Increasing Effect by Simultaneous Use of Levocabastine and Pemirolast on Experimental Allergic Conjunctivitis in Rats

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The effect of the simultaneous use of 0.025% levocabastine hydrochloride eye drops (levocabastine) and 0.1% pemirolast potassium ophthalmic solution (pemirolast) on experimental allergic conjunctivitis in rats was investigated. Levocabastine and pemirolast significantly inhibited allergic conjunctivitis compared with the control group when separately administered. In addition, the simultaneous use of both drugs inhibited allergic conjunctivitis more potently than the original activity of levocabastine or pemirolast. Furthermore, the simultaneous use of levocabastine and pemirolast also significantly inhibited increased vascular permeability induced by antigen compared with levocabastine or pemirolast alone, respectively. Levocabastine and pemirolast inhibited histamine release from the rat conjunctiva in correlation with a decrease in histamine content in tears. When levocabastine and pemirolast were simultaneously applied to the eyes, histamine release from the conjunctiva was greater than for the original activities of both drugs. Similar to histamine release from the conjunctiva, the histamine content in tears induced by the simultaneous use of both drugs was significantly decreased compared with levocabastine and pemirolast alone, respectively. A potentiating effect induced by the simultaneous use of levocabastine and pemirolast may be attributable to the antihistaminic activity of levocabastine and histamine release inhibition by levocabastine and pemirolast.

Key words allergic conjunctivitis; histamine release; vascular permeability; levocabastine; pemirolast

Levocabastine is a potent H_1 -receptor antagonist which provides long-acting antihistaminic and antiallergic activities in experimental animals.^{1,2)} In experimental allergic conjunctivitis in guinea pigs, levocabastine causes the inhibition of antigen- and histamine-induced conjunctivitis, and these effects are superior to those of cromolyn sodium.¹⁾ Clinical studies also revealed that levocabastine is well-tolerated and is at least as effective as cromolyn sodium for the treatment of pollen-provoked conjunctivitis.^{3,4)} Abelson *et al.*⁵⁾ also found that levocabastine is significantly more effective than cromolyn sodium in inhibiting itching, hyperemia, eyelid swelling, chemosis and tearing in allergic conjunctivitis induced by ocular allergen challenge.

On the other hand, the treatment of conjunctivitis associated with pollinosis is at present carried out by antihistamines either alone or in combination with alpha-adrenergic agents and mast cell stabilizers.⁶⁾ Pemirolast is a drug that exhibits potent antiallergic activity compared with cromolyn sodium.⁷⁾ In addition, it inhibits histamine release induced by immunological and non-immunological stimuli, and this effect is more potent than that of cromolyn sodium and tranilast. Therefore, it is possible to obtain a more effective medical treatment of allergic conjunctivitis if levocabastine and pemirolast are administered simultaneously. In the present study, we investigated the effect of the simultaneous use of levocabastine and pemirolast on eye scratching behavior and allergic symptoms in allergic rat conjunctivitis models which we recently developed.⁸⁾

MATERIALS AND METHODS

Animals Six weeks-old male Wistar rats were obtained from Japan SLC, Inc., Shizuoka, Japan. The animals were housed in an air-conditioned room maintained at $24 \pm 2^\circ\text{C}$ with humidity of $55 \pm 15\%$. They were given standard laboratory rodent chow (Oriental Yeast, Tokyo, Japan) and water *ad*

libitum.

Reagents The following reagents were obtained from the sources shown in parentheses: egg albumin (Grade VII; crystallized and lyophilized, essentially salt-free, Sigma, St. Louis, MO, U.S.A.), aluminum hydroxide hydrate gel (alum, LSL, Tokyo, Japan), and Evans blue (Wako, Tokyo, Japan). *Bordetella pertussis* inactive microorganism suspension (*B. pertussis*) was kindly provided by the Kitasato Institute Research Center for Biologicals, Saitama, Japan. The following drugs were purchased from the companies indicated: 0.025% levocabastine hydrochloride eye drops (levocabastine, Livostin eye drops, Santen Pharmaceutical Co., Osaka, Japan) and 0.1% pemirolast potassium ophthalmic solution (pemirolast, Alegysal ophthalmic solution, Santen Pharmaceutical Co., Osaka, Japan). Levocabastine and pemirolast were instilled at 5 and 15 min, respectively, before antigen challenge into the bilateral eyes at $5 \mu\text{l}/\text{site}$.

Sensitization The rats were sensitized by injection of 0.6 ml of physiological saline containing egg albumin (1 mg), alum (2 mg) and 10^{10} *B. pertussis* into the four footpads on the first day. Five days later, they were boosted by subcutaneous injection of 1 ml of physiological saline containing egg albumin (0.5 mg) at 10 sites on the back. Then, local sensitization was performed every day from day 14 to day 20 by instilling egg albumin in physiological saline (10 mg/ml, $5 \mu\text{l}/\text{site}$) into the bilateral eyes using a micropipette.

Evaluation of Conjunctival Symptoms Before the experiment, the animals were placed in an observation cage ($32 \times 22 \times 10$ cm) for about 10 min for acclimatization. After the instillation of $5 \mu\text{l}/\text{site}$ of egg albumin dissolved in physiological saline solution (10 mg/ml) into the bilateral eyes, they were placed into the observation cage (one animal/cage), and the number of eye scratches was counted for 20 min. Eye scratching behavior was defined as fore-limb movements over 2 times directed to the ocular surface, and the number of instances. Allergic symptoms (hyperemia and edema of the

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Table 1. The Scoring System Used for Estimating the Severity of Conjunctivitis

Score	Symptoms	
	Hyperemia	Edema
0	No symptoms	No symptoms
1	Slight hyperemia in one-eye	Slight edema in one-eye
2	Slight hyperemia in bilateral eyes	Slight edema in bilateral eyes
3	Severe hyperemia in one-eye and Slight hyperemia in the other eye	Severe edema in one-eye and Slight edema in the other eye
4	Severe hyperemia in bilateral eyes	Severe edema in bilateral eyes

Hyperemia and edema were evaluated 5 min and 20 min after antigen challenge, respectively.

conjunctiva) were observed using the scoring system shown in Table 1. Hyperemia and edema were evaluated at 5 and 20 min, respectively, after topical antigen challenge.

Vascular Permeability of the Conjunctiva On the day following antigen challenge, 2% Evans blue solution was intravenously injected. Fifteen minutes later, the rats were anesthetized with diethylether, bled, and the conjunctiva was removed. Then, Evans blue was extracted with 0.5 ml of 1 N KOH solution for 12 h at 37 °C, and 4.5 ml of H₃PO₄-acetone (0.6 N H₃PO₄:acetone=5:13) was added and mixed well. After centrifugation at 400×g for 20 min, the amount of extracted dye was determined using a spectrophotometer (Model U-2000, Hitachi, Tokyo, Japan) at 620 nm.⁹⁾

Histamine Release from the Conjunctiva Thirty minutes after antigen challenge, the conjunctiva was carefully excised, weighed and washed twice with saline. The tissues were homogenized with 0.4 N perchloric acid and placed in an ice-bath for 1 h. After centrifugation at 1000×g for 10 min at 4 °C, histamine content in the supernatant was determined by an automated fluorometric assay. The percentage of histamine release was calculated by the following equation:

$$c = (a - b) / a \times 100$$

c : % histamine release

a : histamine content of the conjunctiva in immunized animals

b : histamine content of the conjunctiva (antigen challenged) in immunized animals

Histamine Content in Tears Fifteen minutes after antigen challenge, 50 µl saline was applied to both eyes. This procedure was repeated 3 times and a 200 µl sample was carefully collected from both eyes. The sample and the same quantity of 0.8 N perchloric acid were then mixed together. After centrifugation at 10000×g for 10 min at 4 °C, the histamine content of the supernatant was determined by high performance liquid chromatography using a fluorometric detector (CCP & 8010 Series, Tosoh, Tokyo, Japan).¹⁰⁾

Statistical Analysis All data were represented as the means ± S.E.M. Statistical analysis was performed using one-way ANOVA and Dunnett's test for eye scratching behavior, vascular permeability, histamine release and histamine content, and the Kruskal-Wallis and Mann-Whitney *U* tests were used for allergic symptoms. A probability value of less than 0.05 was considered to be statistically significant.

Fig. 1. The Effect of the Simultaneous Use of Levocabastine and Pemirolast on Conjunctival Symptoms Induced by Antigen in Sensitized Rats

Antigen was applied to the bilateral eyes of rats, and the number of instances of eye scratching was counted for 20 min. Furthermore, an allergic symptom scoring was also performed. Levocabastine and pemirolast were instilled at 5 and 15 min before antigen challenge into the bilateral eye at 5 µl/site. The results show (A) eye scratching behavior and (B) allergic symptoms induced by antigen. Each column and vertical bar show the means ± S.E.M. of 10 experiments. ** Significantly different from the control group (C) at *p* < 0.01. ## Significantly different from the levocabastine group (L) at *p* < 0.01. †† Significantly different from the pemirolast group (P) at *p* < 0.01.

RESULTS

Effect of the Simultaneous Use of Levocabastine and Pemirolast on Eye Scratching Behavior and Allergic Symptoms Induced by Antigen Figure 1 shows the effect of the simultaneous use of levocabastine and pemirolast on eye scratching behavior and allergic symptoms induced by antigen. Levocabastine and pemirolast significantly inhibited eye scratching behavior and allergic symptoms. In addition, the simultaneous use of levocabastine and pemirolast significantly inhibited eye scratching behavior and allergic symptoms compared with levocabastine or pemirolast alone, respectively.

Effect of the Simultaneous Use of Levocabastine and Pemirolast on Vascular Permeability Increase Induced by Antigen Figure 2 shows the effect of the simultaneous use of levocabastine and pemirolast on vascular permeability increase induced by antigen in rats. Levocabastine and pemirolast significantly inhibited the vascular permeability increase in the conjunctiva. The simultaneous use of levocabastine and pemirolast also resulted in the significant inhibition of increased vascular permeability. This effect was more potent than for levocabastine or pemirolast when used separately.

Effect of the Simultaneous Use of Levocabastine and Pemirolast on Histamine Release from the Conjunctiva Figure 3 shows the effect of the simultaneous use of levocabastine and pemirolast on histamine release from the rat conjunctiva. Levocabastine and pemirolast significantly inhibited histamine release. The simultaneous use of levocabastine and pemirolast significantly inhibited histamine release, and this effect was more potent than for levocabastine or pemirolast alone, respectively.

Effect of the Simultaneous Use of Levocabastine and

Fig. 2. The Effect of the Simultaneous Use of Levocabastine and Pemirolast on Increased Vascular Permeability Induced by Antigen in Sensitized Rats

Conjunctival dye content was measured 30 min after antigen application. Levocabastine and pemirolast were instilled at 5 and 15 min before antigen challenge into the bilateral eye at 5 μ l/site. Each column and vertical bar shows the means $\pm$ S.E.M. of 8 experiments. ** Significantly different from the control group (C) at $p < 0.01$. ## Significantly different from the levocabastine group (L) at $p < 0.01$. †† Significantly different from the pemirolast group (P) at $p < 0.01$.

Fig. 3. The Effect of the Simultaneous Use of Levocabastine and Pemirolast on Histamine Release Induced by Antigen in Sensitized Rats

Histamine release from the conjunctiva was measured 30 min after antigen application. Levocabastine and pemirolast were instilled at 5 and 15 min before antigen challenge into the bilateral eye at 5 μ l/site. Each column and vertical bar shows the means $\pm$ S.E.M. of 8 experiments. *, ** Significantly different from the control group (C) at $p < 0.05$ and $p < 0.01$, respectively. ## Significantly different from the levocabastine group (L) at $p < 0.01$. †† Significantly different from the pemirolast group (P) at $p < 0.01$.

Fig. 4. The Effect of the Simultaneous Use of Levocabastine and Pemirolast on Histamine Content in Tears Induced by Antigen in Sensitized Rats

Histamine content in tears was measured 30 min after antigen application. Levocabastine and pemirolast were instilled at 5 and 15 min before antigen challenge into the bilateral eye at 5 μ l/site. Each column and vertical bar shows the means $\pm$ S.E.M. of 8 experiments. *, ** Significantly different from the control group (C) at $p < 0.05$ and $p < 0.01$, respectively. ## Significantly different from the levocabastine group (L) at $p < 0.01$. †† Significantly different from the pemirolast group (P) at $p < 0.01$.

Pemirolast on Histamine Content in Tears Induced by Antigen Figure 4 shows the effect of the simultaneous use of levocabastine and pemirolast on histamine content in tears induced by antigen. Although the histamine content was 0.25 ± 0.2 μ g/ml ($n=8$) before antigen challenge, it increased 20 times (5.2 ± 0.7 μ g/ml, $n=8$) after antigen challenge. Levocabastine and pemirolast caused a decrease in histamine content. In addition, the simultaneous use of levocabastine and pemirolast significantly decreased histamine content

compared with levocabastine or pemirolast when used separately.

DISCUSSION

The objective of the present study was to investigate the potentiating effect of levocabastine and pemirolast when administered together. For this purpose, the doses of levocabastine and pemirolast that are already clinically used were employed in the experiments. We found in the present study that the simultaneous use of levocabastine and pemirolast significantly inhibited eye scratching behavior and allergic symptoms compared with levocabastine or pemirolast when used separately. This indicates that the inhibitory effect of levocabastine on allergic conjunctivitis was augmented by pemirolast or *vice versa*. As previously shown, levocabastine is a potent H_1 -receptor antagonist.¹⁾ On the other hand, pemirolast inhibits antigen-induced histamine release from peritoneal exudates cells containing mast cells and lung fragments of rats.¹¹⁾ Therefore, the potentiation of levocabastine induced by pemirolast or *vice versa* can be accounted by the observation that histamine release from mast cells, which could not completely inhibited by pemirolast, was antagonized by levocabastine.

To make another attempt at elucidating the mechanism responsible for the potentiating effect of levocabastine by pemirolast or *vice versa*, we studied the effect of the simultaneous use of levocabastine and pemirolast on increased conjunctival vascular permeability induced by antigen-antibody reaction. As a result, the simultaneous use of levocabastine and pemirolast also significantly inhibited increased vascular permeability compared with levocabastine or pemirolast alone, respectively. Vascular permeability is mainly related to histamine action, and an increase in vascular permeability is generated by the contraction of endothelial cells after the binding of histamine H_1 receptors located in these cells.¹²⁾ Nakahara *et al.*¹³⁾ showed using histamine H_1 receptor deficient mice that vascular permeability in the conjunctiva in allergic conjunctivitis is regulated through histamine H_1 receptors. Levocabastine has potent and specific H_1 antagonistic activity.¹⁴⁾ Therefore, it inhibits the levocabastine-induced increase in vascular permeability. On the contrary, pemirolast has no binding affinity toward H_1 receptors.¹⁵⁾ However, pemirolast also exerted a significant inhibitory effect on increased vascular permeability in the conjunctiva. Therefore, we thought that the inhibition of increased vascular permeability due to pemirolast is attributable to the inhibition of histamine release from mast cells induced by the drug. The potentiating effect of the simultaneous use of levocabastine and pemirolast on the inhibition of increased vascular permeability can be also accounted for by the antihistaminic activity of levocabastine and the inhibition of histamine release induced by pemirolast.

From the present results it was revealed that levocabastine significantly inhibits histamine release from the rat conjunctiva. On the other hand, Lau and Pearce¹⁶⁾ reported that levocabastine is not capable of inhibiting histamine release from rat peritoneal mast cells induced by antigen. However, there are a few reports that suggest levocabastine inhibits histamine release similar as our present results do. For instance, Tasaka *et al.*²⁾ and Goldschmidt *et al.*¹⁷⁾ found that levocabas-

tine inhibits histamine release from lung pieces from guinea pigs and human leukocytes, respectively. In contrast, pemirolast inhibits histamine release from connective tissue mast cells (dermal and peritoneal mast cells) and mucosal mast cells (lung pieces) in rats.^{7,11,18} Potentiation by the simultaneous use of levocabastine and pemirolast in the inhibition of histamine release may be responsible for an additional effect induced by these drugs. Levocabastine, pemirolast or the simultaneous use of both drugs caused a decrease in histamine content in the tears induced by antigen. This finding indicates that both drugs or their combination inhibited histamine release in *in vivo* experiments.

As shown in the text, simultaneous use of levocabastine and pemirolast completely prevent the increase of histamine content in the tears induced by antigen. Although the simultaneous use of these drugs caused an inhibition of eye scratching behavior and allergic symptoms, did not completely inhibit them. For this reason, our view is as follows; in chronic allergic conjunctivitis, not only histamine but also other chemical mediators are responsible for the allergic symptoms.¹⁹ Accordingly, we assume that in the present study, it is possible to get results where histamine content in the tears is decreased completely even though allergic symptoms are not completely blocked.

From these findings, the potentiating effect of the simultaneous use of levocabastine and pemirolast may be attributable to the antihistaminic activity of levocabastine and histamine release inhibition by both drugs.

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