Abbreviations used
alum: Aluminum hydroxide
cPGES: Cytosolic prostaglandin E synthase
EAC: Experimental allergic conjunctivitis
mPGES: Membrane-bound prostaglandin E synthase
PGE_2: Prostaglandin E_2
PGES: Prostaglandin E synthase
RT: Room temperature
RW: Short ragweed pollen
TARC: Thymus and activation-regulated chemokine
X-gal: 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

reactions.[4] In the latest analysis significantly more pronounced allergic inflammation developed in EP3-deficient (*Ptger3*$^{-/-}$) mice than in wild-type mice, and the EP3-selective agonist suppressed the inflammation. Intriguingly, EP3 is expressed in airway epithelial cells but not infiltrating cells.[4] On the other hand, nonsteroidal anti-inflammatory drugs, which reduce the increase in both prostaglandin D_2 and PGE_2 levels, are used not only for prevention of posterior capsular opacification[5] but also at times for the treatment of allergic conjunctivitis.[6]

In this study we tested a hypothesis that ocular-surface epithelial cells express EP3 and regulate the inflammation of allergic conjunctivitis through the PGE_2–EP3 pathway, and we addressed this issue by examining ocular-surface EP3 expression and analyzing its role in murine experimental allergic conjunctivitis (EAC) by using *Ptger3*$^{-/-}$ mice and a selective EP3 agonist, ONO-AE-248.

METHODS

Mice, compounds, and reagents

BALB/c mice were purchased from CLEA (Tokyo, Japan) and used at 6 to 12 weeks of age for sensitization. *Ptger3*$^{-/-}$ mice were generated as previously described,[7] back-crossed more than 10 generations to BALB/c mice, and subjected to EAC at 9 to 15 weeks of age with age-matched, wild-type BALB/c mice as control animals. Mice were maintained on a 12-hour/12-hour light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto Prefectural University of Medicine, Kyoto, Japan. All studies were performed in accordance with the Association for Research in Vision and Ophthalmology "Statement for the use of animals in ophthalmic and vision research."

ONO-AE-248, a selective EP3 agonist, was supplied by ONO Pharmaceutical Co, Ltd (Osaka, Japan); the ligand-binding specificities of the compounds for each prostaglandin E receptor subtype have previously been described.[8] Short ragweed pollen (RW), RW extract, and aluminum hydroxide (alum) were purchased from Polysciences, Inc (Warrington, Pa); LSL Co, Ltd (Tokyo, Japan); and Sigma (St Louis, Mo), respectively.

RT-PCR analysis

By using TRIzol (Invitrogen, Carlsbad, Calif), total RNA was isolated from mouse kidney, corneal, and conjunctival tissues, according to the manufacturer's instructions. For the RT-PCR, we used the SuperScript preamplification system (Invitrogen). Amplification was performed with DNA polymerase (Takara, Shiga, Japan) for 40 cycles at 94°C for 1 minute, 66°C for 1 minute, and 72°C for 1 minute for mouse *EP3* (GeneAmp; PE Applied Biosystems, Foster City, Calif). The primers for mouse *EP3* were as follows: forward 5′-ATCCTCGTGTACCTGTCACAGCGACGCTGG-3′ and reverse 5′-TGCT CAACCTACATCTGATTGAAGATCATT-3′. For mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT), they were as follows: forward 5′-GTTGGATACAGGCCAGACTTTGTT-3′ and reverse 5′-GAGGGTAG GCTGGCCTATAGGCT-3′. A kidney, conjunctiva, or cornea was isolated from wild-type mice.

Histochemistry and histologic analysis

For staining for β-galactosidase activity, freshly isolated eye tissue with the eyelids and conjunctiva was embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and then flash-frozen in liquid nitrogen and cut into 8-μm-thick sections with a cryostat. The sections were fixed for 10 minutes at room temperature with 4% paraformaldehyde in PBS; washed with PBS with 1 mmol/L $MgCl_2$; incubated for 24 hours at 37°C with a solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 1 mg/mL), 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$, and 1 mmol/L $MgCl_2$; and then counterstained with hematoxylin and eosin.

For staining of eosinophils, the whole eyeball, together with the eyelids and conjunctiva, were dissected and fixed in 10% neutral buffered formalin and then embedded in paraffin blocks. Vertical 6-μm-thick sections were affixed to microscope slides and deparaffinized. The slides were then stained with the Luna method (stain in a working 1% Biebrich scarlet solution, dip in lithium carbonate solution, wash in running water, counterstain in a working hematoxylin solution, wash in running water, and dehydrate), and eosinophils were examined with a light microscope. The number of infiltrating eosinophils in the lamina propria mucosae of the tarsal of the conjunctivas in the entire section was counted. The sections used were those from the central portion of the eye, which included the pupil and optic nerve head. Because the cell numbers vary depending on the counting area, the cell-count data are expressed as infiltrating eosinophil numbers divided by the area (in square millimeters), as measured with Scion Image (Scion Corp, Frederick, Md). The data are presented as means ± SEMs of all the mice examined.

Immunohistochemistry

The whole eyeball, together with the eyelids and conjunctiva, was embedded in OCT compound (Sakura Finetek) and then flash-frozen in liquid nitrogen. Sections 6 μm in thickness were cut and fixed with 100% acetone at 4°C for 10 minutes and blocked for 30 minutes with 10% normal donkey serum in PBS. Rabbit polyclonal antibody was to EP3 or to membrane-bound prostaglandin E synthase (mPGES) 1 (Cayman Chemical Co, Ann Arbor, Mich). Nonspecific rabbit IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) was used as the negative control. For EP3 staining, *Ptger3*$^{-/-}$ mice also were used as the negative control. The secondary antibody (Biotin-SP–conjugated AffiniPure F[ab′]2 Fragment Donkey Anti-Rabbit IgG[H+L], 1:500 dilution; Jackson Immuno Research, Baltimore, Ms) was applied for 30 minutes. VECTASTAIN ABC Reagents (Vector Laboratories, Inc, Burlingame, Calif) were used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector Laboratories, Inc) as a chromogenic substrate.

Sensitization and challenge

Mice were immunized by means of subcutaneous injection into their left hind footpads of RW adsorbed on alum (200 μg of RW and 2.6 mg of alum in a total volume of 200 μL) on day 0, followed by intraperitoneal injection of RW adsorbed on alum on day 7. On day 18, the eyes of the immunized mice were challenged with RW in PBS (500 μg in 5 μL per eye) or with PBS alone (5 μL per eye). For histologic analysis, the eyes were collected 24 hours after challenge.[9] For RT-PCR analysis of *eotaxin-1*–specific mRNA, the upper and lower eyelids were isolated 6 hours after challenge. For measurement of *COX* values, *prostaglandin E synthase (PGES)*–specific mRNA, and PGE_2 content, the upper and lower eyelids were harvested with a time course of 0, 1, 3, 6, and 12 hours after challenge.

Quantitative RT-PCR of eotaxin-1, COXs, and PGES-specific mRNA in eyelids

Quantitative RT-PCRs of *eotaxin-1, COXs,* and *PGES*-specific mRNA in the eyelids were performed as previously reported.[9] Briefly, the upper and lower lids were collected with the previously specified time course after RW challenge and homogenized in liquid nitrogen. Total RNA was extracted with the RNeasy mini kit (Qiagen, Tokyo, Japan). ReverTra Ace (TOYOBO, Otsu, Japan) was used for reverse transcription. The primers and probes for mouse *eotaxin-1, COX-1, COX-2, mPGES-1, mPGES-2, cytosolic PGES (cPGES),*

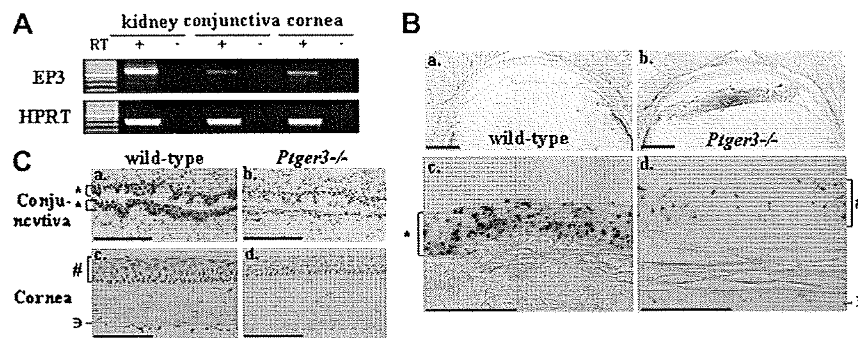

FIG 1. Expression and localization of EP3 in the conjunctiva and cornea. **A,** RT-PCR analyses of the expression of *EP3*-specific mRNA in the cornea and conjunctiva of wild-type mice. **B,** Histochemical staining for EP3 (X-gal): wild-type mice *(a)* and *Ptger3*$^{-/-}$ mice *(b-d)*. Data are representative of 3 experiments. **C,** Immunohistologic analysis for EP3. Each *bar* represents a length of 500 (*B, a, b*), 50 (*B, c, d*), or 100 *(C)* μm. Data are representative of 3 experiments. *Conjunctival epithelium, #corneal epithelium, ϶Corneal endothelium.

and *glyceraldehyde-3-phosphate dehydrogenase (GADPH)* were from Applied Biosystems. The results were analyzed with sequence-detection software (Applied Biosystems); the expression level of *eotaxin-1*, *COXs*, and *PGES*-specific mRNA was normalized to the expression of the mouse housekeeping gene *GADPH*. The time after RW challenge, 6 hours, was optimal for maximum induction of *eotaxin-1* mRNA expression.

Measurement of PGE_2

PGE_2 measurement in eyelids were assessed by means of ELISA with the Prostaglandin E2 Biotrak Enzyme-immunoassay System (Amersham Biosciences, Buckinghamshire, United Kingdom), according to the manufacturer's recommendation.

Data analysis

Data were expressed as the mean ± SEM, and statistical analyses were performed by means of ANOVA and the Student *t* test.

RESULTS

Expression and localization of EP3 in the ocular surface

To examine the expression of *EP3* in the ocular surface, we performed RT-PCR analyses. As a positive control, we used mRNA isolated from the kidney. The expected length of PCR products (608 bp) was obtained from kidney, corneal, and conjunctival samples, suggesting that the ocular-surface tissues, both cornea and conjunctiva, express *EP3* mRNA (Fig 1, *A*). PCR products were isolated and sequenced to confirm the specificity of *EP3* mRNA detection. The sequences obtained from these PCR products were identical to the mouse EP3 cDNA sequence. We next examined EP3 localization using *Ptger3*$^{-/-}$ mice in which the β-galactosidase gene was "knocked in" at the *EP3* gene locus and with immunohistochemistry of wild-type mice. In *Ptger3*$^{-/-}$ mice X-gal staining of ocular-surface tissue revealed dense positive signals in conjunctival epithelia and scattered positive signals in corneal epithelia (Fig 1, *B, b-d*), suggesting a strong EP3 expression in conjunctival epithelial cells and a slight expression in corneal epithelial cells. Interestingly, a few positive signals were seen in corneal endothelia (Fig 1, *B, d*). In other eye compartments, such as the lens, retina, uvea, and sclera, of the *Ptger3*$^{-/-}$ mice, there were no positive signals (Fig 1, *B, b*). X-gal staining of ocular-surface tissue did not reveal positive signals in wild-type mice (Fig 1, *B, a*). Immunohistochemistry of conjunctival and corneal tissue revealed that conjunctival epithelial cells expressed EP3 protein (Fig 1, *C, a*), although we could not find the positive signal in corneal epithelial and endothelial cells, presumably because of decreased expression of EP3 protein (Fig 1, *C, c*).

Antibody responses to RW sensitization

We next examined whether immunization of RW induced immune responses specific to RW equally in wild-type and *Ptger3*$^{-/-}$ mice (see the Methods section of the Online Repository at www.jacionline.org). RW sensitization significantly increased the serum levels of total IgE, anti-RW IgE, and anti-RW IgG1 in both wild-type and *Ptger3*$^{-/-}$ mice compared with levels seen in the unsensitized control mice. There were no significant differences in serum levels of total IgE, anti-RW IgE, and anti-RW IgG1 between the sensitized wild-type and *Ptger3*$^{-/-}$ mice (see Fig E1 in this article's Online Repository at www.jacionline.org). These results suggest that wild-type and *Ptger3*$^{-/-}$ mice had been equally sensitized to RW.

Eosinophil accumulation in the lamina propria mucosae of the conjunctivas

To investigate whether EP3 plays a role in the late-phase reaction of EAC, we examined conjunctival tissue from wild-type and *Ptger3*$^{-/-}$ mice. Histologic analyses of conjunctivas revealed that the RW challenge on RW-sensitized mice led to inflammatory cell infiltrations in the lamina propria mucosae of the conjunctivas at 24 hours after challenge in our EAC model (Fig 2). The infiltrated cells consisted predominantly of eosinophils; only a few lymphocytes and neutrophils were detected. Both wild-type and *Ptger3*$^{-/-}$ mice had eosinophil-dominant inflammatory cell infiltrations in the lamina propria mucosae at 24 hours after challenge; however, the numbers of eosinophils in *Ptger3*$^{-/-}$ mice were significantly greater than in wild-type mice (Table I). Either RW sensitization or RW challenge alone did not significantly increase the numbers of eosinophils in wild-type and *Ptger3*$^{-/-}$ mice (data not shown). These results suggest that the PGE_2–EP3 pathway negatively regulates EAC development in the late-phase reaction, which cause the pronounced allergic inflammation in *Ptger3*$^{-/-}$ mice.

Expression of *eotaxin-1*–specific mRNA in eyelids

Given the critical roles of eotaxin-1 in eosinophil recruitment,[10-12] we examined the *eotaxin-1*–specific mRNA expression

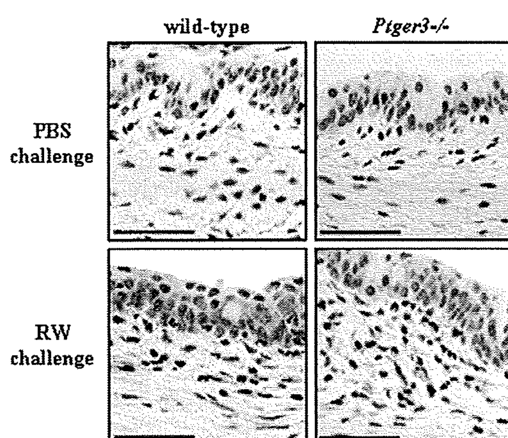

FIG 2. Histologic analyses of eosinophil inflammation in conjunctiva. Infiltration of eosinophils into the conjunctiva of wild-type and *Ptger3*$^{-/-}$ mice were detected by using the Luna method, which stained eosinophil granules with a distinctive red. *Scale bars* = 50 μm.

TABLE I. Number of eosinophils in the lamina propria mucosae of the tarsal conjunctiva

	Wild-type mice		*Ptger3*$^{-/-}$ mice	
Sensitization	+		+	
Challenge	−	+	−	+
No. of mice	19	19	19	19
No. of eosinophils, mean ± SEM/0.1 mm^2	4.6 ± 1.1	42.4 ± 3.1	4.2 ± 0.8	86.5 ± 5.4*

*$P < .0005$ (42.4 ± 3.1 vs 86.5 ± 5.4).

in the eyelids from RW-sensitized wild-type and *Ptger3*$^{-/-}$ mice at 6 hours after RW challenge.

Quantitative real-time RT-PCR analyses demonstrated that the RW challenge significantly increased the *eotaxin-1* mRNA expression in both genotypes compared with that in vehicle-treated control animals. The *eotaxin-1* mRNA expression increase in RW-challenged *Ptger3*$^{-/-}$ mice is significantly larger than that seen in wild-type mice (Fig 3), which is consistent with pronounced eosinophil inflammation in *Ptger3*$^{-/-}$ mice.

Upregulation of *COX-2*, *PGES*-specific mRNA expression, and PGE_2 contents in eyelids during EAC

Because COX and PGES action is necessary for PGE_2 synthesis,[13] we examined the expression of mRNA for *COX* isoforms and *PGES* isoforms in eyelids during EAC. After RW challenge, the relative expression levels of *COX-2* mRNA increased and peaked at 1 hour (about 9 times higher than the basal level at 0 hour) and then gradually decreased (Fig 4, *A*), whereas the expression of *COX-1* mRNA stayed at an almost basal level (data not shown). After RW challenge, the relative expression levels of *mPGES-1* mRNA also increased and peaked at 3 hours and then gradually decreased (Fig 4, *A*), and similar gene expression patterns of *mPGES-2* and *cPGES* were also observed (data not shown). We also examined the PGE_2 contents in eyelids during EAC. After RW challenge, the PGE_2 contents in eyelids increased time dependently until 12 hours (Fig 4, *B*). These results suggest that RW challenge increased PGE_2 synthesis in eyelids through upregulation of inducible enzymes, such as COX-2 and

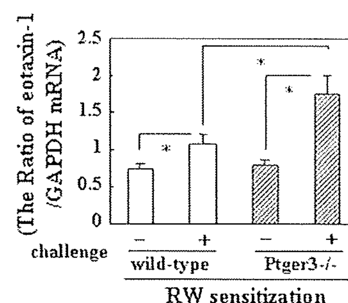

FIG 3. Real-time PCR analyses of the expression of *eotaxin-1* mRNA in the eyelids 6 hours after RW challenge. Relative mRNA expression levels for *eotaxin-1* normalized by *GAPDH* are shown. Data are shown as the mean ± SEM of 7 samples per group. *$P < .05$.

mPGES-1, during the elicitation phase of EAC. Furthermore, we examined the localization of PGE_2 synthesis by using immunohistochemistry of mPGES-1. Immunohistologic analysis of ocular-surface tissue, which was obtained from RW-sensitized wild-type mice at 6 hours after RW challenge, revealed that conjunctival epithelial cells expressed mPGES-1 protein (Fig 4, *C*), suggesting that PGE_2 synthesis through mPGES-1 might occur in conjunctival epithelium during the elicitation phase of EAC.

Effect of an EP3-selective agonist

To investigate whether allergic inflammation can be suppressed by stimulating the PGE_2–EP3 pathway in EAC, we next examined the effects of an EP3-selective agonist, ONO-AE-248. We topically administered ONO-AE-248 to the eyes of RW-sensitized mice 3 times at 1 hour before and at 3 and 7 hours after RW challenge. First, to determine the optimal dose, ONO-AE-248 was administered at various doses (0.01, 0.1, 1, 10, and 100 ng) in 5 μL of PBS per eye to wild-type mice. ONO-AE-248 suppresses eosinophil infiltration dose dependently until 1 ng, and then at the higher doses of 10 ng and 100 ng, its suppression becomes weak (see Fig E2 in this article's Online Repository at www.jacionline.org). We therefore determined that the optimal dose of AE248 was 1 ng in 5 μL of PBS (0.02%). In wild-type mice topical administration of 1 ng of ONO-AE-248 significantly inhibited the antigen-induced infiltration of eosinophils compared with that seen in the vehicle-treated groups (Fig 5 and see Fig E3 in this article's Online Repository at www.jacionline.org). Furthermore, this inhibitory effect of ONO-AE-248 was absent in *Ptger3*$^{-/-}$ mice, confirming that it was mediated by EP3 (Fig 5 and see Fig E3). Although vehicle treatment mildly decreased eosinophil infiltration, it is very likely that a 3-time vehicle administration washed out the challenged RW antigen and resulted in reduced allergic responses. These results suggest that allergic inflammation can be suppressed by stimulating EP3 with a selective agonist.

DISCUSSION

In this study we investigated the roles of the PGE_2–EP3 pathway in allergic conjunctivitis by using RW-induced EAC as a model in mice. First, we examined EP3 expression in ocular tissues and found that EP3 is expressed in the conjunctival epithelium on the ocular surface in mice. Because *Ptger3*$^{-/-}$ mice have allergic inflammation that was much more pronounced than that seen in wild-type mice, negative regulation of the

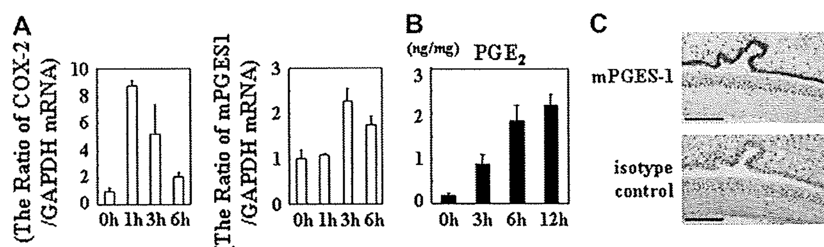

FIG 4. RW challenge increased PGE_2 synthesis in eyelids. **A,** Time course of *COX-2* and *mPGES-1* mRNA expression in eyelids during EAC. The *y-axis* shows the increase of specific mRNA over that seen in 0-hour samples. The *x-axis* shows the time after the RW challenge. Data are shown as the mean ± SEM of 3 samples. **B,** Time course of PGE_2 content in eyelids during EAC. The *x-axis* shows the time after the RW challenge. Data are shown as the mean ± SEM of 4 samples. **C,** Localization of mPGES-1 on the ocular surface. Each *bar* represents a length of 100 μm.

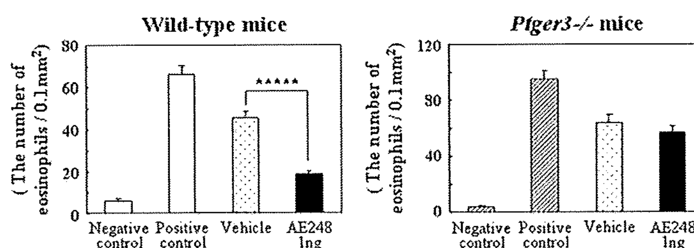

FIG 5. The effects of an EP3-selective agonist on eosinophil infiltration in conjunctiva. Data are shown as the mean ± SEM of samples from all the mice examined. Negative control (RW sensitization without challenge), positive control (RW sensitization with challenge) only vehicle, and AE248 are shown. (Wild-type mice: negative control, n = 20; positive control, n = 20; vehicle only, n = 25; AE248, n = 30; *Ptger3*$^{-/-}$ mice: negative control, n = 11; positive control, n = 11; vehicle only, n = 14; AE248, n = 16). *****$P < .0005$.

development of EAC through the PGE_2–EP3 pathway was suggested. Consistently, significantly greater *eotaxin-1* mRNA expression was observed in *Ptger3*$^{-/-}$ mice. Conversely, treatment with an EP3-selective agonist resulted in significantly decreased eosinophil infiltration in wild-type mice, which was blunted in *Ptger3*$^{-/-}$ mice. We examined the expression of mRNA of *COX* isoforms, *PGES* isoforms, and PGE_2 content in eyelids during EAC and found that *COX-2*, *PGES* mRNA, and PGE_2 content in eyelids increased after RW challenge. Interestingly, the expression of mPGES-1 was localized in conjunctival epithelium, suggesting that PGE_2 synthesis through mPGES-1 might occur in conjunctival epithelium. These results suggest not only the critical roles of the PGE_2–EP3 pathway in allergic conjunctivitis but also a new strategy for treating allergic conjunctivitis by modifying the epithelial cell functions in a similar way to the PGE_2–EP3 pathway.

The allergic response in conjunctivitis is typically elicited by ocular exposure to an allergen, such as grass or tree pollen, that causes cross-linkage of membrane-bound IgE, which triggers mast cell degranulation, releasing a cascade of allergic and inflammatory mediators. Thus mast cells play an important role in the early-phase reactions. Given the predominant role of mast cells in the early-phase reaction, most patients presenting with allergic conjunctivitis are treated with eye drops containing mast cell stabilizers or antihistamines. It is also suggested that mediators released by mast cells during the early-phase reactions contribute to the development of the late-phase reaction.[14] However, we previously demonstrated that mast cells do not play an essential role in the development of a late-phase reaction of eosinophilic conjunctival inflammation by showing that mast cell–deficient mice similarly had eosinophilic conjunctival inflammation compared with congenic littermates with EAC.[9] In this study we showed that conjunctival epithelial cells might be also implicated in the eosinophilic conjunctival inflammation seen in allergic conjunctivitis.

Late-phase reaction is characterized by eosinophil-dominant infiltrations in the local tissue.[9,12] The increase of eosinophil infiltration correlates well with both the severity and condition of this disease.[10-12] For eosinophil recruitment, eotaxin-1 is now accepted as the central mediator, and it was reported that its deficiency ablates eosinophilic responses in allergic conjunctivitis.[12] Hence we examined the *eotaxin-1*–specific mRNA expression in the eyelids from RW-sensitized wild-type and *Ptger3*$^{-/-}$ mice at 6 hours after RW challenge. Because we observed a large number of eosinophils in our EAC at 24 hours after RW challenge, this condition is thought to mimic the late-phase reaction in human allergic conjunctivitis. Recently, T cells[15] and fibroblasts[16] were also reported to contribute to the development of the late-phase reaction. Although various cell types can contribute to orchestrate the allergic response and various mechanisms are thought to develop the late-phase reaction, our findings in this study strongly suggest that conjunctival epithelium, which predominantly expresses EP3 among ocular tissues, substantially contributes to the late-phase reaction in our EAC.

In an allergic asthma model PGE_2 was reported to act at EP3 on airway epithelial cells and regulate the extent of the late-phase reaction by attenuating the expression of chemokine genes, such as *eotaxin-1* and *thymus and activation-regulated chemokine (TARC)*.[4] Given that RW challenge–induced *eotaxin-1* mRNA increase in eyelids was much more pronounced in *Ptger3*$^{-/-}$ mice, it was suggested that the PGE_2–EP3 pathway negatively regulates allergic reactions by suppressing chemokine gene expression in

EAC. With regard to the clinical relevance of eotaxin-1 in allergic conjunctivitis, it was reported that eotaxin-1 was expressed in the conjunctival epithelium of patients with vernal keratoconjunctivitis,[17] although we failed to show the eotaxin-1 or TARC production by cultured conjunctival epithelial cells stimulated with combinations of TNF-α and IL-4 (data not shown). The difference between *in vivo* and *in vitro* conditions might contribute to the different cell responses. On the other hand, cultured conjunctival fibroblasts could release eotaxin-1 or TARC in response to stimulation with combinations of TNF-α and IL-4 (data not shown). The mechanisms of eosinophilic conjunctival inflammation at the late-phase reaction are still elusive. Further investigations are required to identify the precise molecular mechanisms of allergic conjunctivitis.

It is well known that prostaglandins are produced in substantial amounts during allergen exposure and disease development[18,19] and that COX enzymes are involved in the synthesis of prostaglandins.[20] COX-1 is an ubiquitous housekeeping enzyme and thought to produce a basal level of prostaglandins, whereas COX-2 is an inducible enzyme and thought to be involved in pathologic conditions. Consistently, we detected unchanged *COX-1* and markedly increased *COX-2* mRNA expression in conjunctiva after RW challenge. Furthermore, we also detected increased *PGES* mRNA values and PGE_2 contents in eyelids and mPGES-1 expression in conjunctival epithelium. Of 3 PGESs, cPGES and mPGES-2 are constitutive enzymes, whereas mPGES-1 is an induced enzyme and upregulated by proinflammatory stimuli.[21] Because the functional coupling of COX-2 to mPGES-1 for PGE_2 biosynthesis has been suggested,[22] the coordinate increased expression of COX-2 and mPGES-1 might contribute to an increase in PGE_2 production in the conjunctival epithelium during the elicitation phase of our EAC. Thus we propose the endogenous negative-feedback regulation of allergic inflammation; the PGE_2–EP3 pathway is activated on challenge and functions to suppress development of EAC. Notably, this endogenous feedback system can be augmented by stimulating EP3 with a selective agonist.

It is possible that the PGE_2–EP3 pathway might function to suppress the development of human allergic conjunctivitis because we have confirmed the expression of EP3 in human conjunctival epithelium at both the mRNA and protein levels (see Fig E4 in this article's Online Repository at www.jacionline.org).

In summary, we demonstrated that EP3 is expressed in the ocular surface and that the PGE_2–EP3 pathway in conjunctival epithelium works as a negative regulator for allergic conjunctivitis. Although species differences between human subjects and mice should be considered cautiously and extrapolation from the mouse models to human pathologies must be performed with some reservation, stimulating the PGE_2–EP3 pathway with a selective agonist might be useful for treating allergic conjunctivitis in human subjects. Furthermore, we propose a new strategy for treating allergic conjunctivitis by modifying the epithelial cell functions in a similar way to the PGE_2–EP3 pathway.

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Clinical implications: EP3 in conjunctival epithelium can be a target for drug development to treat allergic conjunctivitis.

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METHODS

Measurement of immunoglobulins in serum

Twenty-four hours after RW challenge, blood was collected, and serum was prepared. Serum total IgE levels were assessed by means of ELISA with the OptEIA mouse IgE ELISA set (BD Biosciences PharMingen, San Diego, Calif), according to the manufacturer's recommendation. RW-specific immunoglobulin levels in the serum were assessed as previously reported (Kweon, 2000). Briefly, EIA plates (Costar, Corning, NY) were coated with RW extract (5 μg/mL) at 4°C overnight. After blocking with 1% BSA in PBS, serum samples were added and incubated for 4 hours at room temperature. The plates were washed with PBS plus 0.05% Tween 20 (Wako, Osaka, Japan) and incubated for 2 hours at room temperature with goat anti-mouse IgG1–horseradish peroxidase–conjugated antibody (Southern Biotechnology Associates, Inc, Birmingham, Ala) for RW-specific IgG1. In the case of IgE, biotin-conjugated rat anti-mouse IgE mAb (BD Bioscience PharMingen) was added to each well for 1 hour at room temperature, and then after washing, avidin–horseradish peroxidase conjugate (BD Bioscience PharMingen) was added to each well for 30 minutes at room temperature. After washing, the color reaction was developed with 3, 3′, 5, 5′-tetramethyl-benzidine (Moss, Inc, Pasadena, Calif). End point titers of RW-specific immunoglobulins were expressed as the reciprocal log2 of the last dilution that showed a level of OD units 2-fold higher than the background.

Human conjunctival epithelial cells

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the principles set forth in the Helsinki Declaration. The purpose of the research and the experimental protocol were explained to all patients, and their informed consent was obtained.

For RT-PCR, we obtained human conjunctival epithelial cells from healthy volunteers by means of impression cytology. For immunohistochemistry, serial sections of human conjunctivas were prepared from samples obtained at conjunctivochalasis surgery.

For the RT-PCR, the primers for human *EP3* and human *GADPH* were as follows: forward 5′- GCG CGC TGG TGC TGC GTC TGT ACA CTG CGG -3′ and reverse 5′- AGT GGC CGC TGC AGG GAG GTA GAG CTC CAG -3′ and forward 5′- CCA TCA CCA TCT TCC AGG AG-3′ and reverse 5′- CCT GCT TCA CCA CCT TCT TG-3′, respectively.

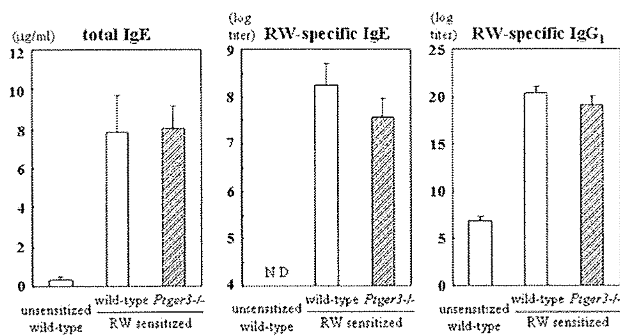

FIG E1. Antibody responses to RW sensitization and challenge. The serum concentration of total IgE *(left)* and the log titer of RW-specific IgE *(middle)* and RW-specific IgG1 *(right)* assessed by means of ELISA are shown. Sera were prepared from sensitized wild-type and *Ptger3*$^{-/-}$ mice at 24 hours after RW challenge. Sera from unsensitized wild-type mice were used as controls. Data are shown as the means ± SEMs of 8 samples.

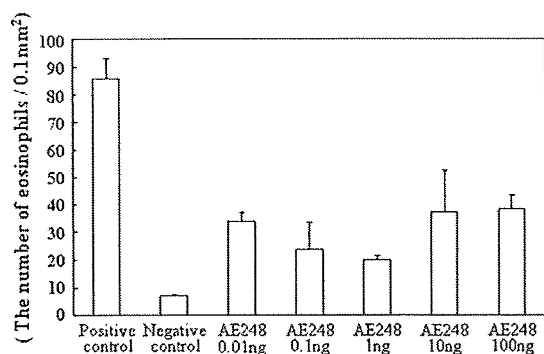

FIG E2. The optimal dose of ONO-AE-248. ONO-AE-248 was administered at various doses (0.01 ng, 0.1, 1, 10, 100 ng) in 5 μL of PBS per eye to wild-type mice at 1, 3, and 7 hours after RW challenge. The quantified eosinophil numbers in conjunctivas of wild-type mice were shown. The data from the mice without both RW challenge and topical treatment and the mice without topical treatment were used as negative and positive controls, respectively. Data are shown as the means ± SEMs of samples from all mice examined. Positive control (RW sensitization with challenge), n = 19; negative control (RW sensitization without challenge), n = 19. AE248: 0.01 ng, n = 18; 0.1 ng, n = 6; 1 ng, n = 27; 10 ng, n = 6; 100 ng, n = 17.

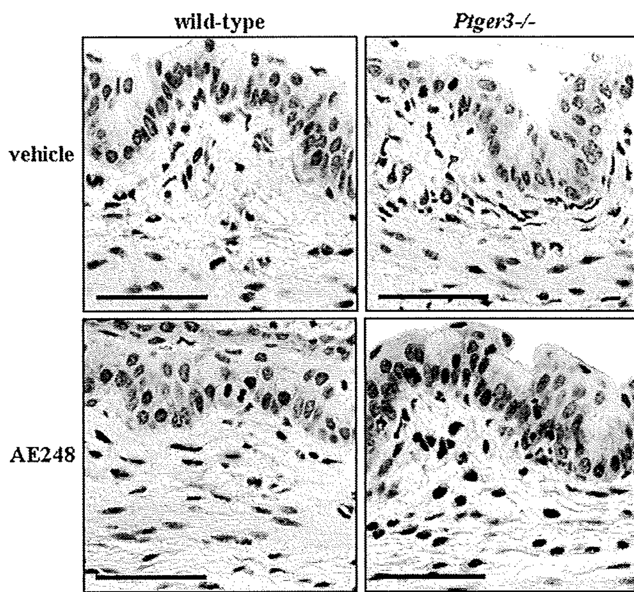

FIG E3. Infiltration of eosinophils into the conjunctiva treated with an EP3-selective agonist. Infiltrations of eosinophils were detected by using the Luna method, which stained eosinophil granules with a distinctive red. *Scale bars* = 50 μm.

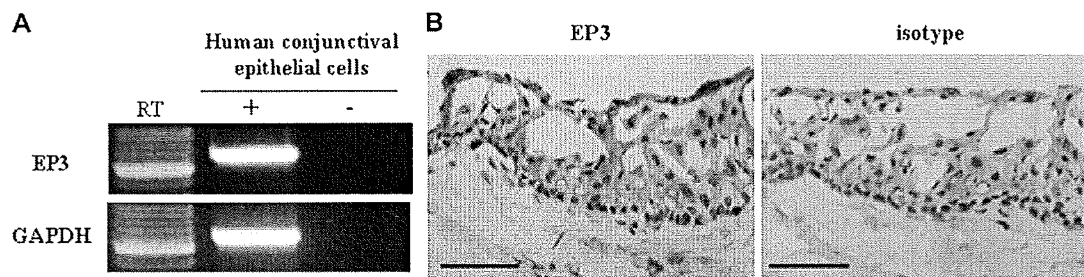

FIG E4. The expression of EP3 in human conjunctival epithelium. **A,** RT-PCR analyses of the expression of *EP3*-specific mRNA. *GAPDH,* Glyceraldehyde-3-phosphate dehydrogenase. **B,** Immunohistologic analysis for EP3. Each *bar* represents a length of 50 μm.

This is the first report of the expression of T_H17-related cytokines in the airway tissues in severe asthma. Although we did not perform colocalization studies, the pattern of the immunoreactive cells in the submucosa suggests that this new subset of T cells may be involved in the inflammatory process in severe asthma. IL-17 has been associated with the activation of epithelial cells *in vitro* and the induction of IL-6 and IL-8 with downstream effects on neutrophil recruitment and activation.[7] We and others have reported an upregulation of IL-8 in severe asthma.[8] Neutrophils were also shown to be increased in severe asthma by many groups,[9] and this phenomenon may be IL-17–driven. We have also previously reported that IL-17 is increased in chronic sinusitis and that its expression is resistant to steroids.[4] Steroid unresponsiveness in severe asthma has been attributed to the presence of neutrophilic inflammation and an upregulation of the glucocorticoid receptor β isoform. T_H17-related cytokines have been implicated in the pathogenesis of a number of diseases that do not respond well to corticosteroids. Recently McKinley et al[10] have shown in a murine model that T_H17 cells not only are proinflammatory cells but also may induce steroid resistance. It is possible that steroid hyporesponsiveness in subjects with severe asthma may also relate to the presence of IL-17A and IL-17F. IL-17 has also been reported to affect structural cells and to stimulate the production of profibrotic cytokines and extracellular matrix proteins. This feature of airway remodeling in severe asthma may be attributable to an excess of these cytokines. If so, targeting IL-17 cytokines may be of value in the therapy of severe asthma, in which steroid resistance, neutrophilic inflammation, and airway remodeling are substantial.

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Toll-like receptor 3 enhances late-phase reaction of experimental allergic conjunctivitis

To the Editor:

Toll-like receptors (TLRs) are well-known key receptors of the innate immune system. *TLR3* recognizes double-stranded RNA, a component of the lifecycle of most viruses, mimicking polyinosinic:polycytidylic acid (polyI:C). Although a relationship between viral infection and allergic inflammation has been reported, the function of *TLR3* in allergic inflammation remains to be defined. Allergic conjunctivitis is an ocular surface inflammation associated with type I hypersensitivity reactions; the degree of eosinophil infiltration in the conjunctiva reflects the degree of its late-phase reaction.[1,2] Using our model of murine experimental allergic conjunctivitis (EAC)[1] and *TLR3* knockout (KO) and *TLR3* transgenic (Tg) mice (*TLR3*KO and *TLR3*Tg mice, respectively), we assessed directly the role of *TLR3* in conjunctival eosinophil infiltration.

BALB/c mice purchased from CLEA (Tokyo, Japan) were sensitized at 6 to 12 weeks of age. *TLR3*KO and *TLR3*Tg mice were generated as previously described,[3,4] back-crossed more than 7 generations to BALB/c mice, and subjected to EAC at 9 to 15 weeks of age. Age-matched wild-type BALB/c mice were used as control animals. The experiments were conducted with a protocol approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine. Short ragweed pollen (RW) was purchased from Polysciences, Inc (Warrington, Pa), and aluminum hydroxide (alum) was purchased from Sigma-Aldrich Corp (St Louis, Mi). The mice were immunized with an intracutaneous injection into the left hind footpad of RW adsorbed on alum (200 μg of RW and 2.6 mg of alum) on day 0. On day 7, they received an intraperitoneal injection of RW adsorbed on alum, and on day 18, their eyes were challenged with RW in PBS (500 μg in 5 μL per eye) or with PBS alone (5 μL per eye).[1] Their eyes, including the conjunctiva, were harvested 24 hours after the last challenge, fixed in 10% neutral buffered formalin, and embedded in paraffin blocks for histologic analysis. Vertical 6-μm-thick sections were mounted on microscope slides, deparaffinized, and stained with Luna stain,[1,2] which identifies erythrocytes and eosinophil granules. Using the entire section from the central portion of the eye, including the pupil and optic nerve head, we counted infiltrating eosinophils in the lamina propria mucosae of the tarsal conjunctiva. Cell counts were expressed as the number of infiltrating eosinophils per unit area (0.1 mm^2) measured with image software (Scion Corp, Frederick, Md).[1,2] Quantitative RT-PCR

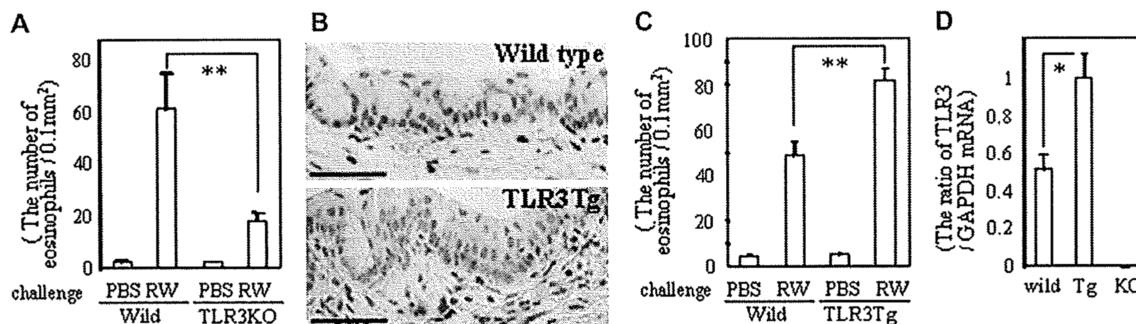

FIG 1. **A,** Eosinophil infiltration in *TLR3*KO mice. **B,** Eosinophil infiltration into the conjunctiva of ragweed-challenged wild-type and *TLR3*Tg mice was detected with Luna's method. Scale bars = 50 μm. **C,** Eosinophil infiltration in *TLR3*Tg mice. **D,** *TLR3* mRNA expression in eyelids. Data are shown as the means ± SEMs of samples from 3 mice. *$P < .05$. In Fig 1, *A* and *C*, data are shown as the means ± SEMs of samples from all 12 mice examined in 3 groups of 4 mice each. **$P < .01$.

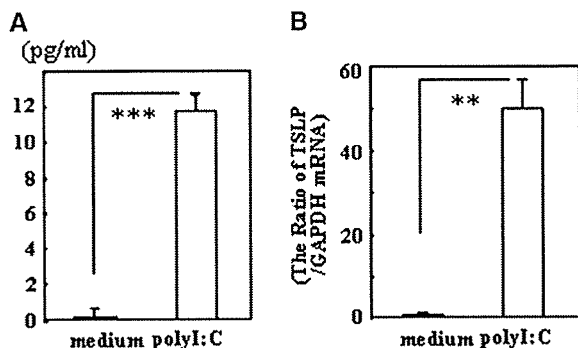

FIG 2. **A,** TSLP production. **B,** *TSLP* mRNA expression. The *y-axis* shows the increase in specific mRNA over that seen in medium samples. Primary human conjunctival epithelial cells were either left untreated or stimulated with 10 μg/mL polyI:C and then incubated for 24 (Fig 2, *A*) or 6 (Fig 2, *B*) hours. The data are representative of 3 independent experiments and shown as the means ± SEMs of 4 samples. ***$P < .0005$, **$P < .01$.

of *TLR3*-specific mRNA in the eyelids was performed as previously reported.[1,2] Briefly, the upper and lower lids were collected 6 hours after the last RW challenge and homogenized in liquid nitrogen. Total RNA was extracted with the RNeasy mini kit (Qiagen, Tokyo, Japan). ReverTraAce (TOYOBO, Otsu, Japan) was used for reverse transcription. The primers and probes for mouse *TLR3* and *glyceraldehyde-3-phosphate dehydrogenase* were from Applied Biosystems (Foster City, Calif). The results were analyzed with sequence detection software (Applied Biosystems). Data were expressed as the mean ± SE, and statistical analyses were performed by means of ANOVA or the Student *t* test, as appropriate.

First, we compared eosinophil infiltration in *TLR3*KO and wild-type mice. Although sensitization without challenge did not affect the number of eosinophils, after sensitization and challenge, their number in the lamina propria mucosae of the conjunctiva was significantly increased in both *TLR3*KO and wild-type mice; however, it was significantly lower in *TLR3*KO than in wild-type mice (Fig 1, *A*). Next we compared eosinophil infiltration in *TLR3*Tg mice and wild-type mice. The numbers of eosinophils in TLR3Tg mice after sensitization and challenge were significantly greater than in wild-type mice (Fig 1, *B* and *C*). Furthermore, we have confirmed that *TLR3* mRNA expression in the eyelids of *TLR3*Tg mice was greater than that of wild-type mice after sensitization with challenge and that *TLR3* mRNA expression in the eyelids of *TLR3*KO mice was undetectable (Fig 1, *D*). These results suggest that TLR3 positively regulates late-phase reaction of EAC, which causes reduced eosinophilic conjunctival inflammation in *TLR3*KO mice and increased it in *TLR3*Tg mice.

We also examined whether sensitization with RW induced RW-specific immune responses equally in wild-type, *TLR3*KO, and *TLR3*Tg mice. It produced an increase in IgE and IgGl antigen–specific antibody responses equally in all 3 groups of mice (data not shown), suggesting that their sensitization to RW was equivalent.

Our results showed that TLR3 could regulate allergic inflammation in the absence of an exogenous viral infection or TLR3 ligand. It is reported that in the absence of viral infection, TLR3 can amplify immune responses during acute inflammatory processes, which might involve stimulation of TLR3 by endogenous RNA from necrotic cells.[5] It is also possible that endogenous RNA from tissue or cells might stimulate TLR3 in our allergic conjunctivitis model. On the other hand, there is a report that a TLR3 ligand can suppress allergic inflammation.[6]

Although the function of TLR3 in allergy remains to be defined, the expression of thymic stromal lymphopoietin (TSLP), which plays a key role in allergic inflammation, is reportedly induced by stimulation with the TLR3 ligand in airway epithelial cells and keratinocytes.[7] TSLP is highly expressed by airway epithelial cells of asthmatic patients and keratinocytes in skin lesions of patients with atopic dermatitis. We previously reported that human ocular surface epithelium expressed TLR3[8,9] and that cytokine production was upregulated by polyI:C, a TLR3 ligand.[9] We also confirmed that TSLP is induced by means of stimulation with the TLR3 ligand polyI:C in human conjunctival epithelial cells (Fig 2 and see the Methods section and Fig El in this article's Online Repository at www.jacionline.org). It is possible that TLR3 positively regulates the late-phase reaction of EAC through the induction of TSLP. Further investigations are required to identify the precise molecular mechanisms of allergic conjunctivitis in the murine model.

Elsewhere, we showed that EP3 is expressed in the ocular surface and that the prostaglandin E_2–EP3 pathway in

conjunctival epithelium works as a negative regulator for allergic conjunctivitis.[1] It is evident that ocular surface epithelial cells regulate the inflammation of allergic conjunctivitis. The actual role of TLR3 in conjunctival inflammation must be further investigated.

In summary, we demonstrated that TLR3 positively regulates late-phase reaction of EAC, which caused reduced eosinophilic conjunctival inflammation in *TLR3*KO mice and pronounced it in *TLR3*Tg mice.

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The carbohydrate galactose-α-1,3-galactose is a major IgE-binding epitope on cat IgA

To the Editor:

Cross-reactive carbohydrate determinants are widely occurring IgE epitopes. Glycan-related IgE reactivity has been demonstrated in most allergen sources, especially in the plant kingdom.[1] The clinical effect of these cross-reactive carbohydrate determinants is debated.

We were recently able to show that IgE Abs to the cat IgA, present in cat-sensitized patients, are mainly directed to a glycan moiety localized on the α-chain.[2] In addition, we have reported that these carbohydrates are present on IgM Abs from cat, as well as on IgM from many different mammalian species, but not human immunoglobulins.[3] Interestingly, IgE antibodies to cat IgM and cat IgA show a complete cross-reactivity, whereas cat IgG does not, suggesting an identical oligosaccharide on the 2 former immunoglobulin classes. Because this is the first mammalian carbohydrate IgE epitope found, it is of major interest to identify the carbohydrate structure responsible for the broad cross-reactivity.

Chung et al[4] have recently investigated subjects with anaphylactic reactions after treatment with the drug cetuximab, a chimeric mouse–human IgG_1 mAb against the epidermal growth factor receptor, which is approved for use in colorectal cancer and squamous-cell carcinoma of the head and neck. The authors found that a carbohydrate epitope on the mouse Fab portion, galactose-α-1,3-galactose, a part of the Galα1,3Galß1,4GlcNAc-R (α-gal) epitope, was responsible for the IgE binding. Furthermore, in most subjects, the IgE antibodies against cetuximab were present in serum before therapy.

The α-gal epitope is expressed on many different glycoproteins in mammals, except for old world monkeys, apes, and human beings. Species lacking the α-gal residues produce large quantities of IgG antibodies to this epitope.[5] Studies have demonstrated that approximately 1% of antibodies in all healthy subjects are directed to α-gal.[6] These antibodies also react with closely related carbohydrate structures in the ABO blood group and are one of the major obstacles in xenotransplantation.

Here we investigated whether α-gal is present on cat IgA and whether it is a major epitope responsible for IgE binding to cat IgA.

Cat IgA was purified from cat serum,[3] and α-gal–human serum albumin was obtained from Dextra Laboratories, Reading, United Kingdom. To investigate the presence of α-gal on cat IgA, a monoclonal anti-Gal antibody was used in ELISA. Plates were coated with 5 μg/mL α-gal, cat IgA, or recombinant Fel d 1,[7] which was included as negative control. Incubation with monoclonal anti-Gal antibodies (Alexis Biochemicals, Lausen, Switzerland), diluted 1:25, was followed by antimouse–IgG-alkaline phosphatase (Dako, Glostrup, Denmark) and substrate solution (Sigma, Steinheim, Germany). We found that the anti-Gal reactivity to α-gal and cat IgA was almost identical, whereas no reactivity was detected to recombinant Fel d 1 (Table I).

TABLE I. Comparison of monoclonal antigalactose reactivity to solid phase bound α-gal, cat IgA, and recombinant Fel d 1 (rFel d 1) by ELISA

Antigen	OD (450 nm)
α-gal	0.69
Cat IgA	0.67
rFel d 1	0.05

Twenty sera from the United States, 9 from patients who were found to have IgE antibodies to the α-gal epitope on cetuximab by using the streptavidin CAP technique,[8] (range, 0.79 to >100 kilo

METHODS

Primary human conjunctival epithelial cells

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the principles set forth in the Declaration of Helsinki. The purposes of our research and the experimental protocol were explained to all patients, and their prior written informed consent was obtained.

For ELISA and real-time quantitative PCR, we harvested primary human conjunctival epithelial cells from conjunctival tissue obtained at the time of conjunctivochalasis surgery. Cells were cultured by using a modification of previously described methods.[E1] Briefly, conjunctival tissues were washed and immersed for 1 hour at 37°C in 1.2 U/mL purified Dispase (Roche Diagnostic Ltd, Basel, Switzerland). Epithelial cells were detached, collected, and cultured in low-calcium k-SFM medium supplemented with 0.2 ng/mL human recombinant epidermal growth factor (Invitrogen, Carlsbad, Calif), 25 mg/mL bovine pituitary extract (Invitrogen), and 1% antibiotic-antimycotic solution. Cell colonies usually became obvious within 3 or 4 days. After reaching 80% confluence in 7 to 10 days, the cells were seeded, and after reaching subconfluence, they were used in subsequent procedures.

ELISA

Primary human conjunctival epithelial cells were either left untreated or stimulated with 10 μg/mL polyI:C and then incubated for 24 hours. The amount of TSLP proteins was determined by using ELISA. TSLP release into culture supernatants was quantitated by using the Human TSLP DuoSet (R&D Systems, Inc, Minneapolis, Minn), according to the manufacturer's instructions.

Real-time quantitative PCR

Real-time quantitative PCR was performed on an ABI-prism 7700 (Applied Biosystems), according to previously described procedures.[E2] The initial amount of RNA used for reverse transcription to cDNA was approximately 1 μg. The cDNA was used at the original concentration for quantitative PCR. The primers and probes for human *TSLP* and human *glyceraldehyde-3-phosphate dehydrogenase* were from Perkin-Elmer Applied Biosystems. Quantitative PCR was used to measure the expression of *TSLP* mRNA in primary human conjunctival epithelial cells treated for 0, 1, 3, or 6 hours with 10 μg/mL polyI:C. The quantification data were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

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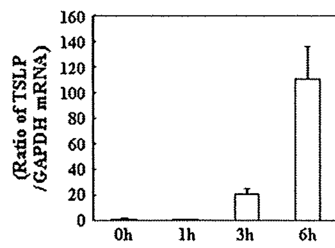

FIG E1. Increased *TSLP* mRNA expression by TLR3 stimulated with polyI:C. The *y-axis* shows the increase in specific mRNA over 0-hour samples or medium samples. The x-axis shows the time after stimulation. The data are presented as the means ± SEMs of 3 samples.

Examination of *Staphylococcus aureus* on the Ocular Surface of Patients With Catarrhal Ulcers

Mayumi Ueta, MD, PhD,† Chie Sotozono, MD, PhD,* Junko Takahashi, MD,* Kentaro Kojima, MD,* and Shigeru Kinoshita, MD, PhD**

Purpose: The purpose of this study was to investigate the role of *Staphylococcus aureus* in the onset of ocular catarrhal ulcers.

Methods: We examined the colonization by *S. aureus* of the ocular surface (conjunctival sac, upper and lower lid margins) of 3 patients with catarrhal ulcers and analyzed the *S. aureus* isolates by pulsed-field gel electrophoresis.

Results: *S. aureus* organisms were found on the lid margin of all eyes affected by catarrhal ulcers. The contralateral eye without ulcers harbored *S. aureus* exhibiting a pulsed-field gel electrophoresis pattern identical to that of the affected eye.

Conclusions: Although *S. aureus* on the lid margin plays an important role in the onset of catarrhal ulcers, its presence is one among several risk factors.

Key Words: *Staphylococcus aureus*, catarrhal ulcer, pulsed-field gel electrophoresis (PFGE)

(*Cornea* 2009;28:780–782)

INTRODUCTION

Catarrhal ulcers are usually a complication of long-standing staphylococcal blepharitis, conjunctivitis, or meibomitis,[1,2] which might sometimes be subclinical, and cultures from the lid margins of affected patients usually yield colonies of *Staphylococcus aureus*,[2] although lid margins of normal eyes also sometimes, but not usually, have *S. aureus*.[3,4] Because corneal cultures are usually negative for the organisms, it has been suggested that catarrhal ulcers are not the result of direct infection of the cornea, but rather derive from an antigen–antibody reaction with complementary activation and neutrophil infiltration in patients sensitized to staphylococcal antigens.[1,5,6]

Catarrhal infiltrates and ulcers are frequently seen by ophthalmologists and because they readily respond to adequate treatment, they do not attract much attention in the literature. To the best of our knowledge, this is the first pulsed-field gel electrophoresis (PFGE) analysis of the relationship between catarrhal ulcers and the presence of *S. aureus*.

MATERIALS AND METHODS

The diagnosis of catarrhal ulcer in our 3 patients was based on ocular surface manifestations. The patients were 15- (Case 1, Fig. 1A), 81- (Case 2, Fig. 1B), and 55-year-old (Case 3, Fig. 1C) females. In all patients, the right eye was involved. Clinical examinations revealed oval infiltrates and ulcers separated from the limbus by a distinct lucid border and adjacent conjunctival inflammation. We examined 3 ocular sites (the conjunctival sac and the upper and lower lid margins) for the presence of bacteria; in cases 1 and 2, we examined both eyes and in case 3 only the affected eye. Using PFGE analysis, we analyzed and compared the *S. aureus* organisms isolated from 2 or more sites in each patient.

The isolates obtained were stored (–20°C) in ANAport BIKEN culture medium (BIKEN, Osaka, Japan) at the Department of Ophthalmology of Kyoto Prefectural University of Medicine; they were sent to The Research Foundation for Microbial Diseases of Osaka University the next day. The isolates were cultured in both methods, direct culture and enrichment culture, as previously reported.[3,7]

We used the GenePath system (Bio-Rad Laboratories, Hercules, CA) to perform PFGE according to the manufacturer's instructions (GenePath Group I Reagent Kit; Bio-Rad) and visually compared the DNA banding patterns as described by Tenover et al.[8]

RESULTS

The colonization by *S. aureus* is shown schematically in Figure 2A. In case 1, *S. aureus* was detected in the lower lid margin of the affected and the conjunctiva of the unaffected eye. The PFGE patterns of the organisms from both eyes were identical (Fig. 2B-1), suggesting that they derived from the same clone. In case 2, *S. aureus* was detected in the upper lid margin and conjunctiva of the affected and in the lower lid

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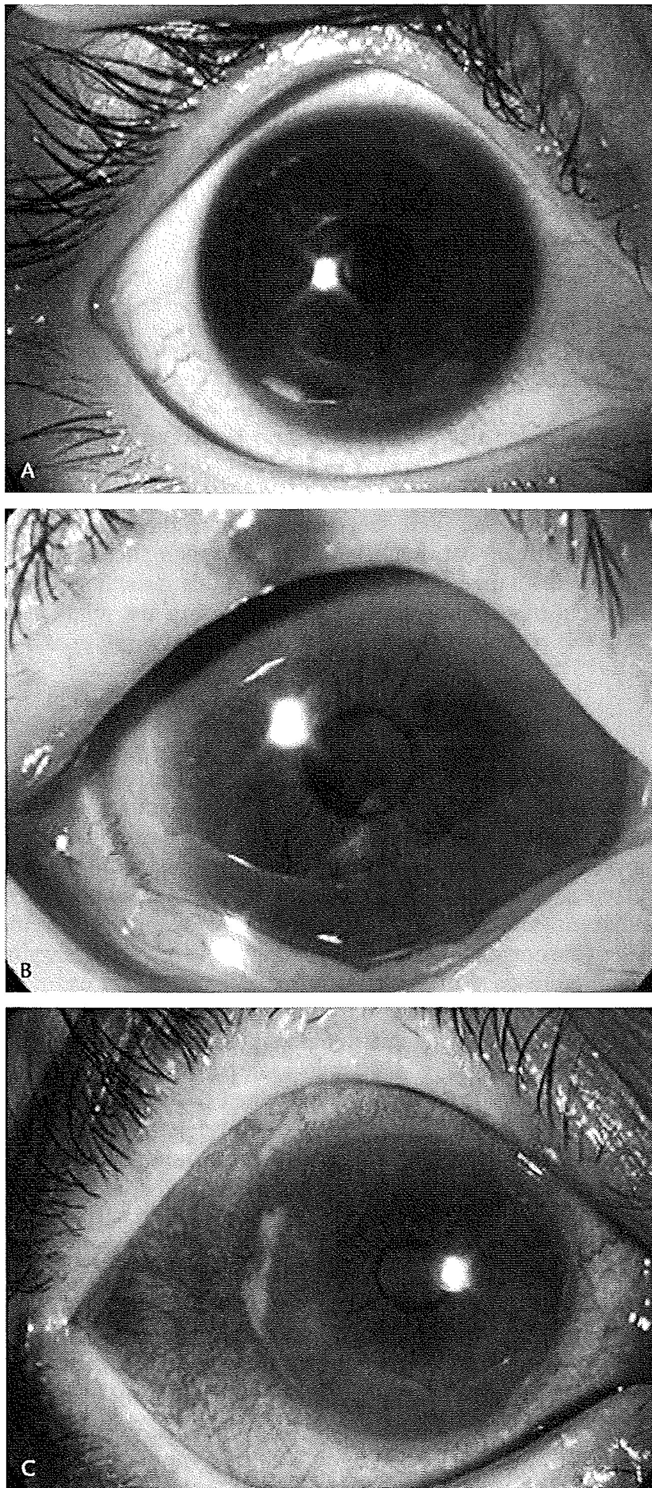

FIGURE 1. Photographs of the affected eyes of patients with catarrhal ulcers. (A) Case 1: The right eye of a 15-year-old girl. (B) Case 2: The right eye of an 81-year-old woman. (C) Case 3: The right eye of a 55-year-old woman.

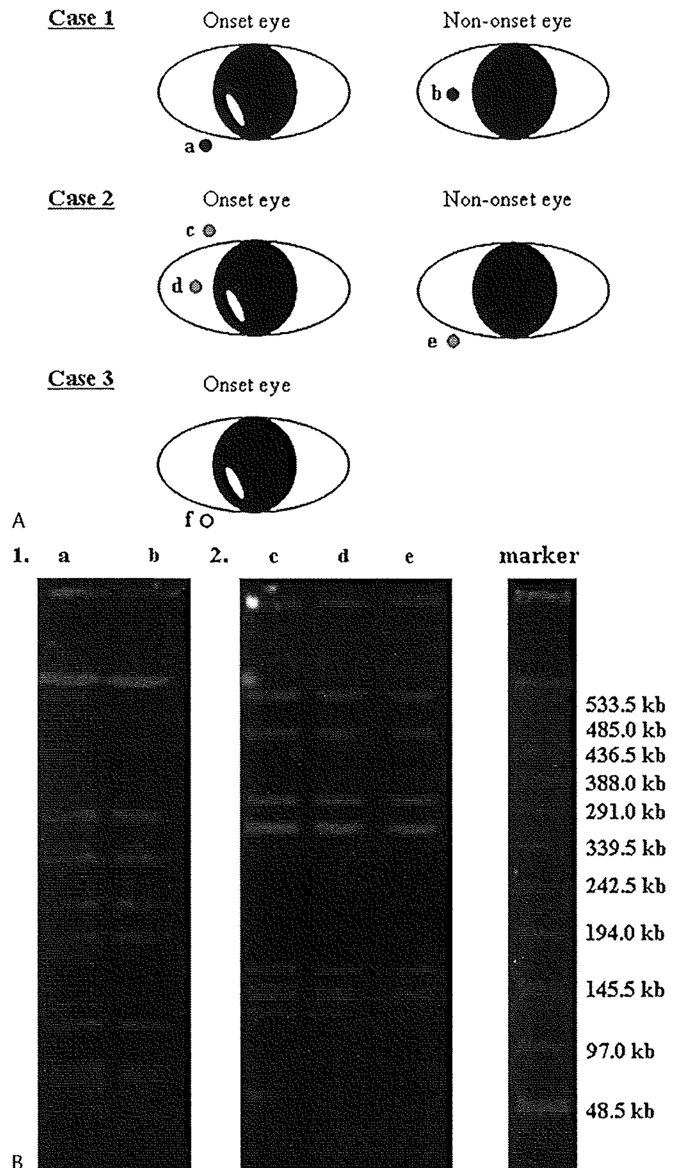

FIGURE 2. Colonization by (A) and pulsed-field gel electrophoresis (PFGE) analysis of (B) *Staphylococcus aureus*. Case 1: *S. aureus* was detected in the lower lid margin (A) of the affected and the conjunctiva (B) of the unaffected eye. The organisms from the 2 sites showed identical PFGE patterns (Fig. 2B-1). Case 2: *S. aureus* was detected in the upper lid margin (C) and the conjunctiva (D) of the affected eye and in the lower lid margin (E) of the unaffected eye. These organisms from the 3 sites exhibited identical PFGE patterns (Fig. 2B-2). Case 3: *S. aureus* was detected in the lower lid margin of the affected eye (F). We used the GenePath System for PFGE. Bacterial chromosomal DNA was cut with SmaI. The PFGE patterns were obtained by running digested DNA on 1% agarose gels in a CHEF-DR Mapper. A lambda ladder was used as the molecular size marker.

margin of the unaffected eye. They also manifested identical PFGE patterns (Fig. 2B-2), suggesting that they originated from the same clone. In case 3, *S. aureus* were detected in the lower lid margin of the affected eye; the unaffected eye could not be examined because the patient gave consent to examine only the affected eye.

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DISCUSSION

Although our study included only a small number of patients, we found *S. aureus* to be present in the lid margin of the eyes affected by catarrhal ulcers. This might suggest that their presence in the lid margin, rather than the conjunctival sac, is important for the development of catarrhal ulcers. Because we were able to detect all *S. aureus* organisms in enrichment cultures, it appears that the development of catarrhal ulcers does not require the presence of large amounts of the bacterium.

Interestingly, in case 2, we also found *S. aureus* in the lid margin of the unaffected eye. It means that if the patient, who was sensitized to staphylococcal antigens, has *S. aureus* on both eyes, the catarrhal ulcer may occur on only one eye but not the fellow eye. Moreover, our PFGE analysis showed that *S. aureus*, which was detected in both eyes, might be derived from the same clone, suggesting that the kind of clone of *S. aureus* is not necessarily important for the initiation of the catarrhal ulcers.

Although our study included only a small number of patients, our findings might suggest that other factors may be necessary for the initiation of the catarrhal ulcers in addition to the existence of *S. aureus* on the lid margin and the patients' sensitivity to staphylococcal antigens. One possible factor may be an immune abnormality of the ocular surface of the affected eye. A second possible factor may be the condition of contact between the cornea and the lid margin such as a subtle difference of pressure and/or angle of the lid margin, although it is not apparent in clinical findings. A third possible factor may be a difference in the amount of bacterium between the affected eye and unaffected eye, although the development of catarrhal ulcers does not require the presence of large amounts of the bacterium. Investigations are underway to shed light on the pathogenesis of catarrhal ulcers.

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Diagnosis and Treatment of Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis with Ocular Complications

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Purpose: To present a detailed clarification of the symptoms at disease onset of Stevens-Johnson syndrome (SJS) and its more severe variant, toxic epidermal necrolysis (TEN), with ocular complications and to clarify the relationship between topical steroid use and visual prognosis.

Design: Cross-sectional study.

Participants: Ninety-four patients with SJS and TEN with ocular complications.

Methods: A structured interview, examination of the patient medical records, or both addressing clinical manifestations at disease onset were conducted for 94 patients seen at Kyoto Prefectural University of Medicine. Any topical steroid use during the first week at the acute stage also was investigated.

Main Outcome Measures: The incidence and the details of prodromal symptoms and the mucosal involvements and the relationship between topical steroid use and visual outcomes.

Results: Common cold-like symptoms (general malaise, fever, sore throat, etc.) preceded skin eruptions in 75 cases, and extremely high fever accompanied disease onset in 86 cases. Acute conjunctivitis and oral and nail involvements were reported in all patients who remembered the details. Acute conjunctivitis occurred before the skin eruptions in 42 patients and simultaneously in 21 patients, whereas only 1 patient reported posteruption conjunctivitis. Visual outcomes were significantly better in the group receiving topical steroids compared with those of the no-treatment group ($P<0.00001$).

Conclusions: Acute conjunctivitis occurring before or simultaneously with skin eruptions accompanied by extremely high fever and oral and nail involvement indicate the initiation of SJS or TEN. Topical steroid treatment from disease onset seems to be important for the improvement of visual prognosis.

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Stevens-Johnson syndrome (SJS) and its more severe variant, toxic epidermal necrolysis (TEN), are acute inflammatory disorders that affect the skin and mucous membranes.[1–4] Although the incidence of SJS and TEN is very low, approximately 0.4 to 1 case per 1 million persons and 1 to 6 cases per 1 million persons, respectively, both can affect anybody at any age, usually as a consequence of adverse drug reactions.[5–7] A variety of drugs including antibiotics, nonsteroidal anti-inflammatory drugs, and antiepileptic medications, that is, any of the popularly used drugs, have been reported to cause severe drug reactions and to induce SJS or TEN.

The mortality rates for SJS and TEN are high: 1% to 5% and 25% to 35%, respectively.[8,9] Ocular complications occur in more than 50% of the patients, and ocular surface inflammation develops rapidly at the acute stage.[10,11] Extensive inflammation of the ocular surface often is accompanied by pseudomembranous formation and corneal or conjunctival epithelial defects, or both. The common pathway after the acute stage includes persistent epithelial defects, ulceration, and perforation, finally developing into corneal cicatricial changes such as neovascularization, opacification, keratinization, and symblepharon.[12,13] Even after the acute-stage impairments subside, permanent visual impairment or blindness remains and conjunctival inflammation prolongs at the chronic stage.[14] Patients with SJS or TEN require life-long management for ocular discomfort and morbidity. Stevens-Johnson syndrome or TEN accompanied by ocular complications, at both the acute and chronic stage, are 2 of the most devastating ocular surface diseases, and both are extremely difficult to treat.

The loss of corneal epithelial stem cells, which are located in the limbal region,[15–18] evidenced by the loss of palisades of Vogt, is the most common ocular feature of SJS.[13] As soon as the corneal epithelial stem cells are lost at the acute stage of SJS or TEN, the corneal epithelium does not regenerate, thus resulting in conjunctival epithelial invasion into the cornea (conjunctivalization) and cicatricial changes of the ocular surface. In contrast, the regeneration of the epidermis develops rather smoothly at the remission of the diseases.

Penetrating keratoplasty (PK) generally is contraindicated for eyes with SJS or TEN because PK does not supply the limbal region of the eye with corneal epithelial stem

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cells. Moreover, PK-initiated, immunologically driven ocular surface inflammation may induce persistent epithelial defects and corneal melting, perforation, or both, ultimately resulting in blindness.[12] Allograft transplantation of healthy limbal tissue is useful for the reconstruction of the ocular surface. However, long-term outcomes are poor in eyes with SJS or TEN.[19] Groundbreaking surgical procedures have been developed over the past 12 years. We first reported the usefulness of cultivated corneal epithelial transplantation for SJS with persistent epithelial defects after the acute stage.[20–23] In another report, we clarified the efficacy of ex vivo expanded autologous oral mucosal epithelial cells to the ocular surface.[24] Cultivated oral mucosal epithelial transplantation and the 2-step surgical combination of cultivated oral mucosal epithelial transplantation and PK have provided the patients with SJS or TEN with a surgical pathway toward restoration of their visual function.[25–27] However, it is impossible for the ocular surface of those patients to be restored to its previously normal state.

Diagnosis of SJS or TEN at disease onset is complex, often confusing, and very difficult. Moreover, the use of steroids for treatment remains controversial.[10,28–30] Our recent reports and those of others indicated the influence of genetic endowment in SJS and TEN.[31–40] For instance, there are statistically significant differences in single nucleotide polymorphisms of toll-like receptor 3, interleukin (IL)-4R/IL-13, and Fas ligand in SJS and TEN; thus, genetic screening may help to deliver a more rapid diagnosis in the future. At present, however, the understanding of the typical clinical picture of SJS and TEN is still a vital aspect of early diagnosis and the initiation of treatment. Therefore, this study investigated the clinical manifestation at disease onset of SJS and TEN with ocular complications and evaluated the relationship between ophthalmic management at the acute stage and the visual outcomes.

Patients and Methods

From November 2005 through May 2008, extensive interviews were conducted with 94 patients (45 males and 49 females) with SJS or TEN with ocular complications seen at the SJS outpatient service at Kyoto Prefectural University Hospital. Of those patients, 88 cases were referral patients from the greater Japan area who had come to the SJS service at the acute stage (n = 14) or at the chronic stage (n = 74). Their ages ranged from 1 to 83 years (mean age±standard deviation, 41.6±18.5 years). At disease onset, the patients' ages ranged from 0 to 77 years (mean age±standard deviation, 26.2±18.8 years), and the duration of the illness ranged from 1 to 48 years (mean±standard deviation, 16.1±15.2 years). The questionnaires used in this study were structured as follows: (1) age of the patient at disease onset; (2) causative drugs; (3) the presence of prodromal symptoms; and (4) the episodes of high fever, conjunctivitis, skin eruptions, fingernail loss, and associated mucous membrane involvements. Medical records also were examined or the patients were asked directly regarding any ophthalmic management, especially the use of topical steroids, during the first week from disease onset. Then, the Mann–Whitney U test was used to analyze the correlation between the use of topical steroids and the visual outcomes. This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan.

The diagnosis of SJS or TEN at the acute stage was based on the acute onset of high fever, serious mucocutaneous illness with skin eruptions, involvement of at least 2 mucosal sites, and the pathologic findings of a skin biopsy that demonstrated necrotic changes of the dermis. The diagnosis of SJS or TEN at the chronic stage was based on ocular cicatricial findings such as symblepharon, severe dry eye, corneal neovascularization, opacification, and conjunctivalization, and a confirmed history of the acute onset of high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least 2 mucosal sites including the ocular surface. In the patients where disease onset occurred before age 10 years or in those who had lost consciousness at the acute stage because the illness, specific details were obtained by directly interviewing members of the immediate family.

Results

Of the 94 patients, drugs were the most commonly associated etiologic factor in 84 patients (89.4%). The causative drugs were cold remedies in 30 patients, antibiotics in 23 patients, nonsteroidal anti-inflammatory drugs in 19 patients, anticonvulsants in 5 patients, and others (anticancer agents, antirheumatic drugs, antimalarial, Chinese medicine, etc.).

Best-corrected visual acuity obtained at the chronic stage was 20/20 or better in 34 eyes (18.3%; Fig 1A), worse than 20/20 and up to and including 20/200 in 55 eyes (29.6%; Fig 1B), worse than 20/200 and up to and including 20/2000 in 53 eyes (28.5%; Fig 1C), and worse than 20/2000 in 44 eyes (23.7%; Fig 1D). Two eyes of 1 boy who was 1 year or age were excluded from the results because his visual acuity could not be assessed.

Characteristics of Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis with Ocular Complications

Of the 94 patients, common coldlike symptoms (general malaise, fever, sore throat, etc.) preceded skin eruptions in 75 patients. Extremely high fever (more than 39° C) was reported by 86 patients, whereas 1 patient reported no fever and the remaining 7 patients could not remember the extent of the fever. Acute conjunctivitis and oral involvements (blisters, erosions, and bleeding of the mouth and lips) occurred in all patients who could recollect their symptoms in detail. Fingernail loss at the acute stage or deformation at present existed in all patients (Table 1; Fig 2). Other mucous membrane involvements included those of the pharynx, respiratory tract, or ear canal.

Forty-two patients reported episodes of acute conjunctivitis several hours to 4 days before the skin eruptions, and 21 patients reported that skin eruptions and conjunctivitis occurred simultaneously. Only 1 patient reported posteruption conjunctivitis (Table 2).

Topical Steroid Instillation and Visual Outcomes

Thirty-three patients (13 males and 20 females; mean age±standard deviation at disease onset, 31.5±18.6 years) began topical steroid treatment during the first week from disease onset, whereas 31 patients (14 males and 17 females; mean age±standard deviation, 27.9±19.5 years) received no topical steroid treatment or any other treatment for their eyes. The remaining 30 patients could not recall the details of ocular management during the first week from disease onset. Visual outcomes were significantly better in the group that received topical steroids at the acute stage compared with those of the no-treatment group (P<0.00001; Fig 3).