

Effect of the smell of Seirogan, a wood creosote, on dermal and intestinal mucosal immunity and allergic inflammation

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Seirogan, a wood creosote, has been used as an antidiarrhetic drug in Asian countries including Japan for many years. This antidiarrhetic has recently been used as a sugar-coated pill because Seirogan has a strong smell. The strong smell of the uncoated form of Seirogan may modulate the defense systems of animals because the sense of smell is important for the detection of toxic metabolites in foods contaminated with pathogens. This study examined the effect of the sugar-coated and uncoated forms of this antidiarrhetic on the immunological response and inflammatory reactions in mice that had been sensitized with either fluorescein isothiocyanate or oxazolone. The sensitization of mice with either FITC or oxazolone markedly increased the plasma levels of tumor necrosis factor- α and mucosal IgA and elicited severe inflammation in the colon by a mechanism that could be suppressed by exposure of animals to the smell of uncoated Seirogan as effectively as the oral administration of the agent. Dermal inflammation in the FITC- and oxazolone-sensitized mice was also suppressed effectively either by the exposure to the smell or oral administration of the agent. Biochemical and histochemical analyses revealed that the elevated levels of plasma tumor necrosis factor- α and mucosal IgA were significantly decreased by exposure to the smell of uncoated Seirogan as well as by oral administration of the agent. Exposure of mice to the smell of Seirogan but not oral administration of the agent selectively increased plasma levels of adrenocorticotropic hormone and cortisol, particularly in the sensitized animals. These observations suggest that exposing the animals to the smell of Seirogan *per se* activated the hypothalamo-pituitary-adrenal axis and systemically modulated immunological reactions to suppress the allergic reactions.

Key Words: wood creosote, TNF- α , FITC, oxazolone, inflammatory bowel disease

Wood creosote (Seirogan) has long been used in Asia to treat both acute and chronic digestive disorders associated with diarrhea. Seirogan is extracted by distillation from the beechwood tree and is a mixture of simple phenolic compounds, including guaiacol, creosol and related compounds, and is chemically distinct from, and should not be confused with, coal tar creosote. In addition, the binding medicine that wood creosote is a main ingredient is not seen in Southeast Asia and China.

Seirogan appears to possess antimotility activity in both the small intestine and colon. Studies in small animals have shown that Seirogan inhibits spontaneous and electrically stimulated contractility in isolated segments of guinea pig small intestine,

and rat ileum in smooth muscle baths.⁽¹⁻³⁾ Seirogan also retards the expulsion of a rectal bead in the rat, suggesting the inhibition of propulsive patterns of colonic motility.⁽⁴⁾

Kuge *et al.*⁽⁵⁾ revealed that administration of Seirogan by oral gavage inhibited stress-induced secretion and normalized basal short-circuit current in the jejunum and colon. In addition, peptide hormones such as corticotrophin-releasing factor participate in these suppressive effects.⁽⁶⁾ This suggests that Seirogan may modulate the mucosal neuro-immune network. The effect of Seirogan on the type IV allergic reactions in the skin and intestine was investigated to test this hypothesis. In addition, the suppressive effect of exposure to the smell was examined because Seirogan has unique smell.

Materials and Methods

Animals. Specific pathogen-free male ICR mice were purchased from SLC (Hamamatsu, Japan). The mice were 8 weeks old at the beginning of each experiment and were housed in filter-protected cages. Ambient light was controlled automatically to produce a 12-h light/12-h dark cycle, and sterile water was provided *ad libitum*. The animals were subjected to experiments according to the animal care regulations of Osaka City University Medical School.

Chemicals. 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) was obtained from Sigma-Aldrich Chemical (Milwaukee, WI), fluorescein isothiocyanate (FITC) isomer-I was supplied by Dojindo (Kumamoto, Japan). Oxazolone solution was dissolved in 1:1 v/v ethanol:distilled water and FITC solution was dissolved in 1:1 v/v acetone:dibutyl phthalate. Solutions were prepared freshly immediately prior to dosing.

Contact sensitization. The skin on the back of each mouse was shaved with electric clippers and used as a sensitizing area. The mice were sensitized by applying 50 μ l of 0.5% FITC or 0.5% oxazolone solution. The mice were challenged for 5 days after sensitization by applying the FITC or oxazolone solution (4 μ l) to both the dorsal and ventral surfaces of the right ear. Ear thickness was measured with a micrometer, and contact hypersensitivity (CHS) was measured as the difference between the ear thickness measured before challenge and that measured 24 h after challenge. Either FITC or oxazolone solution was administered to the colon through the rectum via a 3.5 F catheter equipped with a 1 ml syringe. The catheter was inserted so that the tip was 4 cm proximal

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to the anal verge and the oxazolone or FITC was injected with a total volume of 100 μ l. The mice were held in a vertical position for 30 s after the injection to ensure distribution of the oxazolone or FITC within the entire colon. The colon was removed 24 h after challenge and the morphological and immunological changes were observed.

Seirogan treatment. Sugar-coated and uncoated Seirogan were supplied by Taiko Pharmaceutical Co. Ltd. (Osaka, Japan). Seirogan is coated with white soft sugar (sucrose) which prevents any smell from being noticed during oral ingestion. One group received 30 mg/day of coated Seirogan in solution orally throughout the experiment period. Only distilled water was administered orally to the control animals. The other group was exposed to the uncoated Seirogan for 2 h per day throughout the experimental period. The uncoated Seirogan smell was diluted with ultrapure water to 0.03% (v/w). This concentration of uncoated Seirogan smell has been shown to be effective in reducing the increase in the plasma concentration of adrenocorticotropic hormone (ACTH) induced by acute immobilization stress (data not shown).

Quantification of cytokines by enzyme-linked immunosorbent assay (ELISA). Blood samples were taken from the heart 5 h after sensitization, and then the plasma was fractionated. Skin samples were removed 1 day after the challenge and immediately frozen in liquid nitrogen and stored at -80°C . For extraction, the skin samples were homogenized, then the supernatant after centrifugation was used for the analysis. The plasma tumor necrosis factor- α (TNF- α), cortisol, and ACTH concentration and the colonic level of IgA were determined using a commercial ELISA kit (TNF- α , Pierce Biotechnology, IL; ACTH, Phenix Pharmaceuticals Inc., CA; Cortisol, Oxford Biochemical Research Inc., MI; IgA, BETHYL Laboratories Inc., TX) according to the manufacturer's instructions.

Histological analysis. The colon specimens were fixed in phosphate-buffered paraformaldehyde (4%), embedded in frozen Tissue Tek, OCT compound, and cut into 5 μ m thick sections. Thin sections were stained with hematoxylin-eosin and analyzed histologically to evaluate the degree of colon inflammation caused by the FITC and oxazolone solution. Other thin sections, were washed with phosphate-buffered saline (PBS), and then were incubated with goat anti-mouse IgA (1:100) polyclonal antibody (ZYMED, San Francisco, CA) overnight at 4°C . The sections were thereafter washed in PBS, and incubated at room temperature for 2 h with FITC-conjugated anti-goat immunoglobulin made from rabbit (1:30; Dako Cytomation, Denmark). The expression of IgA was evaluated immunohistochemically under fluorescence microscopy.

Statistical analysis. All data were expressed as the mean \pm SD. The results obtained from the three animal groups were analyzed by either Student's *t* test or an ANOVA using a computer software. Differences were considered to be significant when $p < 0.05$.

Results

Effect of Seirogan on systemic suppression of CHS ear thickness by FITC and oxazolone. CHS induced by FITC or oxazolone was suppressed by 55% and 68%, respectively, following oral administration of coated Seirogan. The group that smelled uncoated Seirogan, showed a greater suppression of CHS by FITC in comparison to the oral administration group (Fig. 1).

Seirogan reduced TNF- α , cortisol and ACTH production induced by FITC or oxazolone. The plasma level of TNF- α , cortisol and ACTH increased with either FITC or oxazolone sensitization (Fig. 2). The oral administration of coated Seirogan after FITC or oxazolone sensitization decreased the cytokine and hormone levels in this study in comparison to the levels in the mice not treated with Seirogan. In addition, uncoated Seirogan smell

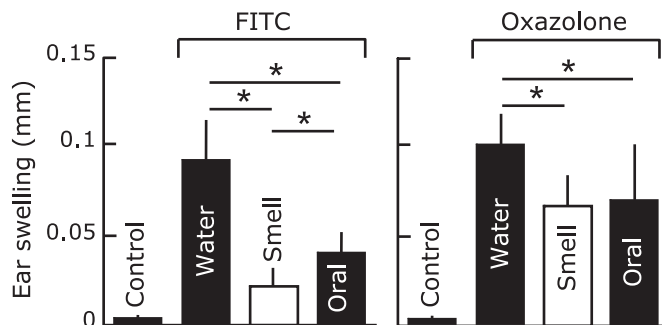


Fig. 1. Effects of Seirogan treatment on FITC- or oxazolone-induced CHS. CHS is expressed as the amount of ear swelling 24 h after FITC or oxazolone challenge. The data are presented as the mean \pm SD from ten animals. * $p < 0.05$.

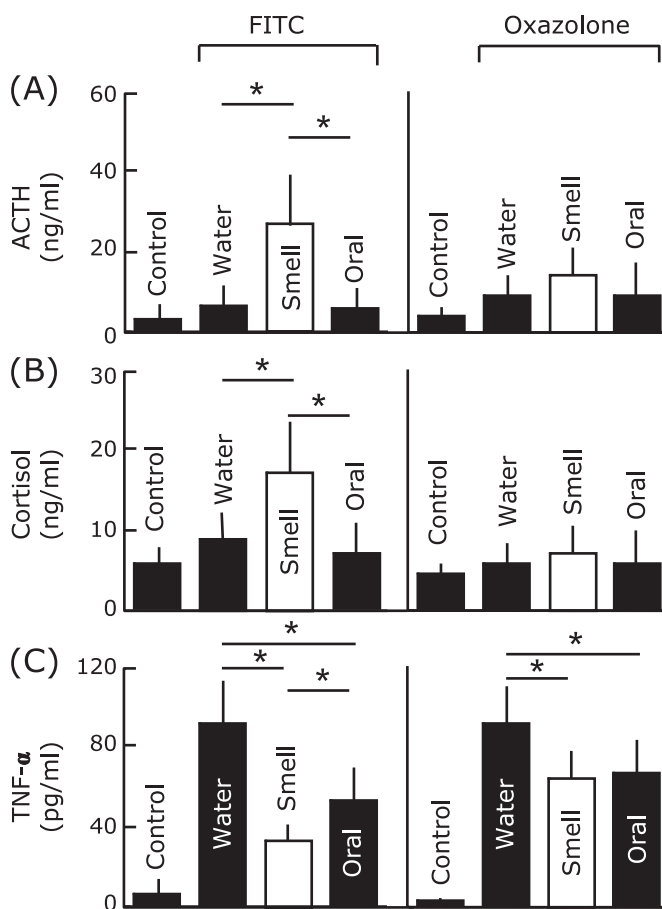


Fig. 2. Effects of Seirogan treatment on the plasma concentration of TNF- α (A), ACTH (B), or cortisol (C). The plasma concentration of each mediator was determined 24 h after challenge with FITC or oxazolone challenge. The data are presented as the mean \pm SD from ten animals. * $p < 0.05$.

treatment after FITC sensitization decreased the TNF- α , cortisol and ACTH levels in comparison to the levels in the coated Seirogan oral administration group.

Morphological and histopathological changes in Seirogan treated mice. Biopsy specimens were taken from the regions of positive colon reactions. The morphological observations are shown in Fig. 3. There was remarkable edema and congestion due

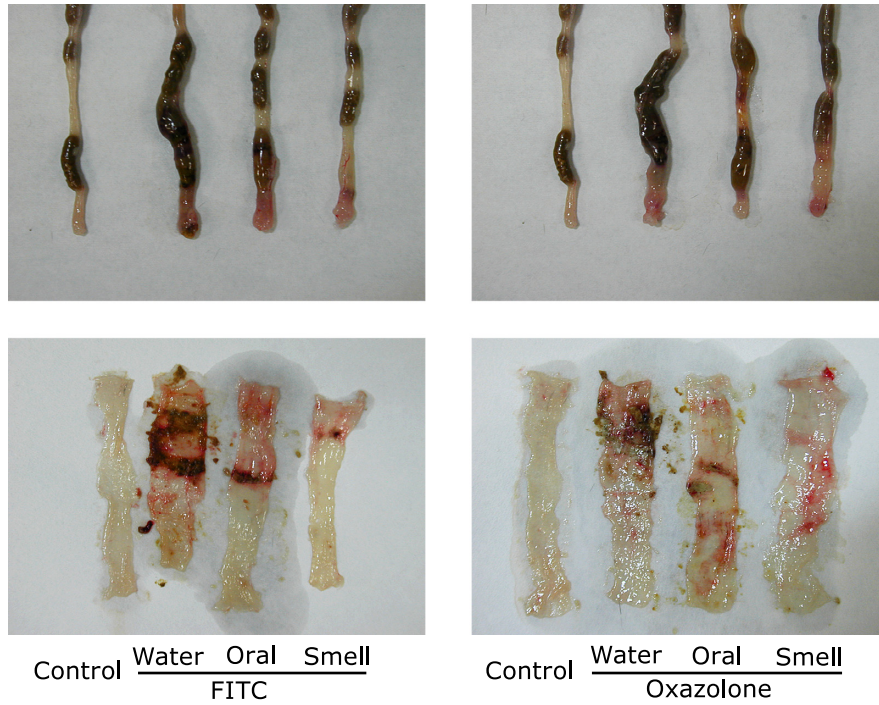


Fig. 3. Effects of Seirogan treatment on FITC- or oxazolone-induced CHS causing colon edema 24 h after FITC or oxazolone challenge. The data are from one typical experiment involving ten animals.

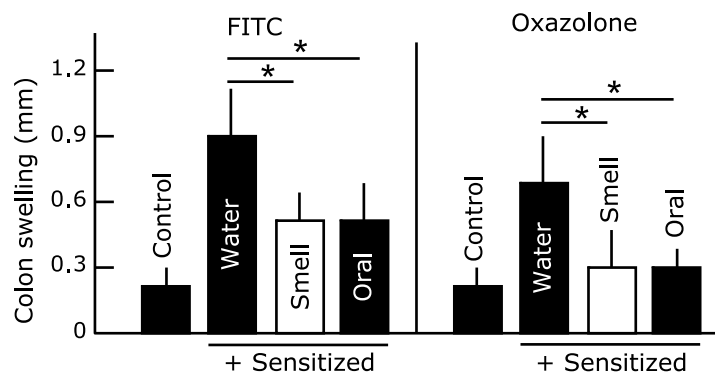
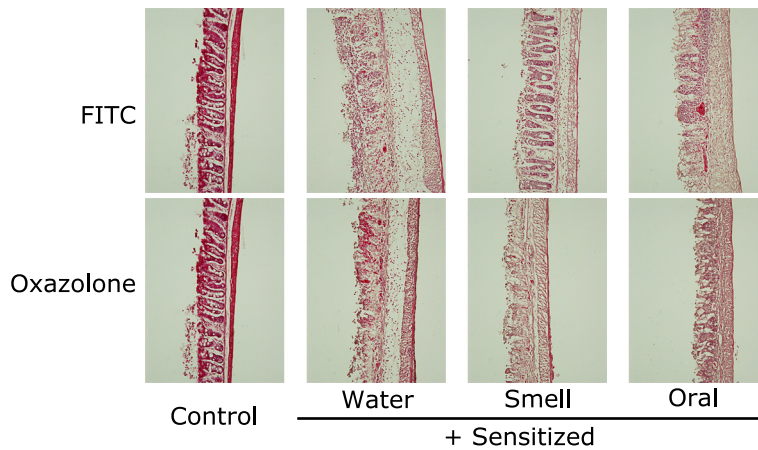


Fig. 4. Effects of Seirogan treatment on FITC- or oxazolone-induced CHS. The mice were killed 24 h after FITC or oxazolone challenge and colon specimens were frozen, cut into thin sections, and then were stained with HE. The data show one typical experiment involving ten animals. Magnification, 100 \times . The data are presented as the means \pm SD from ten animals. * $p < 0.05$.

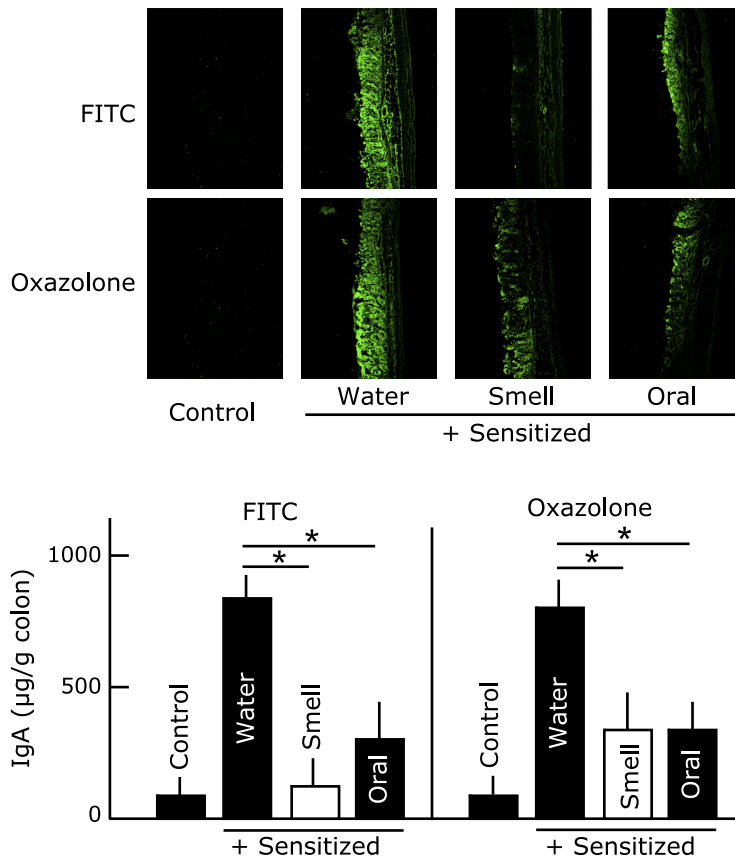


Fig. 5. Effects of Seirogan treatment on FITC- or oxazolone-induced CHS. The mice were killed 24 h after the FITC or oxazolone challenge, and colon specimens were frozen, cut into thin sections, treated with anti-IgA antibody and then stained with FITC-conjugated second antibody. The data show one typical experiment involving ten animals. Magnification, 200 \times . The data are presented as the means \pm SD from ten animals. * p <0.05.

to CHS by FITC or oxazolone. Microscopic observation of colon samples stained with hematoxylin and eosin revealed infiltration of large numbers of lymphocytes and neutrophils in response to CHS by FITC or oxazolone (Fig. 4). The infiltration of smaller numbers of lymphocytes and neutrophils was observed in the colons from the Seirogan treated mice.

Immunohistological changes in the Seirogan treated mice. The expression of IgA was observed in the colon from Seirogan treated animals. The colonic expression of IgA markedly increased after either the FITC or oxazolone challenges. Fig. 5 shows that the expression of IgA in the CHS groups decreased in the Seirogan treated mice. In addition, there was little expression in the animals that smelled uncoated Seirogan in comparison to those that received the oral administration of coated Seirogan.

In addition, all the results (Fig. 1–5) of the sham-stressed mice were equal to those of the control mice.

Discussion

Delayed-type allergic reactions, such as type IV allergies, are often accompanied by serious inflammation. The present study found that both FITC and oxazolone successfully increased ear and colon thickness at 24 h after challenge. Furthermore, histopathological observations of the ear and the colon sections revealed that edema and infiltration of inflammatory cells occurred simultaneously in these mice.

This study reported that the overproduction of TNF- α is suppressed by administration of Seirogan. TNF- α is a pleiotropic cytokine, originally identified by its anti-tumor activity,⁽⁷⁾ and is

believed to play a role in many immunological and inflammatory reactions.^(8,9) In addition, blocking TNF- α inhibits allergic skin dermatitis. For example, treatment with an anti-TNF- α antibody can inhibit CHS,⁽¹⁰⁾ arthus reaction⁽¹¹⁾ and type I allergies.⁽¹²⁾ Mediators, such as TNF- α , are induced by acetylcholine, initiate and amplify inflammatory response.^(12,13) Seirogan also inhibits spontaneous longitudinal contractions was induced by acetylcholine.^(3,14) It is therefore possible that oral administration of Seirogan can thus improve the inflammatory and allergic conditions induced by FITC or oxazolone.

The current results demonstrated that allergic inflammation was completely suppressed by inhalation of the smell of Seirogan. This suggested that smell stimulation acted through the hypothalamic pituitary adrenal axis (HPA axis) because the plasma level of the ACTH and cortisol increased. The cortisol increases noninflammatory cytokines such as IL-10 or IL-4 and represses inflammatory cytokines such as TNF- α or interferon gamma (IFN- γ).^(15,16) Furthermore, the smell of Seirogan affects the autonomic nervous system because it activates the HPA axis (stress axis). As a result, the acetylcholine level in plasma decreases,^(17,18) and a reduction of TNF- α is induced,⁽¹⁹⁾ because the parasympathetic system is controlled. The two pathways that use this stress axis may act as the mechanism of the allergic restraint by the smell of Seirogan. Furthermore, when rats were burdened with stress, the excretion of feces aggravated the condition. In this situation of stress-induced feces excretion, corticotropin-releasing factor (CRF) participates as a neurotransmitter in the brain.⁽²⁰⁾ This CRF promotes the release of the ACTH, and as a result, increases the secretion of cortisol. In this study, it is

thought that the anti-allergic actions and the strengthening of the gastrointestinal peristalsis change in parallel. It has been reported that a serotonin receptor participates in the transmission of CRF.⁽²¹⁾ In addition, the serotonin-stimulated neurons have nerve terminals to mast cells, and induced the activation of the mast cells by nerve stimulation.⁽²²⁾ The gastrointestinal tract then shifts to the Th2 type response following the activation of the mast cells, and as a result, may act on allergic suppression. It is thought that the CRF-serotonin neuron-mast cell system may explain why the blood levels of ACTH and cortisol increase, and why an anti-allergic reaction is induced by the smell of seirogan.

An opioid receptor participates in the normalization of the peristaltic movement of the bowels, but Seirogan itself does not affect the opioid receptor.⁽²³⁾ On the other hand, the smell of the compound acts on an opioid receptor through sensory nerves, and induces an anti-allergic action. In addition, there is an opioid receptor to the Th cells of the bowels, which induces the differentiation to Th2 cells and reinforces the expression of Th2 cytokines.⁽²⁴⁾ Therefore, the smell of Seirogan acts on the opioid receptor of the bowels and may be responsible for the immunomodulation.

In addition, chemical contact allergy induced by dinitrofluorobenzene, trinitrofluorobenzene and oxazolone is a cell-mediated immune response mediated by IFN- γ producing Th/Tc1 cells.⁽²⁵⁾ However, Tang *et al.*⁽²⁶⁾ found that the FITC-induced CHS

response is Th2-dominant, while the oxazolone response is Th1-dominant. Therefore, the expression of IL-4 and/or mast cells plays an important role in the FITC-induced CHS response.^(26,27) One of the factors associated with the increase in mast cells expression is the increase of acetylcholine.⁽²⁸⁾ However the smell of the Seirogan suppressed the increase of acetylcholine. Therefore, the smell of Seirogan restrained the increase in the number of mast cells. The reduced TNF- α level and reduced expression of the mast cells may be why the allergic reaction caused by the FITC-induced CHS was reduced in comparison to the oxazolone-induced CHS.

In summary, the current study suggested that the FITC- and oxazolone-induced CHS response could be inhibited by oral administration of coated Seirogan and the smell of uncoated Seirogan. Therefore, the FITC-induced CHS allergic response was low in comparison with the oxazolone-induced CHS. The FITC-induced CHS model shows an inflammatory skin and colon pathology reproducing all the key features of human atopic dermatitis, including what appears to be a Th2 driven immune response. Therefore, the smell of the Seirogan may effectively prevent and/or cure the symptoms of atopic dermatitis.

Conflict of Interest

No potential conflicts of interest were disclosed.

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