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Immunosuppressive Effect of Cholera Toxin B on Allergic Conjunctivitis Model in Guinea Pig

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Purpose: To investigate the new method of immunotherapy using cholera toxin B (CTB) in experimental allergic conjunctivitis.

Methods: We used 21 white Hartley guinea pigs. The animals were sensitized by intraperitoneal injection of ovalbumin (100 μg/mL) and albumin hydroxide (5 mg/mL) repeated after an interval of 2 weeks. One week after the second injection, conjunctivitis was induced by topical instillation of ovalbumin (5 mg/mL). The animals were divided into two groups, CTB group and control group. The CTB group underwent pretreatment of topical instillation of CTB (4μg/30 mL) and ovalbumin (10 μg/30 mL), three times a day for 3 days, 1 week before the intraperitoneal injection. The control group did not undergo the pretreatment. Clinical examination was performed at 0.5, 6, and 24 hours after the development of conjunctivitis. Histological examination was performed at 6 and 24 hours.

Results: Both groups developed palpebral and bulbar edema with hyperemia 30 minutes after instillation of ovalbumin. The allergic reaction score was significantly less in the CTB group than in the control group (Mann-Whitney *U*-test: $P < .01$). The clinical reactions subsided after 6 hours. The CTB group showed less eosinophilic infiltration in the conjunctiva and the limbus, particularly in the conjunctival epithelium, than the control group at 6 and 24 hours.

Conclusion: Pretreatment with topical CTB and antigen suppresses clinical and histological findings in experimentally induced allergic conjunctivitis. **Jpn J Ophthalmol 2001;45:332–338**

Key Words: Experimental allergic conjunctivitis, cholera toxin B, mucosal immunity.

Introduction

Mucosal immunity is one of the immunological defense mechanisms against invasion of antigens and infectious microorganisms on the mucosal surface causing antigen-specific secretory immunoglobulin A (IgA) production.[1] The IgA antibody production is induced by the involvement of lymphatic tissues called mucosa-associated lymphoid tissue (MALT) present in the mucosal tissue.[1] Transmucosal antigen administration induces and increases antigen-specific secretory IgA in the mucosal immune system, and immunological tolerance is induced in the systemic immune system. This response is reported as the immunological characteristic of mucosal immunity.[1] Transmucosal antigen administration is performed through the nose and mouth by inhalation. Depending on the properties of antigen substances, however, not enough elevation of the antibody titer can be obtained by administration of the antigen alone. In order to induce mucosal immunity effectively, therefore, concurrent administration of an adjuvant with an antigen is reported to be effective.[2] The adjuvant that induces mucosal immunity efficiently is called a mucosal immunity adjuvant. Cholera toxin (CT) and cholera toxin B (CTB) have been reported as representative of such adjuvants.[1–7]

Cholera toxin, an exotoxin produced by *vibrio cholerae*, is a pathogenic substance that causes diarrhea. Comprised of 1 molecule of A subunit (CTA)

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and 5 molecules of B subunit (CTB), it consists of hexamer protein with a molecular weight of about 84 kDa. The CTA is the substance that causes diarrhea and dehydration in patients with cholera, and CTB connects with GM1 ganglioside on the surface layer of nucleated cells of animals via B subunit and is transmitted into the cytoplasm.[8] When this CT is administered orally with an antigen, antigen-specific secretory IgA shows a marked increase. However, the A subunit of CT (CTA) is toxic and harmful to animals and humans. McKenzie and Halsey[4] reported a high level of antibody production in the intestinal mucosa and serum when they administered a mixture of CTB and horseradish peroxidase (HRP) to mice orally, and also reported for the first time that CTB had a potent adjuvant activity even when administered alone. Jertborn et al[7] and Ogawa et al[9] reported that CTB was a useful mucosal immunity adjuvant and caused little toxicity in man. In the present study, we used CTB as the mucosal immunity adjuvant and studied it experimentally.

Mucosal tissue is the place where allergic reactions, in addition to biophylactic reactions, occur. Elucidation of the pathophysiology and treatment for allergic conjunctival disorders have been studied from the point of view of mucosal immunity. In the conjunctiva, as in other mucosal tissues, conjunctiva-associated lymphoid tissues (CALT), corresponding to MALT, and secretory IgA exist in high concentration in tears.[10–13] Therapies utilizing orally induced immunological tolerance have been studied in the field of ophthalmology.[14] However, there has been no report of a method whereby mucosal immunity is induced by topical application of antigen to the eye to suppress allergic disorders. We investigated the possibility of using this new method of immunotherapy utilizing mucosal immune response by topical application of antigens and CTB to the eye in an experimentally induced allergic conjunctivitis model.

Materials and Methods

Experimental Model of Allergic Conjunctivitis

We used 21 female white Hartley guinea pigs, each weighing 480–550 g. All the studies were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Ovalbumin (OVA; Sigma Chemical, St Louis, MO, USA) was used as antigen. The animals were sensitized by two intraperitoneal injections of OVA (100 ug/30 mL) and albumin hydroxide (5 mg/mL) at an interval of 2 weeks. One week after the second injection, conjunctivitis was induced by topical instillation of OVA (5 mg/mL) by the method explained in previous reports.[15–17] The animals were divided into two groups, CTB group and control group. The CTB group underwent pretreatment with topical instillation of CTB (List Biological, Campbell, CA, USA) (4 ug/30 mL) and OVA (10 ug/30 mL), three times a day for 3 days, 1 week before the first intraperitoneal injection. The control group did not undergo the pretreatment.

Clinical Findings

Clinical examination was performed at 0.5, 6, and 24 hours after the development of conjunctivitis by topical application of OVA. At these three examination times, we examined 6 guinea pigs in the CTB group and 5 in the control group. Hyperemia of palpebral conjunctiva, chemosis of bulbar conjunctiva, and lid swelling were scored using the assessment criteria shown in Table 1. The clinical score for each finding was evaluated by the Mann-Whitney *U*-test. The total of clinical scores for hyperemia of palpebral conjunctiva, chemosis of bulbar conjunctiva, and lid swelling was also evaluated as an overall clinical score.

Histological Study

After the 24-hour histological examination, the animals in each groups that underwent clinical evaluation were sacrificed at 24 hours after the clinical examination. After the 6-hour examination, 5 animals in each group had been sacrificed. The animals were sacrificed by administration of overdoses of pentobarbital sodium (Nembutal®; Dainabot, Osaka) intraperitoneally. Then, eyelids and eyeballs were excised. At 24 hours, the animals (12 eyes of 6 animals in CTB

Table 1. Scoring for Clinical Findings

A. Hyperemia of palpebral conjunctiva
0: none
1: mild
2: moderate
3: severe
B. Chemosis of bulbar conjunctiva
0: none
1: mild
2: moderate
3: severe
C. Lid swelling
0: none
1: mild
2: moderate
3: severe
Total clinical score = A + B + C

Jpn J Ophthalmol
Vol 45: 332–338, 2001

group and 10 eyes of 5 animals in control group) were sacrificed by the same method after assessing the clinical score, and eyelids and eyeballs were excised. In all animals in this study, tissues of 1 eye were used for light microscopic study and tissues of the contralateral eye, for transmission electron microscopic study. Samples for light microscopy were fixed in a Zamboni solution for one hour, then freeze-embedded in OCT compound (Tissue-Tek®; Miles, Elkhart, IN, USA) and cut into 7-μm frozen sections with a cryostat. To examine eosinophil infiltration in this study, cryostat sections were stained with acid Giemsa (Diff Quick®; Kokusai Shiyaku, Kobe).

Samples for transmission electron microscopy were fixed in 2.5% glutaraldehyde (0.2 mol/L-cacodylate buffer, pH 7.4) for 12 hours, and postfixed in 1% osmium acid for 1 hour. After dehydration with an ascending alcohol series, they were embedded in epoxy resin (EPOK812®; Ohken-shouji, Tokyo) and cut into ultrathin sections. The sections were subjected to double staining with lead acetate and uranyl acetate, and observed under a transmission electron microscope (JEM1200EX®; Nihon Denshi, Tokyo).

Results

Study of Clinical Findings

Figure 1 shows the overall clinical score for all cases. The overall clinical score showed differences between the two groups at 30 minutes and 6 hours. Figures 2a–c illustrate each clinical score in the statistical analysis. Hyperemia of palpebral conjunctiva ($P < .01$), chemosis of bulbal conjunctiva ($P < .05$) and lid swelling ($P < .01$) were significantly suppressed (Figures 3a,b) in the CTB group compared with the control group at 30 minutes.

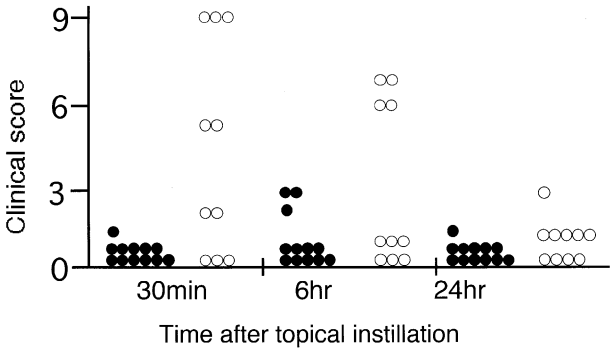

Figure 1. Total clinical score of experimental allergic conjunctivitis. Clinical score is significantly higher in control group than in cholera toxin B (CTB) group eyes. CTB group ●, control group ○.

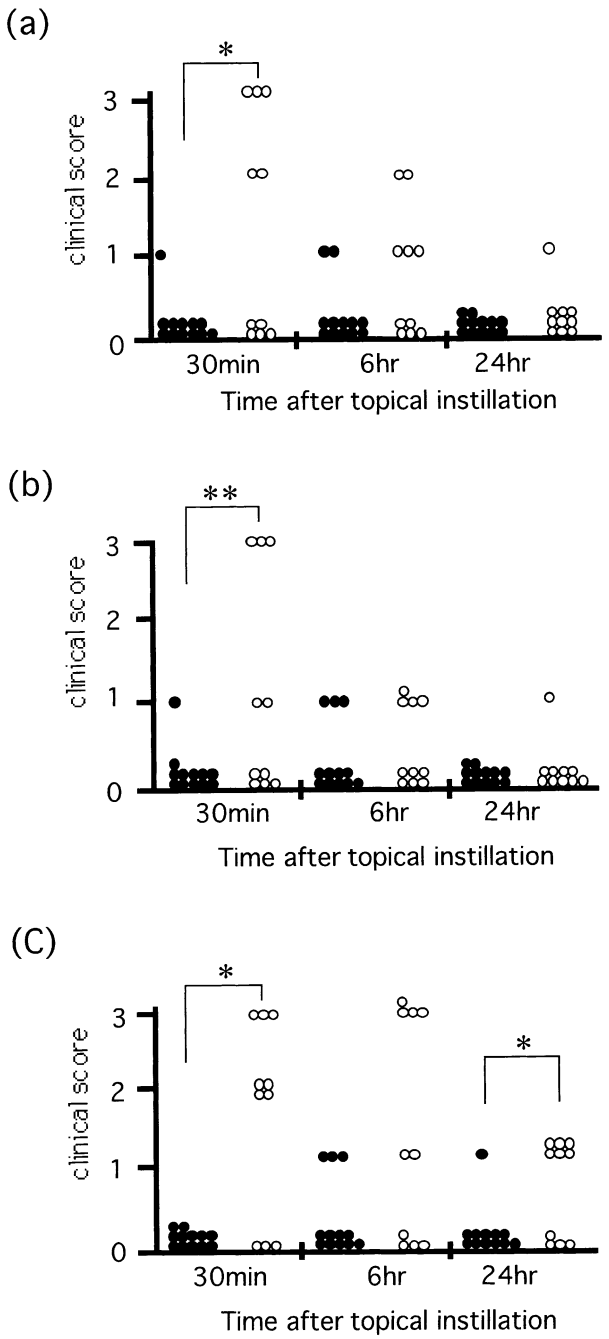

Figure 2. (**a**) Clinical score of hyperemia of palpebral conjunctiva. *Mann-Whitney *U*-test: $P < .01$. Cholera toxin B (CTB) group, ●: control group, ○.(**b**) Clinical score of chemosis of bulbar conjunctiva. **Mann-Whitney *U*-test: $P < .05$. ●: CTB group, ○: control group. (**c**) Clinical score of lid swelling. *Mann-Whitney *U*-test: $P < .01$. CTB group, ●: control group, ○.

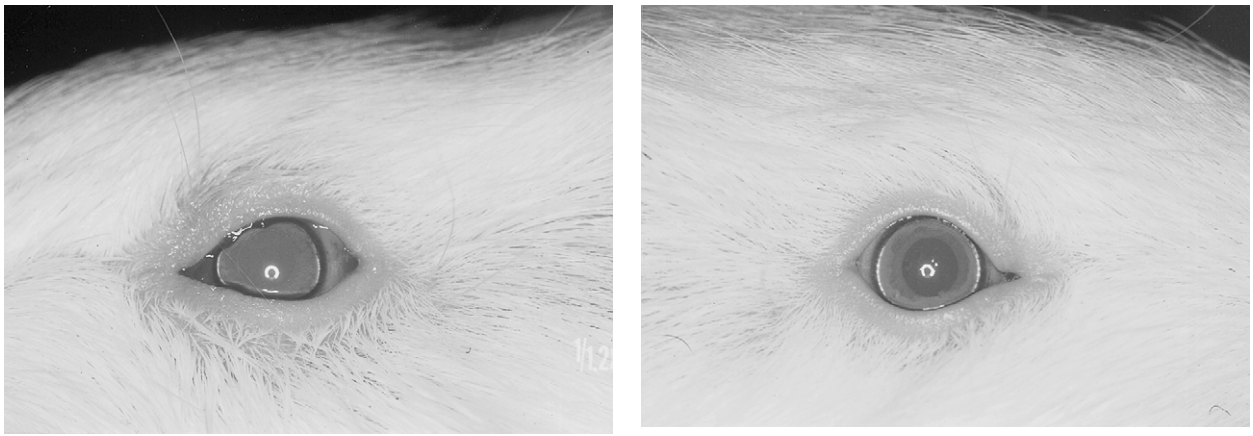

Figure 3. (**left**) Representative photograph of clinical score 5. (**right**). Representative photograph of clinical score 0.

Histological Study

Light microscopic study. At 6 hours after application of OVA, cell infiltration beneath the conjunctival epithelium was mild in the CTB group, while cell infiltration mainly of numerous eosinophils was seen beneath the conjunctival epithelium in the control group (Figures 4a,b).

At 24 hours, subconjunctival tissues showed dilatation of vessels with a congestive pattern filled with erythrocytes and an increase of eosinophils in both groups. However, infiltration of eosinophils was mild in the CTB group compared to that seen in the control group. In the control group, conjunctival epithelium showed loss of goblet cells and epithelial impairment. In the CTB group, however, goblet cells were maintained and the epithelium was not severely impaired (Figures 5a,b).

Transmission electron microscopic study. Eosinophils could be differentiated from other granulocytes by their segmented nucleus and a specific granule shaped like a coffee bean in the cytoplasm. At 6 hours, marked infiltration of eosinophils and neutrophils beneath the conjunctival epithelium was seen in the control group, but the cell infiltration beneath the epithelium was mild in the CTB group. At 24 hours, infiltration of eosinophils in the conjunctival epithelium with an enlargement of the intercellular space and loss of conjunctival epithelial cells around the cell infiltration were observed in the control group. Subconjunctival tissues showed marked infiltration

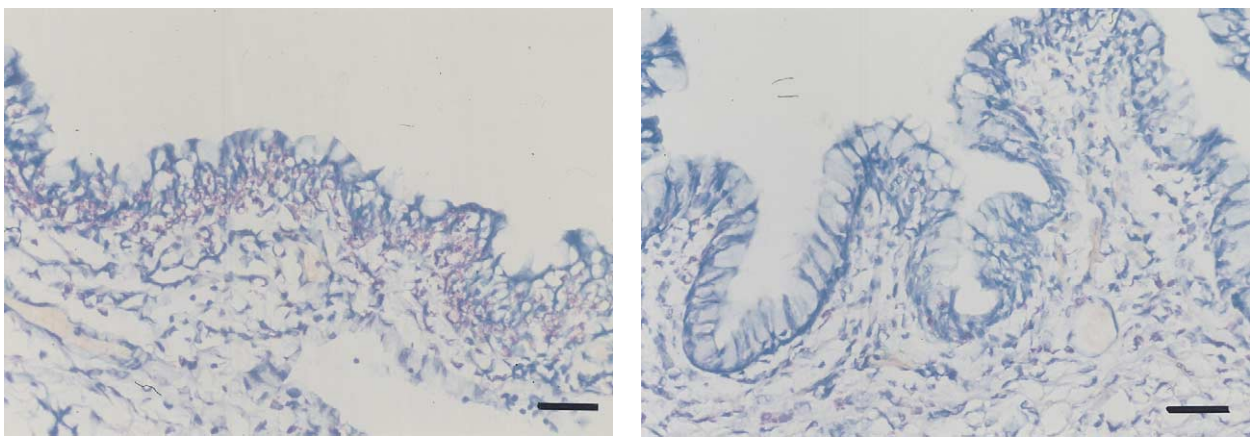

Figure 4. (**left**) Light micrograph of palpebral conjunctiva at 6 hours in control group. Numerous eosinophil infiltrations are observed in conjunctival epithelium and subconjunctival tissue. (light micrograph, Giemsa staining; Bar = 50 μm). (**right**) Light micrograph of palpebral conjunctiva at 6 hours in CTB group. Subconjunctival tissue in some eosinophil infiltrations. (light micrograph, Giemsa staining; Bar = 50 μm).

Jpn J Ophthalmol
Vol 45: 332–338, 2001

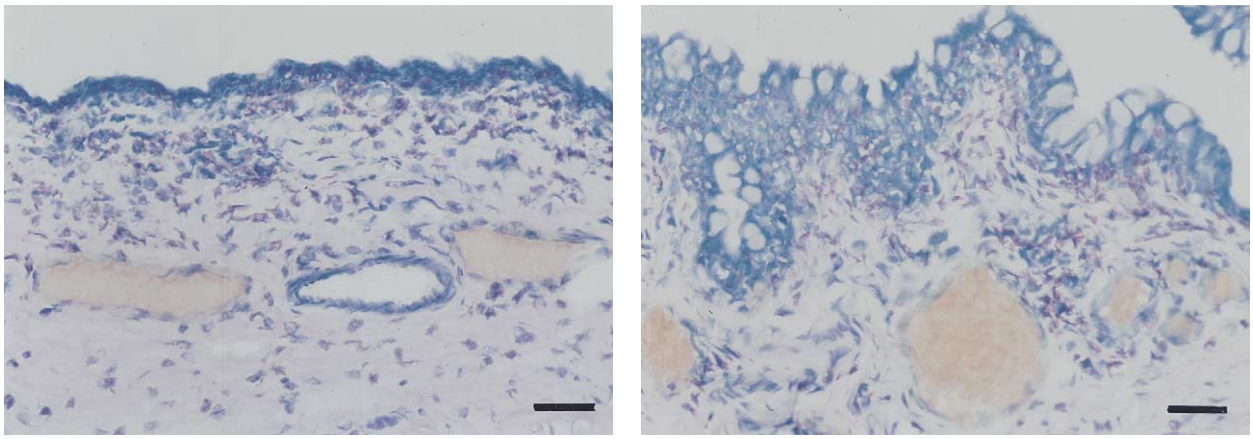

Figure 5. (**left**) Light micrograph of palpebral conjunctiva at 24 hours in control group. Conjunctival epithelium is damaged and goblet cells have disappeared. Numerous eosinophil infiltrations are observed in subconjunctival tissue. (Giemsa staining; Bar = 50 μm). (**right**) Light micrograph of palpebral conjunctiva at 24 hours in CTB group. Structure of conjunctival epithelium and subconjunctival tissue are maintained despite infiltration of eosinophils. Goblet cells are intact. (Giemsa staining; Bar = 50 μm).

of eosinophils and lymphocytes. In the CTB group, while epithelial cells and intercellular space remained almost unchanged, a slight infiltration of eosinophils occurred beneath the epithelium, but few eosinophils were seen in the epithelium (Figures 6a,b).

Discussion

Mucosal vaccine[18,19] against viral infections, oral immunotherapy[20–22] against allergic disease, and oral immunological tolerance against autoimmune diseases[23] have been reported as the clinical utilization of mucosal immunity. We studied the development of clinical methods to suppress allergic conjunctivitis by induction of mucosal immunity in conjunctiva.

The experimental allergic conjunctivitis model in the guinea pig is reported to be an active sensitization model.[16] The model showed a biphasic reaction: the early phase reaction consisting mainly of marked chemosis at 30 minutes after the application of antigen, and the late phase reaction with conjunctival swelling caused by cell infiltration, mainly of eosinophils and lymphocytes, 6 to 24 hours after antigen application, corresponding to the clinical findings of allergic conjunctivitis.[15–17] In the present experimental model, allergic conjunctivitis was induced coincidental to an increase in the anti-OVA antibody titer in the conjunctiva,[11] so that the sensitization was performed 4 weeks before the induction of allergic conjunctivitis. The observation time was set at 30 minutes (early phase reaction), and 6 and 24 hours (late phase reaction), because previous reports[16,17] showed no remarkable change in histological and clinical findings between 30 minutes and 6 hours. The guinea pigs were mongrel and showed individual differences, which is similar to the reaction in man. Therefore, it was necessary to score clinical symptoms for individual cases and to conduct a statistical study. For the experimental allergic conjunctivitis model in guinea pig, we compared clinical findings and histological findings between the groups, with and without pretreatment of a mixed solution of OVA, an antigen, and CTB. Clinical findings at 30 minutes after antigen application were significantly suppressed in the CTB group. In the histological examination at 6 to 24 hours, infiltration of eosinophils and impairment of conjunctival epithelium were mild in the CTB group compared to the control group. These results suggest that a series of allergic reactions from the early phase to the late phase was suppressed by the pretreatment with the mixed solution of antigen and CTB in this experimental allergic conjunctivitis model.

Regarding the method of administering antigen and CTB through mucosal tissue rather than the intestinal route, Hirabayashi et al[19] applied a mixed solution of influenza virus and CTB nasally in mice as a nasal influenza mucosal vaccine, reporting that nasal antigen administration resulted in a significant increase of antigen-specific IgA antibodies in serum and bronchial washes, compared with intraperitoneal or percutaneous vaccine administration. Wakamori[24] hypothesized that an actively increased local IgA antibody plays the role of blocking antibody against allergen. He reported that in an experiment of concurrent administration of CTB and anti-

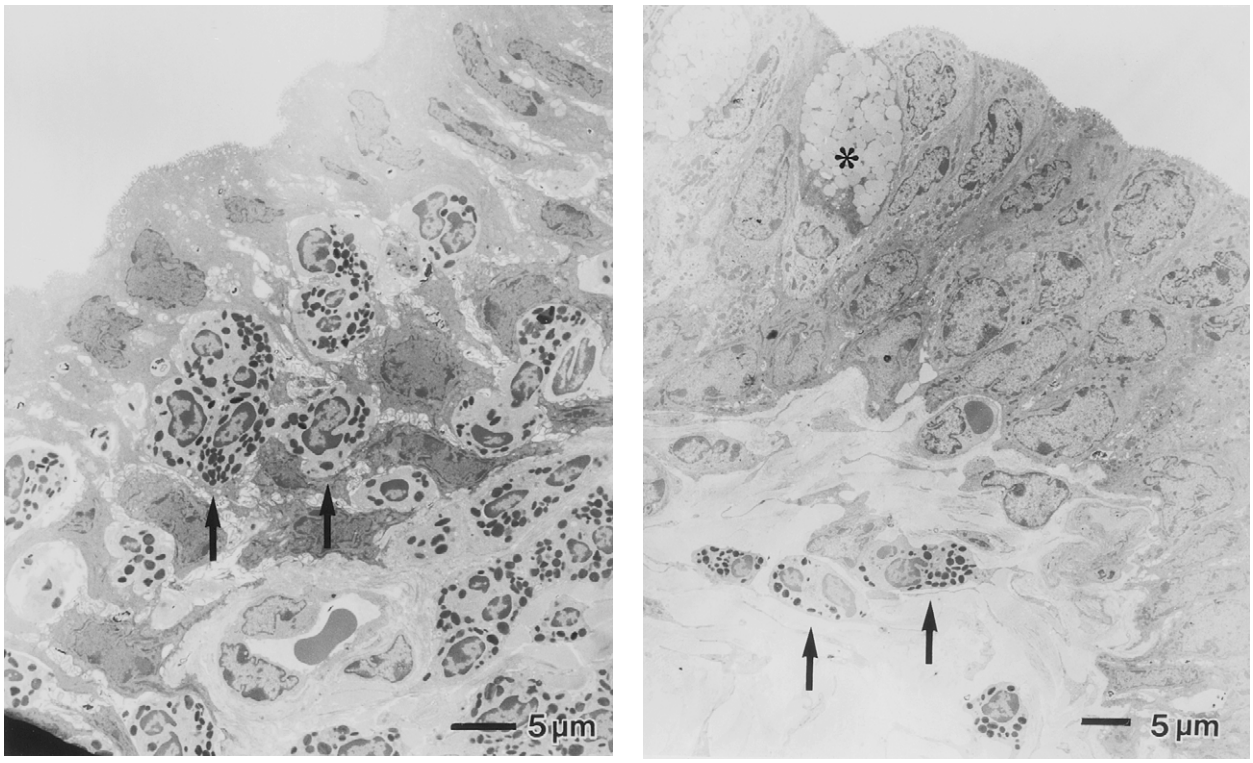

Figure 6. (**left**) Electron micrograph of conjunctival epithelium at 24 hours in control group. Observed eosinophils infiltrated into conjunctival epithelium and subconjunctival tissue (arrows). Intracellar space of conjunctival epithelium enlarged remarkably. (Bar = 5 μm). (**right**) Electron micrograph of conjunctival epithelium at 24 hours in CTB group. Conjunctival epithelium is intact without infiltration of eosinophils. Only a few eosinophils are observed in subconjunctival tissue (arrows). (Bar = 5 μm).

gen, the production of serum immunoglobulin E (IgE) antibody was suppressed by re-exposure to antigen. With respect to conjunctival tissues, Inada et al[11] applied HRP, with Freund's complete adjuvant, to guinea pig eyes, and observed by immunohistochemical analysis that antigen-specific IgA antibody producing plasma cells appeared in the conjunctival tissue. They reported that mucosal immunity could be induced by transconjunctival administration of antigens. These reports suggest that application of antigen and CTB to the eye induces antigen-specific IgA antibody that functions as a blocking antibody in the eye, thereby suppressing an allergic reaction. Moreover, because the secretory IgA has an important role in the biological defense mechanism to prevent antigens from invading the immune tissue, it is possible that the entire course of allergic reaction, including the early phase reaction, could be suppressed.

The usefulness of immunotherapy utilizing the mucosal immunity mechanism in the treatment of allergic diseases has been reported recently.[20–22] Oral therapy is one of the possible or potential immunotherapies whereby immunological tolerance is induced by oral administration of allergen to suppress the development of allergic diseases. Suko et al[22] administered crude tick antigen to adult patients with asthma and reported a suppressive effect on not only both early and late phase asthmatic reaction induced by inhalation of tick antigen, but also on reduction of eosinophils in peripheral blood and on production of interleukin-5 in lymphocytes. Koizumi and Abe[14] induced immunological tolerance orally in rats and reported the suppression of IgE antibody titer in serum and conjunctiva in allergic conjunctivitis. However, it is reported that the induction of immunological tolerance depends on the amount of antigen administered.

In the present study, an experiment with pretreatment by OVA alone was not performed. Therefore, it is still unknown whether the suppression mechanism of allergic conjunctivitis by the method used in the present study is due to induction of immunological tolerance or due to the effect of blocking antibodies related to secretory IgA. The sensitization procedure employed in this study is similar to the previously reported method for mucosal vaccine. We

Jpn J Ophthalmol
Vol 45: 332–338, 2001

consider that the concurrent application of CTB and antigen to the eye is a useful clinical method for treating allergic conjunctivitis. Elucidation of the suppression mechanism of the experimental allergic conjunctivitis model and development of a clinically applicable transconjunctival vaccine method will be the subjects of future study.

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