum IgM, IgG1, IgG2b, IgG2a, IgE and IgA specific to SBP.

Figure 1 shows the results of the titration experiment of sera collected 3 weeks (Day 49) after the fifth sensitization for various Ig subclasses. IgM responses were induced to an almost equal level in all groups. In contrast to IgM responses, marked levels of IgG1 antibody responses were detected in mice sensitized with the co-administration of JCP/CT. The responses were dependent on doses of CT. No mice treated with JCP/r-CTB or JCP alone showed IgG1 antibody response.

A slight increase of IgG2b or IgG2a response was detected in mice sensitized with JCB in combination with 1 μg CT. In the case of serum IgA, it was detected at very low but significant levels only in mice sensitized with JCP/CT:1. IgA in lung fluid was examined at the time of histopathological analysis. Very low levels of IgA were detected in mice sensitized with JCP/CT:1 (data not shown).

The Cry j 1 or Cry j 2-specific IgG1 antibodies were also examined in these mice sera by ELISA. Cry j 1-specific IgG1 antibody was 54% of SBP-specific IgG1

ODs, and Cry j 2-specific IgG1 antibody was 17% of SBP-specific IgG1 ODs.

IgE Response to SBP

We attempted to detect the SBP-specific IgE by ELISA. It was barely detected in any group at the time of the fifth sensitization. A significant but slight increase of IgE production was detected 3 weeks after the fifth sensitization in mice treated with JCP and 1 or 0.3 μg CT (Fig. 1). All four mice showed IgE responses in the case of JCP/CT:1, while two of four mice produced IgE responses in the case of JCP/CT:0.3. A very weak IgE

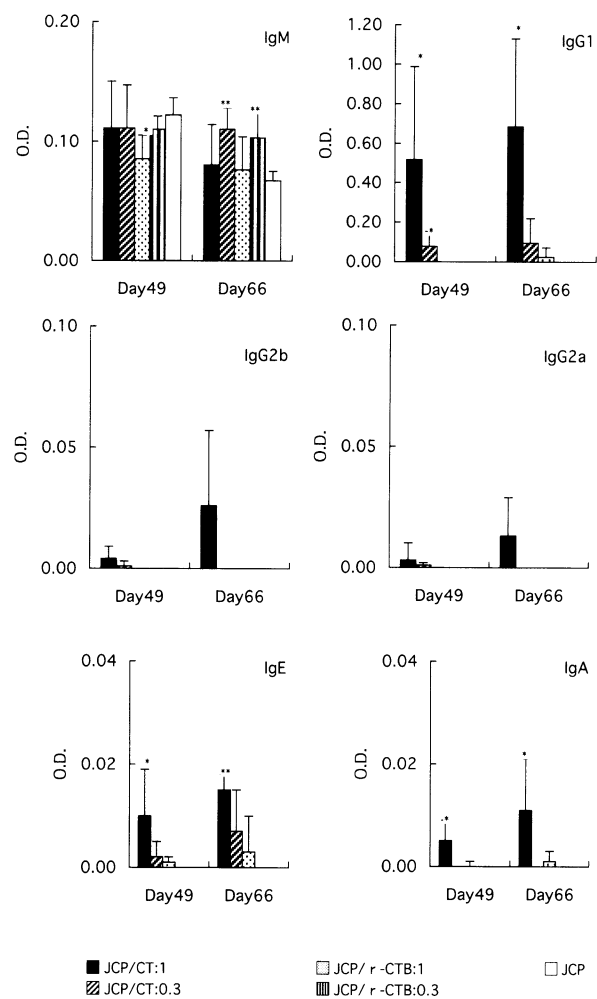


Fig. 1. SBP-specific Ig antibodies of mouse sera. Mice were sensitized with JCP/CT:1, JCP/CT:0.3, JCP/r-CTB:1, JCP/r-CTB:0.3, or JCP alone. Each group consisted of four mice. Mouse sera were collected on Days 49 and 66. The results were indicated as the mean value $\pm$ standard deviation. Serum dilutions were 1:4 (IgE), 1:50 (IgM, IgG2b, IgG2a, IgA) or 1:150 (IgG1). Optical density was measured at a wavelength of 450 nm. The experiments were repeated three times. Representative results are presented. * $P < 0.05$; ** $P < 0.01$ versus JCP control. No SBP-specific antibodies were detected in mouse sera sensitized with CT or r-CTB alone throughout the experimental protocol.

response was detected in one of four mice sensitized with JCP/r-CTB:1.

These responses were confirmed by PCA reactions using rats (Fig. 2). Consistent with the results of ELISA, the combined sera of JCP/CT:1-sensitized mice exhibited clear positive reactions at dilutions of 1:2, 1:6 and 1:18, whereas the sera from JCP/r-CTB:1 showed a very weak positive response at a 1:2 dilution (Fig. 2, a and b). The positive responses were negated by heating the sera at 60 °C for 30 min, indicating IgE-mediated reactions (data not shown). No reaction was detected in mice treated with JCP alone (Fig. 2b).

T-Cell Proliferation Specific to Cry j 1 or Cry j 2

In order to determine whether the sensitization was established in response to the major allergenic molecules, Cry j 1 and Cry j 2, as shown in human cases, a T-cell proliferation assay was performed *in vitro* by stimulating cells (JCP/CT:0.3) with Cry j 1 or Cry j 2. As shown in Table 1, stimulation of T-cell proliferation by

Cry j 1 at 5 µg/ml was approximately 5 times greater than the non-stimulated control response. In the same concentration range, Cry j 2 induced strong proliferation, 6 to 19-fold greater than the control response. Thus, even if the content of Cry j 1 or Cry j 2 per sensitization was extremely low, in the order of nanograms, the repetitive administration of these molecules in combination with CT enabled them to stimulate the immune system as described in human cases. In the r-CTB treatment group, Cry j 1 or Cry j 2 induced no T-cell proliferation.

Cytokine Production

To investigate the effects of CT or r-CTB on cytokine production, we attempted to amplify the immune responses by repeating the same treatment at Days 56 and 66. Immediately before sensitization on Day 66, the features of antibody responses were tested and it was found that they had not substantially changed, with only a slight appearance of IgG2b and IgG2a (Fig. 1). Using these mice, spleen cells were stimulated with Cry j 1 (5

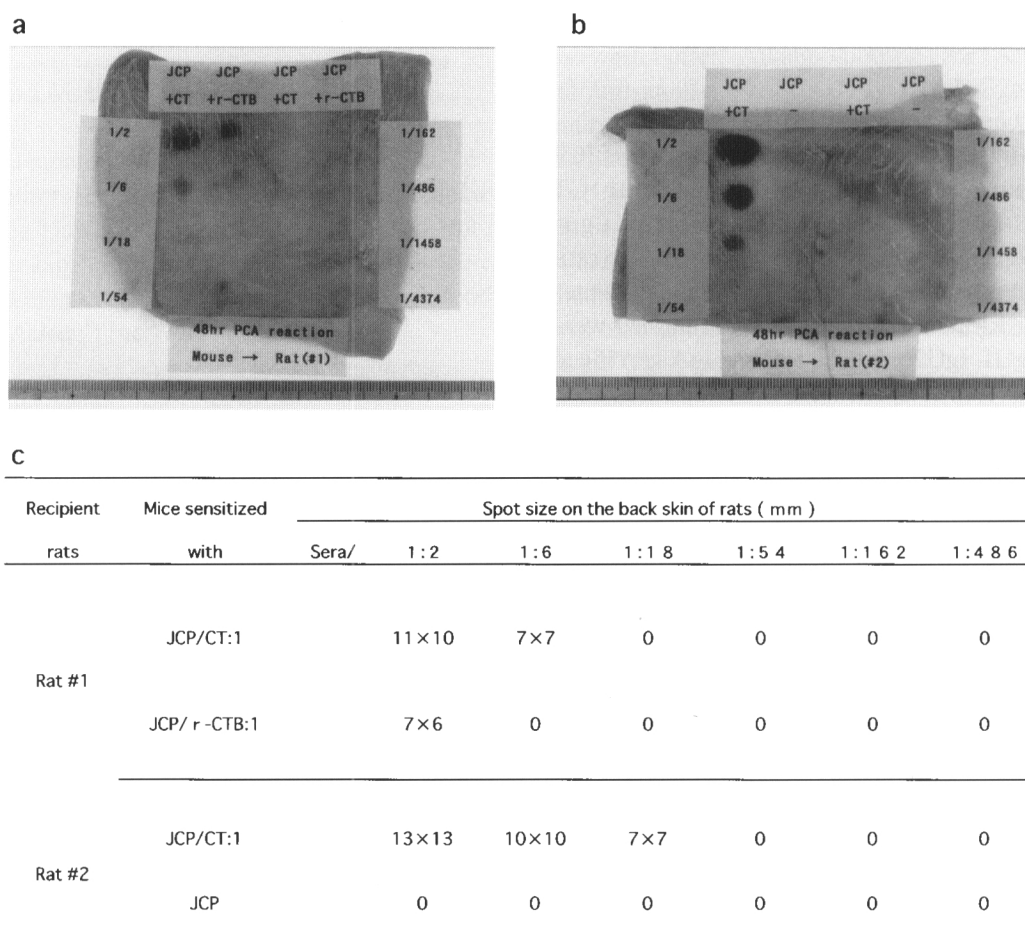


Fig. 2. PCA reaction of rats treated with mouse sera. PCA reactions were performed as described in "Materials and Methods." (a) PCA reaction on the back skin of rat sensitized with the pooled sera treated with JCP/CT:1 or JCP/r-CTB:1. (b) PCA reaction on the back skin of rat sensitized with the pooled sera treated with JCP/CT:1 or JCP alone. (c) PCA reaction of rats treated with murine sera. Mouse sera were diluted 1:2 to 1:486 with saline.

µg/ml), Cry j 2 (5 µg/ml), SBP (5%) or Concanavalin A (2.0 µg/ml) and cultured for 72 hr. The concentrations of IFN-γ, IL-4 and IL-5 in the culture supernatants were determined by ELISA (Table 2). Concanavalin A induced marked and comparable production of IFN-γ, IL-4 and IL-5 from each cell culture. SBP strongly induced

IFN-γ production from cells of the JCP/CT-, JCP/r-CTB- and JCP-sensitized mice, indicating the establishment of strong T-cell sensitization in these mice. JCP/CT-sensitized cells showed relatively higher responses than JCP/r-CTB production of IFN-γ and IL-4. Cry j 2 showed relatively stronger inducing activity than Cry j

Table 1. Proliferation of mouse spleen cells stimulated with Cry j 1 or Cry j 2 (dpm ± S.D.)

Stimulation ^{a)}	Concentration (µg/ml)	Exp. 1 ^{b)}			Exp. 2 ^{c)}	
		JCP/CT:1	JCP/r-CTB:1	JCP	JCP/CT:0.3	JCP/r-CTB:0.3
Cry j 1	5				1,633 ± 532	351 ± 149
	1.67	4,982 ± 542	2,447 ± 550	2,561 ± 427	1,065 ± 449	248 ± 65
	0.56	4,717 ± 561	2,198 ± 283	2,078 ± 156	458 ± 244	222 ± 78
	0.19	4,217 ± 87	2,683 ± 304	2,425 ± 143	470 ± 241	206 ± 106
Cry j 2	5				4,940 ± 1,519	274 ± 102
	1.67	6,704 ± 3,350	2,904 ± 683	3,003 ± 300	5,819 ± 1,744	243 ± 30
	0.56	8,764 ± 2,313	3,181 ± 982	3,021 ± 225	4,954 ± 1,197	295 ± 159
	0.19	7,629 ± 336	2,835 ± 413	2,918 ± 125	1,961 ± 476	197 ± 23
Concanavalin A	2				34,675 ± 27,426	43,636 ± 2,192
Non-stimulation	0	3,281 ± 1,148	2,836 ± 485	2,896 ± 129	300 ± 23	241 ± 56

^{a)} Proliferation assay was performed using spleen cells as described in "Materials and Methods."

^{b)} Mice were sensitized with JCP/CT:1, JCP/r-CTB:1 or JCP alone. Ten days after final sensitization, spleen cells were tested for proliferative responses.

^{c)} Mice were sensitized with JCP/CT:0.3 or JCP/r-CTB:0.3. Experiments were done in triplicate cultures using a protocol identical to Exp. 1.

Table 2. IFN-γ, IL-4, and IL-5 production of spleen cell cultures^{a)}

Mice sensitized with	Stimulants	Concentration (µg/ml)	IFN-γ (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml) ^{c)}
JCP/CT:1	Cry j 1	5	4 ± 5	n.d.	6 ± 0
	Cry j 2	5	113 ± 2	2 ± 1	7 ± 1
	SBP	(5%)	725 ± 90	9 ± 0	13 ± 0
	Concanavalin A	2	872 ± 17	832 ± 1	389 ± 5
	None	0	4 ± 4	n.d.	5 ± 0
JCP/CT:0.3	Cry j 1	5	34 ± 1	n.d.	6 ± 0
	Cry j 2	5	347 ± 2	12 ± 0	10 ± 0
	SBP	(5%)	856 ± 6	1 ± 2	29 ± 1
	Concanavalin A	2	912 ± 4	918 ± 8	536 ± 9
	None	0	4 ± 2	n.d.	1 ± 0
JCP/r-CTB:1	Cry j 1	5	1 ± 2	n.d.	2 ± 0
	Cry j 2	5	3 ± 1	n.d.	2 ± 1
	SBP	(5%)	396 ± 8	1 ± 0	22 ± 1
	Concanavalin A	2	782 ± 4	818 ± 12	415 ± 11
	None	0	3 ± 4	n.d.	3 ± 0
JCP/r-CTB:0.3	Cry j 1	5	13 ± 8	n.d.	5 ± 2
	Cry j 2	5	27 ± 10	n.d.	7 ± 1
	SBP ^{b)}	(5%)	609 ± 27	n.d.	21 ± 0
	Concanavalin A	2	974 ± 24	992 ± 15	474 ± 3
	None	0	188 ± 41	n.d.	2 ± 1
JCP	SBP	(5%)	891 ± 51	n.d.	36 ± 1
	Concanavalin A	2	857 ± 39	622 ± 7	278 ± 4
	None	0	n.d. ^{b)}	n.d.	3 ± 1

^{a)} Cytokine assays were performed as described in "Materials and Methods." Experiments were repeated three times and a representative result is presented.

^{b)} n.d.: Not detected below 1 pg/ml.

^{c)} In the case of IL-5 production, no statistically significant differences were observed in any corresponding group ($P > 0.05$) when the mean value ± S.D. of three independent experiments was compared to JCP/r-CTB:0.3.

1, particularly in IFN- γ but also in IL-4 production. In IL-5 production, there was little difference between the effects of CT and r-CTB.

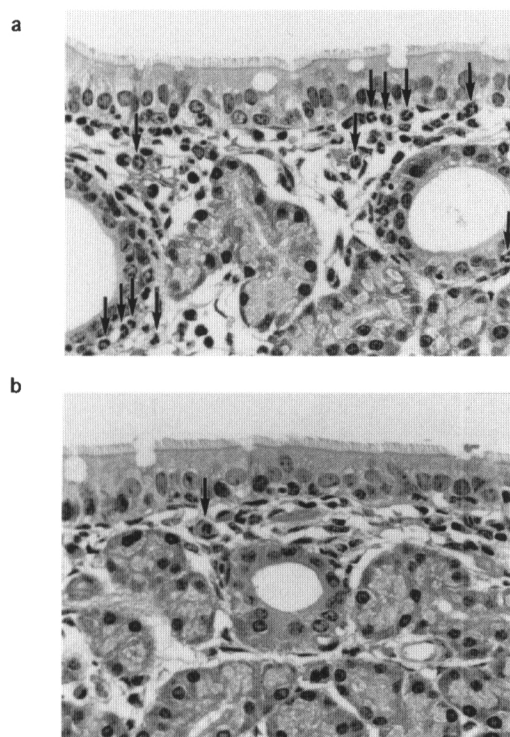
Although the IL-4 production of JCP/CT-sensitized cells was very low, the amount of Cry j 2-induced IL-4 was almost comparable or rather higher than those responses stimulated with SBP. These results suggested that IL-4 might be preferentially produced by Cry j 2-specific T cells. Conversely, Cry j 1-specific T cells showed weak production of IFN- γ and almost no production of IL-4 in spite of remarkable Cry j 1-specific T-cell proliferation.

The low dose (0.3 μ g) of CT was more effective than the high dose (1 μ g) in the sensitization to produce these cytokines.

Regarding the effects of r-CTB, there appears to be significant suppression of IFN- γ production against SBP. The response of JCP/r-CTB:1 and JCP/r-CTB:0.3-sensitized cells was 44 and 68%, respectively, of the response of JCP-sensitized cells. Little effect was shown on the IFN- γ response stimulated with SBP in JCP/CT:1 or JCP/CT:0.3-sensitized cells.

Histopathological Analysis

In order to examine the development of allergic reactions in mice, we performed a histopathological analysis of the nasal mucosa. Ten days after the seventh sensitization with JCP/CT:1, mice were challenged with JCP and 1 μ g CT (JCP/CT:1), BSA and 1 μ g CT (BSA/CT:1) or 1 μ g CT (CT:1) by administration into the nostrils. Naive mice were also challenged with JCP/CT:1 (Fig. 3). When sensitized mice were challenged with JCP/CT:1, strong eosinophil infiltration was elicited in the nasal mucosa (Fig. 3a). The infiltration was particularly severe in the respiratory epithelium area. In contrast, only slight infiltration of eosinophil was observed when sensitized mice were challenged with BSA/CT:1 (Fig. 3b) or CT:1. To examine the involvement of the CT-specific antibody, we tested CT-specific IgG1 antibody in mice sera for sensitization to CT (Fig. 3c). High levels of CT-specific IgG1 antibody response were detected in JCP/CT:1-sensitized mice. These results indicate that the degree of eosinophil infiltration elicited with BSA/CT or CT might reflect the response due to the involvement of anti-CT antibodies. Allergic responses to JCP might further increase the degree of allergic reactions as shown



Mice sensitized with	Challenge	Hisopathology	CT-specific IgG1 antibody
		finding	
	JCP/CT:1	eosinophil infiltration (particularly severe in the respiratory epithelium area) Grade : 4	
JCP/CT:1	BSA/CT:1	eosinophil infiltration Grade : 2	0.363 $\pm$ 0.160
	CT:1	eosinophil infiltration Grade : 2	
Naive	JCP/CT:1	eosinophil infiltration Grade : 1	0.007 $\pm$ 0.009

Fig. 3. Histopathological findings of mouse nasal mucosa. A histopathological analysis was performed as described in "Materials and Methods." Three mice were examined in each experimental group, and representative results are shown in the figure. (a) Eosinophil infiltration of nasal mucosa after challenge with JCP/CT:1. (b) Eosinophil infiltration of nasal mucosa after challenge with BSA/CT:1. (a, b) Magnification was 1:200 by light microscope. Arrows indicate eosinophil. (c) Histopathological findings in nasal mucosa after antigen challenge. Histopathological findings criteria are as follows: 1, very slight; 2, slight; 3, moderate; and 4, marked. On CT-specific IgG1 antibody measurement, mouse sera were collected 5 hr after challenge. CT-specific IgG1 antibodies in mouse sera were measured by ELISA at a serum dilution of 1:150. The absorbance at 405 nm was measured 1 hr after development.

in the case of JCP/CT elicitation. No infiltration of eosinophil was observed in naive mice after challenge with JCP/CT:1.

Discussion

We studied here the adjuvant effects of CT and r-CTB on the generation of allergic sensitization in mice followed by the intranasal administration of allergens. The sensitizing dose of JCP, 6 μ l of PBS suspension containing 600 μ g of JCP, was the maximum attainable preparation for intranasal administration because of its viscosity, due in turn to contaminated lipids and glucoses. A single administration of 6 μ l of JCP suspension contained 289 ng Cry j 1 and 58 ng Cry j 2. The mice were therefore repeatedly sensitized in the intranasal mucosa with this dose of JCP alone, or JCP in combination with 1 μ g CT, 0.3 μ g CT, 1 μ g r-CTB or 0.3 μ g r-CTB.

We examined the features of antibody responses, T-cell proliferation and cytokine production of these mice. Sensitization by JCP alone or JCP/r-CTB only induced an IgM response and no IgG1, IgG2b, IgG2a or IgE serum antibodies. In contrast, treatment with JCP/CT produced a marked level of SBP-specific IgG1 and low levels of IgG2b or IgG2a. It is notable that the co-administration of JCP with CT induced a significant level of IgE antibody, which was detected by SBP-specific IgE-ELISA. These results were confirmed by PCA tests *in vivo* (Fig. 2). On the other hand, r-CTB showed no adjuvant activity.

To examine whether these mice were sensitized with Cry j 1 or Cry j 2, which are known to be major allergenic molecules in humans (8, 16), the spleen cells from these mice were stimulated with purified molecules *in vitro*. Cells sensitized with JCP alone showed very weak proliferative responses to Cry j 1 and Cry j 2. In contrast, cells from JCP/CT-sensitized mice showed strong T-cell proliferation in response to Cry j 1 or Cry j 2 as well as a SBP induced response. Recombinant CTB did not show adjuvant activity for Cry j 1- or Cry j 2-specific T-cell proliferation or antibody production.

Cytokine assays indicated that Cry j 2 was a stronger cytokine inducer than Cry j 1 when JCP/CT-sensitized spleen cells were tested. In the case of cells sensitized with JCP/CT:0.3, a significant increase of IL-4 and marked IFN- γ production were induced by *in vitro* stimulation with Cry j 2. On the other hand, Cry j 1 induced IFN- γ production but not IL-4. These results suggested the potential role of CT as an adjuvant, particularly skewing the immune response to Cry j 2 toward Th2-type IgG1 and IgE antibody responses (1) although JCP/CT-sensitized cells showed significant increases in IFN- γ and

IL-4. In the case of Cry j 1-specific response, CT enhanced the IgG1 response, which also suggested that CT might be an adjuvant for Th2-type response. However, it showed only a weak adjuvant effect on the production of IFN- γ and none for that of IL-4. Furthermore, IL-5 production did not appear to be influenced by CT or r-CTB. As the profile of cytokine production was derived from an *in vitro* assay system using spleen cells and not the regional lymph nodes, it might not accurately reflect the *in vivo* situation. Thus, judging from the pattern of antibody production, particularly regarding IgG1 antibody response, it is conceivable that CT preferentially potentiates Th2-type responses in mice *in vivo*.

The experiment of multiple sensitization by aerosol of 4 mg birch pollen extract with 5 μ g CT showed that CT mixed with the aerosolized birch pollen extract induced a Th1-like immune response (19). In our experiments, the lower dose of CT (0.3 μ g) was more effective than the higher dose (1 μ g) regarding the adjuvant effect of CT on IFN- γ or IL-4 production. Thus, the dose of CT may also be critical for the preference of Th1/Th2-type responses.

The adjuvant activity of r-CTB is still a subject of controversy. Blanchard et al reported that r-CTB is not an effective mucosal adjuvant for oral immunization of mice against *Helicobacter felis* (2), while Tochikubo et al has shown that r-CTB acted as an adjuvant for mucosal and systemic responses of mice to mucosally co-administered bovine serum albumin (18). They employed the multiple administration protocol including 10 μ g r-CTB per administration. Under our experimental conditions using 0.3–1.0 μ g per administration, r-CTB did not show adjuvant activity in our cytokine production assay. Conversely, in the case of IFN- γ production against SBP stimulation, r-CTB appeared to induce suppression in comparison with CT as shown in SBP-stimulated responses. This result may be related to findings that CTB up-regulates TGF- β 1 activity although we did not examine this possibility (10).

Thus, it was demonstrated that repeated intranasal sensitization of JCP in combination with 1 μ g CT enables mice to produce SBP-specific IgE antibody, even if the sensitizing dose of JCP contained pollen allergens, Cry j 1 and Cry j 2, only at the nanogram level. In histopathological findings, strong eosinophil infiltration was observed in the nasal mucosa following JCP/CT challenge to sensitized mice. In contrast, either no or only slight eosinophil infiltration was shown in either sensitized mice challenged by BSA/CT or CT in naive mice challenged by JCP/CT. Thus, the results of histopathological analyses shown in Fig. 3 demonstrate direct evidence of the manifestation of JCP-specific sensitization in the nasal mucosa. The features of cytokine and anti-

body production in the regional nasal mucosa may characterize the JCP-induced immune response more adequately. In this study, we presented the results of serum antibody and cytokine responses of spleen cells stimulated *in vitro*.

With regard to the toxicity of CT, it has been shown that intranasal challenging of mice with CT induces fatal cytotoxic pulmonary lesions that are characterized by suppurative interstitial pneumonia with marked perivascular edema, fibrin deposition, and hemorrhage (7). In this study, we investigated the lungs and bronchi of mice in gross necropsy and histopathology 5 hr after challenge with JCP and 1 µg CT. No abnormal changes except for slight macrophage migration in the alveoli were noted.

Taking these results together, the potential of CT as a mucosal adjuvant in intranasal sensitization was demonstrated. This study protocol may provide a useful mouse allergic model as intranasal administration is equivalent to the invasive route in cedar pollinosis.

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