

Full Paper

The Combined Effect of Topical CX-659S, a Novel Diaminouracil Derivative, With Topical Corticosteroid on the Three Types of Allergic Responses in Mice or Guinea Pigs

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Abstract. CX-659S ((S)-6-amino-5-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)-3-methyl-1-phenyl-2,4(1*H*,3*H*)-pyrimidinedione), a newly discovered anti-inflammatory compound, exerts inhibitory effects against picryl chloride-, oxazolone-, and dinitrochlorobenzene-induced acute contact hypersensitivity responses (CHRs) characterized by Th1-type reactions. Furthermore, this compound suppressed chronic CHRs characterized by Th2-type reactions, which is well known to mimic many, if not all, events occurring within the lesional skin of patients with atopic dermatitis (AD). The present study was conducted to determine the combined effect of topical CX-659S with topical corticosteroid on immediate type (ITR), late type (LTR), and delayed type hypersensitivity (DTHR) allergic reactions that are involved in AD. An ineffective dose of CX-659S (0.03 mg/ear) combined with betamethasone valerate (BV) significantly potentiated inhibitory activity of BV alone (0.1 μ g/ear and 0.3 μ g/ear) on both the ITR and the LTR in mice with the ovalbumin (OVA)-induced biphasic cutaneous reaction. Furthermore, the combined effect of CX-659S with BV was also observed on dinitrochlorobenzene (DNCB)-induced DTHR in guinea pigs. These results indicate that CX-659S has a combined effect with corticosteroids on every ITR, LTR, and DTHR. Proper treatment with corticosteroids for a safe and effective treatment of AD is needed. Thus, the combination therapy of topical CX-659S with topical corticosteroid would be one of the potential approaches for devising a proper treatment with corticosteroids.

Keywords: CX-659S, corticosteroid, combined effect, Th2-type reaction, Th1-type reaction

Introduction

Atopic dermatitis (AD) is a common and distinctive form of allergic skin disease associated with severe eczema, intense pruritus and enhanced IgE production (1, 2). Diagnosis is based on physical examination of the skin condition and family and patient history of other manifestations of atopy. The onset and development of allergic diseases are considered to be caused in individuals with some genetic factors under the influence of environmental factors (3). Topical corticosteroids have been widely utilized for the past

40 years in AD, and their efficacy and safety have been studied extensively. However, their chronic use may be associated with significant adverse effects at the application site. Skin atrophy and other undesirable effects are frequently seen after long-term corticosteroid treatment. These cause anxiety for both patients and clinicians and are the main reason for patients' poor compliance with treatment (4). These drawbacks, in addition, have generated a search for other effective modalities of therapy. The search for such alternative treatments has included chemical alteration of the structure of corticosteroids with the aim of reducing their adverse effects without a loss of potency (5), simultaneous topical application of retinoic acid to diminish steroid-induced skin atrophy (6), and the investigation of cytokine antagonists and inhibitors of inflammatory

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infiltrates (7, 8). To achieve prolonged remission of AD, many dermatologists use potent topical corticosteroids in short bursts followed by a break period with a bland emollient (9). Others advocate a mild preparation, such as 1% hydrocortisone as required, to avoid local adverse effects such as skin atrophy. In addition, recent studies demonstrated additive effects of antibacterial (10) or anti-oxidative agent (11) with topical corticosteroid for patients with AD. Many dermatologists have been making much effort to devise proper treatment with corticosteroids to achieve prolonged remission of AD.

We have recently described the inhibitory efficacy of a novel diaminouracil derivative CX-659S ((S)-6-amino-5-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)-3-methyl-1-phenyl-2,4(1*H*,3*H*)-pyrimidinedione) against picryl chloride (PC)- or oxazolone (OX)-induced acute contact hypersensitivity responses (CHR) in mice when administrated topically. The inhibitory activities of this compound were also confirmed in guinea pigs with dinitrochlorobenzene (DNCB)-induced acute CHR (12). In addition, this compound profoundly reduced the inflammation responsible for the ear swelling reaction and tissue damages comparable to the intact sample in the murine model of chronic CHR without any other adverse effect such as atrophy, alopecia or telangiectasis observed in mice treated with prednisolone (13). This mouse model appears to mimic many, if not all, events occurring within the lesional skin of patients with AD (14–16). On the other hand, this compound has anti-oxidative activity toward two reactive oxygen species (hydroxyl radical and peroxynitrite) and toward lipid peroxidation (17) as well as inhibitory activity against pro-inflammatory cytokines and Th-2 type cytokines mRNA expression (12, 13).

In this study, to evaluate the usefulness of combined therapy between topical CX-659S and topical corticosteroid against AD, we performed studies to clarify the possibility of an additive effect or synergism between CX-659S and corticosteroid betamethasone valerate (BV) against the ovalbumin (OVA)-induced biphasic cutaneous reaction in mice, which shows both the immediate type (ITR) (IgE-dependent, mast cell-mediated) and late type (LTR) (IgE-dependent, Th2-mediated) allergic reactions. We also determined the combined effect of CX-659S with BV against delayed type hypersensitivity (DTHR) (IgE-independent, Th1-mediated) allergic reaction observed in the DNCB-induced acute contact hypersensitivity reaction in guinea pigs.

Materials and Methods

Animals

Male 5-week-old ICR mice were purchased from Clea

Japan, Inc. (Tokyo), and 5-week-old male Hartley guinea pigs, from Japan SLC, Inc. (Hamamatsu); they were each used at the age of 6 weeks. The animals were maintained in a room with a 12-h light / 12-h dark cycle, and the room temperature and humidity were controlled at $23 \pm 2^\circ\text{C}$ and $55 \pm 5\%$, respectively. The animals were provided food and tap water ad libitum. The study protocol was approved by the Japan Energy Corporation Animal Care and Use Committee.

Reagents

CX-659S was synthesized by the method described in the previous report (18). The following reagents were obtained from commercial sources: DNCB (Tokyo Kasei Kogyo Co., Ltd., Tokyo); chicken egg OVA and BV (Sigma, St. Louis, MO, USA). DNCB and test compounds were dissolved in acetone.

OVA-induced biphasic cutaneous reaction in mice

Five or six mice in a group were immunized by i.p. injection of 1 μg of OVA and 1 mg aluminum hydroxide gel (alum) to induce production of IgE antibody. Fourteen days later, mice were challenged with intradermal (i.d.) injection of 20 μL of saline containing 10 μg of OVA to the left ear under slight anesthesia with pentobarbital-Na. Control mice were also injected with saline alone in a volume of 20 μL to analyze the influence of intradermal injection. Forty microliters of 0.075%, 0.25%, 0.75%, or 2.5% CX-659S solution or vehicle (acetone) was applied to the left ear just after the challenge for its dose-dependency study. Also, 40 μL of 0.00025%, 0.00075%, 0.0025%, or 0.0075% BV solution or vehicle was applied to the left ear 5 min after the challenge for its dose-dependency study. In addition, seven randomized groups were designed for determination of the combined effect of CX-659S with BV as described in Table 1. Forty microliters of 0.075% CX-659S solution or vehicle (acetone) was applied to the left ear just after the challenge, and was followed by applying with 40 μL of 0.00025%, 0.00075% BV solution or vehicle to the left ear 5 min after the challenge according to the protocol as described Table 1. The ear thickness was measured by using an engineer's dial thickness gage (Mitsutoyo Corp., Tokyo), before, 1 and 24 h after antigen challenge. Ear swelling was expressed as the increment of thickness between before and after the challenge.

DNCB-induced acute CHR in guinea pigs

Eleven guinea pigs were sensitized by applying 10 μL of 39% DNCB (w/v) solution in acetone to the atrichous area of the root of each ear. Fourteen days later, animals were challenged by applying 10 μL of 0.2% DNCB

Table 1. Design of the study to confirm combined effect of CX-659S with BV on OVA-induced biphasic cutaneous reaction

Immunization	Challenge	N	Treatment	
			Just after the challenge	5 min after the challenge
+	–	6	acetone	acetone
+	+	6	acetone	acetone
+	+	6	CX-659S 0.03 mg/ear	acetone
+	+	6	acetone	BV 0.1 μ g/ear
+	+	6	acetone	BV 0.3 μ g/ear
+	+	6	CX-659S 0.03 mg/ear	BV 0.1 μ g/ear
+	+	6	CX-659S 0.03 mg/ear	BV 0.3 μ g/ear

Table 2. Design of the study to confirm combined effect of CX-659S with BV on DNCB-induced delayed type hypersensitivity reaction

Immunization	Challenge	N	Treatment	
			5 min after the challenge	10 min after the challenge
+	+	11	acetone	acetone
+	+	11	CX-659S 0.1 mg/ear	acetone
+	+	11	acetone	BV 10 μ g/ear
+	+	11	acetone	BV 5 μ g/ear
+	+	11	CX-659S 0.1 mg/ear	BV 10 μ g/ear
+	+	11	CX-659S 0.1 mg/ear	BV 5 μ g/ear

solution in acetone: olive oil (4:1) to the shaved backs. Six sites per animal at intervals of about 2.5 cm were marked on the shaved backs of the animals. Forty microliters of 0.075%, 0.25%, 0.75%, or 2.5% CX-659S solution or vehicle (acetone) was applied to the site 5 min after the challenge for its dose-dependency study. Also, 40 μ L of 0.0075%, 0.025%, 0.075%, or 0.25% BV solution or vehicle was applied to the site 10 min after the site for its dose-dependency study. In addition, 20 μ L of 0.5% CX-659S solution or vehicle was applied to the site 5 min after the challenge and 20 μ L of 0.025%, or 0.05% BV solution or vehicle was applied to the site 10 min after the challenge according to the procedure shown in Table 2. The positions of each test site were randomized in each animal. Sites were examined for erythema and edema at 24 h after the challenge. Erythema and edema were graded on a scale of 0 to 5, where a score of 0 indicated no reaction, a score of 2 indicated a pale pink color and no edema, a score of 3 indicated a pale pink color and edema, a score of 4 indicated a red color and edema, and a score of 5 represented a bright red color and edema.

Data analyses and statistics

The data were presented as the mean $\pm$ S.E.M. of 5 or

6 animals. Statistical significance between the vehicle (acetone) group and CX-659S-treated group or BV-treated group was tested with one-way analysis of variance (ANOVA), and *P* values were corrected by Dunnett's test for multiple comparison. Student's *t*-test was employed for the evaluation of significance between the BV-treated group and CX-659S combined BV-treated group. A *P* value less than 0.05 was considered significant.

Results

Inhibitory effects of CX-659S and BV on OVA-induced biphasic cutaneous reaction

In the first series of experiments, we examined the effect of topical CX-659S and BV against the ITR and the LTR on the OVA-induced biphasic cutaneous reaction. Mice that were immunized 14 days prior to challenge, by i.p. injection with OVA, developed a biphasic reaction that peaked at 1 and 24 h after antigen-challenge (data not shown). As shown in the Figs. 1 and 2, intradermal injection of saline in a volume of 20 μ L/ear to OVA-immunized mice induced the increment of ear thickness to 0.127 ± 0.028 (mean $\pm$ S.D.) mm or 0.021 ± 0.030 mm at 1 or 24 h after the injection, respec-

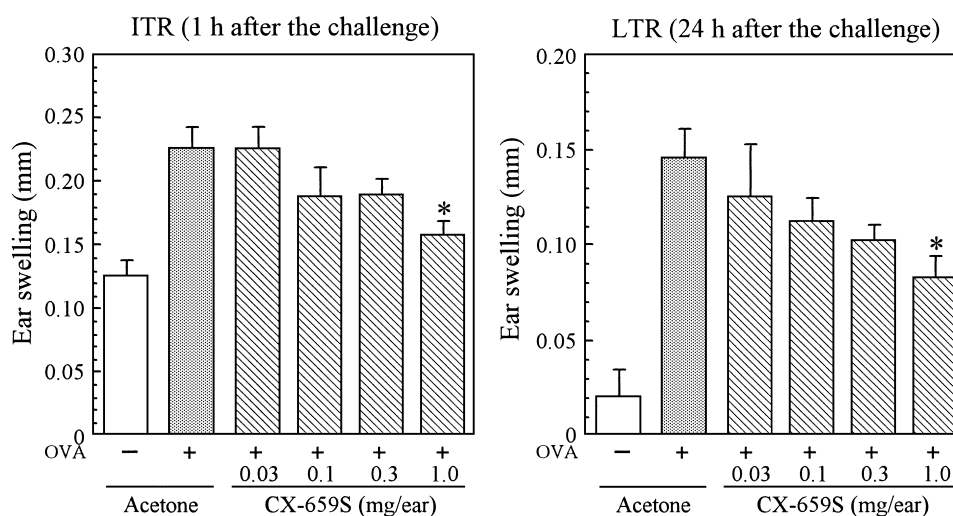


Fig. 1. Effect of CX-659S on OVA-induced biphasic cutaneous reaction in mice. The efficacy of CX-659S was assessed according to the protocol described in Materials and Methods. The results are expressed as the mean $\pm$ S.E.M. of 5 animals. * $P < 0.05$, when compared with the vehicle (acetone)-treated group (Dunnett's test).

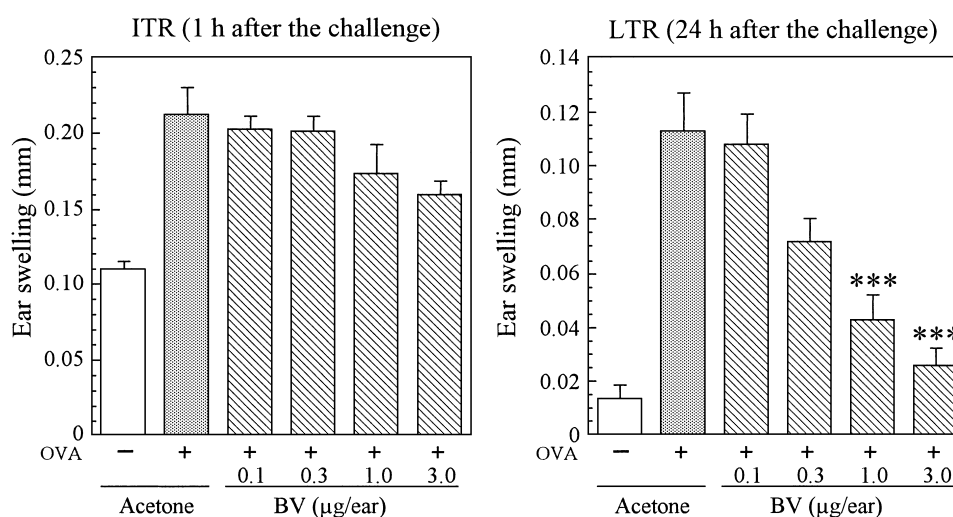


Fig. 2. Effect of BV on OVA-induced biphasic cutaneous reaction in mice. The efficacy of CX-659S was assessed according to the protocol described in Materials and Methods. The results are expressed as the mean $\pm$ S.E.M. of 6 animals. *** $P < 0.001$, when compared with the vehicle (acetone)-treated group (Dunnett's test).

tively; while, the ear thickness of OVA-immunized mice challenged with OVA greatly increased. Thus, the difference of ear thickness between antigen-challenged and saline-injected mice immunized with OVA reflected the allergic reactions. CX-659S showed inhibitory activities on both the ITR and the LTR in a dose-dependent manner (Fig. 1). The inhibitory potency of CX-659S on the ITR was almost the same level compared to that on the LTR. In contrast, BV showed a weak inhibition on the ITR in spite of having a strong inhibitory activity on the LTR (Fig. 2).

Combined effect of CX-659S with BV on OVA-induced biphasic cutaneous reaction

To clarify the possibility of combination therapy between topical CX-659S and topical corticosteroid, we determined the combined effect of CX-659S with BV on the Th2 dominant allergic reactions, the ITR and the LTR. As shown in the Fig. 3, a small amount of CX-659S, which had no inhibition on both the ITR and the LTR, significantly potentiated inhibitory activity of BV alone (0.1 μ g/ear and 0.3 μ g/ear) on both the ITR and the LTR (Fig. 3). Especially, a combination with a small amount of CX-659S (0.03 mg/ear) significantly

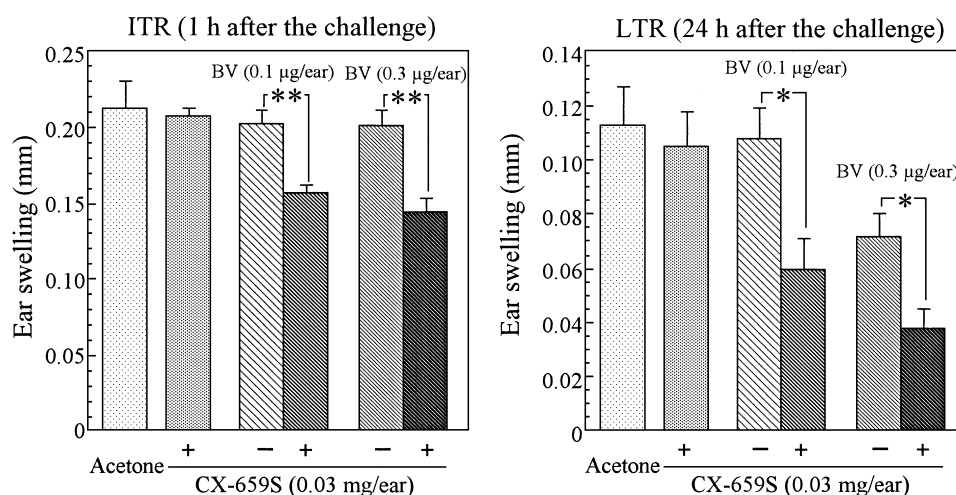


Fig. 3. Combined effect of CX-659S with BV on OVA-induced biphasic cutaneous reaction in mice. Combined effect of CX-659S with BV was assessed according to the protocol described in Materials and Methods. The results are expressed as the mean $\pm$ S.E.M. of 6 animals. * P <0.05 and ** P <0.01, when compared between the BV alone group and the combined CX-659S with BV group (Student's t -test).

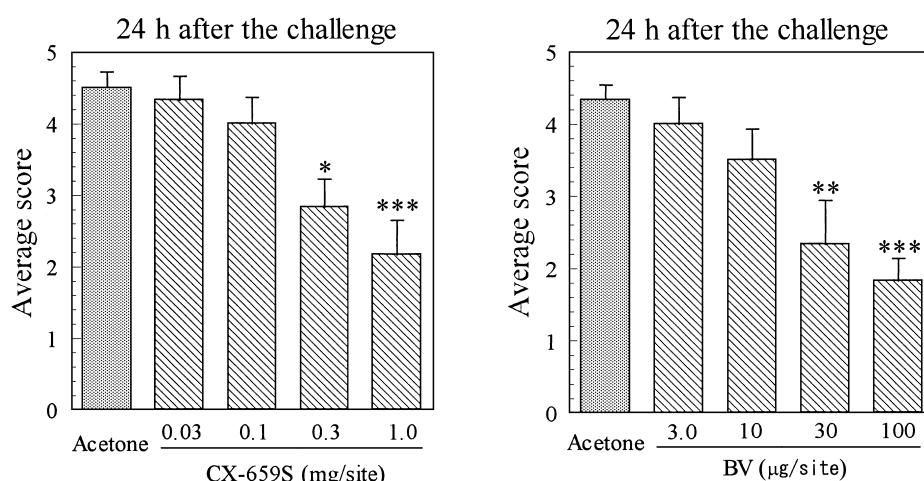


Fig. 4. Effect of CX-659S and BV on DNCB-induced delayed type hypersensitivity reaction in guinea pigs. The efficacies of CX-659S and BV were assessed according to the protocol described in Materials and Methods. The results are expressed as the mean $\pm$ S.E.M. of 11 animals. * P <0.05, ** P <0.01, and *** P <0.001 when compared with the vehicle-treated group (Dunnett's t -test).

potentiated the inhibitory activity of BV on the ITR to more than that of a thirty times higher dose of BV.

Inhibitory effects of CX-659S and BV on DNCB-induced DTHR

We examined the effect of topical CX-659S and BV on the DNCB-induced DTHR, which is a Th1-dominant allergic reaction. As shown in the Fig. 4, CX-659S and BV dose-dependently suppressed erythema and edema at 24 h after antigen-challenge. The inhibitory potency of CX-659S was almost ten times weaker than that of BV.

Combined effect of CX-659S with BV on DNCB-induced DTHR

We further determined the combined effect of CX-659S with BV on the DTHR. Combination with an ineffective dose of CX-659S (0.1 mg/site) significantly enhanced inhibitory activity compared to that of BV alone on the DNCB-induced DTHR (Figs. 5 and 6).

Discussion

Although the precise mechanism underlying AD has

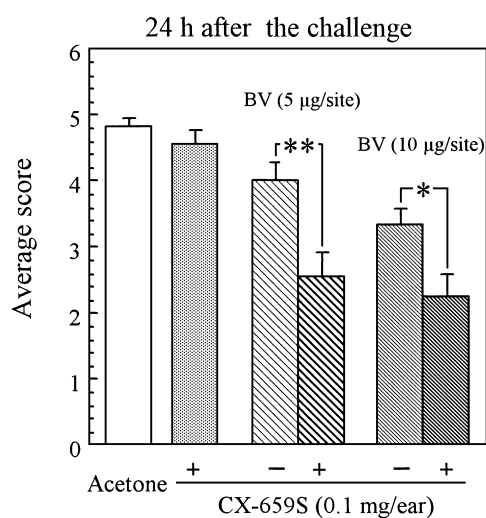


Fig. 5. Combined effect of CX-659S with BV on DNCB-induced delayed type hypersensitivity reaction in guinea pigs. Combined effect of CX-659S with BV was assessed according to the protocol described in Materials and Methods. The results are expressed as the mean $\pm$ S.E.M. of 11 animals. * P <0.05 and ** P <0.01, when compared between the BV alone group and the combined CX-659S with BV group (Student's *t*-test).

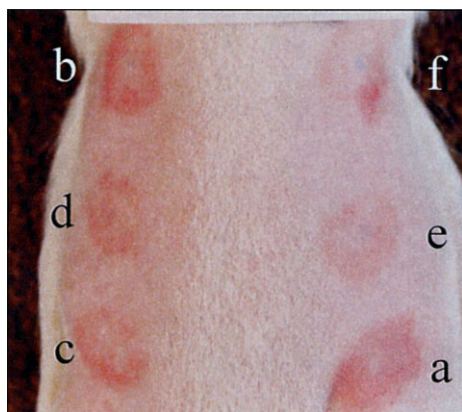


Fig. 6. Representative appearance of the shaved back 24 h after the challenge in a guinea pig. Severe erythema was observed in the site treated with acetone alone (a). A small amount of CX-659S (0.1 mg/site) (b) was ineffective, and BV (5 µg/site) (c) or BV (10 µg/site) (d) showed a weak suppression. Combination between CX-659S (0.1 mg/site) and BV (5 µg/site) (e) or BV (10 µg/site) (f) was more effective against the erythema than BV alone.

remained unclear, it has been proposed that at least three types of allergic reactions, ITR (IgE-dependent, mast cell-mediated), LTR (IgE-dependent, Th2-mediated), and DTHR (IgE-independent Th1-mediated), are involved in AD (19–25): Grewe's and Thepen's groups have suggested that there is a biphasic pattern in AD, starting with a Th2-type allergen-specific reaction, followed by a Th1-type allergic reaction, and that the Th2-type is important in the induction of inflammation,

whereas the Th1-type is responsible for the maintenance and aggravation of the inflammation representing the chronic phase of AD (21, 24). Thus, the drugs that can suppress these three types of allergic reactions would be needed for safe and effective treatment of AD.

The ITR is primarily caused by IgE-mediated activation of mast cells/basophils to release chemical mediators; and it is often followed after several hours by a delayed and sustained local inflammation, termed the LTR, which is characterized by the local accumulation of activated inflammatory cells including eosinophils, monocytes and T lymphocytes (26). According to some investigators, LTR is primarily an IgE- and mast-cell-dependent reaction (27, 28), whereas according to others, LTR mainly corresponds to a CD4 T cell-mediated inflammatory response (29, 30). Interestingly, CD4 T cells, particularly Th2 cells, and mast cells have been shown to produce common cytokines, so-called Th2 cytokines including IL-4 and IL-5, in vitro and in vivo (31, 32). These cytokines have been reported to be involved in eosinophil recruitment, suggesting that either CD4 T cells, mast cells, or both play a role in development of the antigen-induced LTR. The OVA-induced biphasic cutaneous reaction is well documented to show both the ITR and the LTR (33, 34).

Although BV inhibited both the ITR and the LTR, its inhibitory potency on the ITR was very weak compared with that on the LTR (Fig. 2), while it was described that an immunosuppressant such as FK-506 had less-potency on the ITR in spite of having inhibitory activity on the LTR (35). Contrary to the effects of these two compounds, CX-659S showed almost the same inhibitory potencies on both the ITR and the LTR in a dose-dependent manner (Fig. 1).

On the other hand, T cell-mediated DTHR, seen in the DNCB-induced acute contact hypersensitivity response in guinea pigs, are Th1-dominant allergic reactions, and they are also thought to be involved in AD. We previously reported that topical CX-659S has an inhibitory activity against typical DTHR; i.e., PC- or OX-induced acute CHR in mice. The inhibitory activities of this compound were also confirmed in guinea pigs with DNCB-induced acute CHR (12). Thus, these unique activities of CX-659S (i.e., this compound exerts inhibitory activity against every ITR, LTR, and DTHR) makes it desirable as a therapeutic agent for AD.

Although topical corticosteroids remain one of the most efficient treatments available for AD, there is an urgent requirement for proper treatment of these medications because of concern about their potential adverse effects. Combination therapy of topical corticosteroids with an antibacterial or anti-oxidative agent would be one of the potential approaches to devise a proper

treatment with corticosteroids. In the present study, we demonstrated the synergism of topical CX-659S with topical BV on both the OVA-induced biphasic cutaneous reaction in mice and the DNCB-induced delayed type hypersensitivity reaction in guinea pigs. These data indicate that CX-659S had a synergism with BV on every ITR, LTR and DTHR, which thought to be involved in AD. We have recently described that CX-659S inhibits chronic CHR with suppression of serum IgE production under both protective and curative conditions (13). These data suggest that CX-659S is a very useful agent for devising an appropriate corticosteroid therapy against recurring AD.

CX-659S has potent scavenging activity against hydroxyl radicals and peroxynitrite as well as other various pharmacological properties (17). Although both the hydroxyl radical and peroxynitrite play an important role in host defense against infection by virtue of their potent oxidizing ability, they are also responsible for the cellular and tissue damage in inflammation and other various pathological processes (36). The reactive oxygen species may also contribute to cutaneous inflammatory diseases such as psoriasis (37, 38), AD (39), and contact dermatitis (40). Therefore, the anti-oxidative activity of CX-659S, at least partly, accounts for the mechanism of its anti-inflammatory/antiallergic properties. On the other hand, corticosteroids inhibit the generation of the adhesion molecules and the induction of some enzymes responsible for the generation of inflammatory mediators, and they prevent immune and inflammatory cell activation (41–44). Thus corticosteroids collectively exhibit potent anti-inflammatory, antiallergic and immunosuppressive actions. Therefore, synergism of CX-659S and BV on the ITR, LTR, and DTHR shown in this study may be reflected in the difference in their anti-inflammatory/antiallergic mechanism.

As far as the toxicological aspects are concerned, chronic daily treatment of up to 28 days with topical CX-659S produced no symptoms of adverse effects such as atrophic scars, alopecia and telangiectasis, which are observed in chronic treatment with corticosteroid in mice with chronic CHR (13), and the results on several cutaneous toxicity tests such as the skin sensitization test and skin photosensitization test were negative (data not shown). These results suggest that CX-659S will have low toxicity.

Taken together, the present data indicate that combined therapy of topical CX-659S with a topical corticosteroid would be a potential approach to devise an appropriate corticosteroid therapy for patients with recurring AD who require a long-term therapeutic strategy.

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