Antipruritic and Antierythema Effects of Ascomycete *Bulgaria inquinans* Extract in ICR Mice

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Received June 20, 2005; accepted September 14, 2005

The effect of ethanol extract obtained from *Bulgaria inquinans* on the scratching behavior and vascular permeability changes induced by compound 48/80, histamine and serotonin in ICR mice was studied. The extract dose dependently inhibited scratching behavior induced by compound 48/80 and serotonin. A significant inhibition was observed at doses of 300 and 600 mg/kg when *Bulgaria inquinans* extract was administered orally. However, no inhibitory effect was observed on the histamine-induced scratching behavior by the extract, even at a dose of 600 mg/kg. In addition, it also inhibited the increase in the vascular permeability induced by compound 48/80 and serotonin at doses of 300 and 600 mg/kg; however, it failed to inhibit the increased vascular permeability induced by histamine, even at a dose of 600 mg/kg. *Bulgaria inquinans* extract showed a potent inhibitory effect on histamine release induced by compound 48/80. These results suggest that *Bulgaria inquinans* extract is effective in cutaneous pruritus and erythema, which were probably mediated by inhibiting the release of histamine from mast cells and antagonizing the effect on serotonin.

Key words *Bulgaria inquinans*; compound 48/80; scratching behavior; serotonin; histamine; mast cell

Pruritus, or itching, is the main symptom of some dermatological diseases, such as atopic dermatitis, contact dermatitis and urticaria, and the accompanying symptom of some systemic disorders, like chronic renal, cholestasis and diabetes mellitus.1—3) Atopic dermatitis is a chronic inflammatory skin disease that affects 10—20% of all children, and its prevalence has gradually increased over the past few decades.4) It is now well established that endogenous mediators, such as histamine, serotonin, peptides, proteases, prostaglandins and cytokines, are the main pruritogens responsible for itching.5,6) Based on this research, many antipruritic drugs, histamine H1 receptor antagonists, antiallergic drugs and steroidal drugs are developed and widely used in the clinical treatment of itching caused by atopic dermatitis. Unfortunately, there is still no specific remedy available for this disease. In order to improve the quality of life for the dermatitis patients, many efforts have been focused on the screening of herbs or finding new constituents from herbs that exhibit antipruritic effect.7—10)

*Bulgaria inquinans*, a wood-inhabiting ascomycete growing on freshly felled oak, is widely distributed in the area of Changbai Mountain (Northeastern China).11) In the Changbai Mountain area, *Bulgaria inquinans* has been used as a folk medicine for the treatment of dermatitis.11) Phytochemical investigation of the fruit bodies of *Bulgaria inquinans* demonstrated the presence of ergosterol, galactitol, ethanedioic acid,12) azaphilone derivatives,13) palmitic acid, oleic acid and linoleic acid.11) However, very little information is available about the pharmacological findings pertaining to this ascomycete. In the present study, the effect of the ethanol extract of *Bulgaria inquinans* on both the scratching behavior and vascular permeability induced by compound 48/80 in ICR mice was studied.

MATERIALS AND METHODS

**Animals** Female ICR mice (6—10 weeks old) and male Wistar rats (7 weeks old) were obtained from Japan SLC, Shizuoka, Japan. The animals were housed in an air-conditioned room maintained at 24 ± 2 °C with a relative humidity of 55 ± 15%. Standard laboratory rodent food (Oriental Yeast, Tokyo) and water were available *ad libitum*. All procedures involving animals were conducted in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center.

**Preparation of Extract** *Bulgaria inquinans* was collected at Changbai Mountain (Northeastern China) and authenticated by Professor Zhong-Kai Yan, Academy of Traditional Chinese Medicine and Materia Medica of Jilin Province. Five hundred grams of *Bulgaria inquinans* were ground and refluxed with 95% ethanol (600 ml) three times for 2 h. The decoctions were evaporated under reduced pressure and dried. The characteristics of the extract were black in color, odorless and thick syrup (37.5 g, yield 7.5%) like consistency.

**Reagents and Drugs** The following reagents were used in this study and their sources are shown in parentheses: histamine dihydrochloride (Nacalai Tesque, Kyoto, Japan), compound 48/80 (Sigma, St. Louis, MO, U.S.A.), 5-hydroxytryptamine (serotonin) (Sigma) and Evans blue (Wako, Tokyo, Japan). Compound 48/80, histamine and serotonin were dissolved in physiological saline and administered intradermally. The drugs used were tranilast (Kissei Pharmaceuticals Co., Ltd., Nagano, Japan), ketotifen fumarate and cypromeptadine (Sigma). These drugs including *Bulgaria inquinans* extract were suspended 5% arabic gum solution and administered orally.

**Scratching Behavior** Scratching behavior was observed as the same method as Kuraishi et al.14) Ethanol extracts of *Bulgaria inquinans* were suspended in 5% arabic gum and administrated orally 1 h before the start of behavioral observation. Compound 48/80 10 μg/0.02 ml, histamine 100 nmol/0.02 ml and serotonin 100 nmol/0.02 ml were injected intra-dermally into the rostral part of the back of the mice. Immediately after injection, the mice were placed in an observation chamber and their behavior was observed for 60 min. In the
present study, scratching behavior was automatically detected and objectively evaluated with a new apparatus, MicroAct (Neuroscience, Tokyo, Japan). A small magnet (diameter in 1 mm, length in 3 mm) was implanted subcutaneously into the both hind paws of a mouse under ether anesthesia at least 12 h before the measurement of scratching. Mice were placed in an observation chamber (11 cm in diameter, 18 cm high), which was surrounded by a round coil. The electric current induced in the coil by the movement of magnets attached to the hind paws was amplified and recorded.15)

**Vascular Permeability of the Skin** After intradermal injection of 0.5 μg/0.02 ml compound 48/80, 10 nmol/0.02 ml histamine or 10 nmol/0.02 ml serotonin into the rostral part of the back, 2% Evans blue solution was intravenously injected into each animal. The animals were sacrificed 30 min later, and the diameters of the ‘bluing’ reaction at the injection site were measured.16) *Bulgaria inquinans* extract was administered orally 1 h before the experiment.

**Compound 48/80-Induced Histamine Release from Isolated Rat Peritoneal Mast Cells** Peritoneal mast cells of male Wistar strain rats were harvested and purified by percoll density gradient centrifugation.17) The collected mast cells were then incubated with physiological buffered saline (PBS; in mm: NaCl 154, KCl 2.7, CaCl₂ 0.9, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) 5, glucose 5.6; pH 7.4) for 10 min at 37 °C. The test drugs dissolved in PBS were added (0.1 ml) 10 min before compound 48/80 (final concentration: 0.5 μg/ml). The reaction was stopped 10 min later by cooling the tubes in ice water, and the tubes were centrifuged for 15 min at 2000 g. The histamine contents were measured in the supernatant and precipitate using an autoanalyzer.18)

**Statistical Analysis** The data are presented as means±S.E.M. Statistical significance was tested by one-way analysis of variance with Dunnett’s test. A probability value less than 0.05 was considered to be significant.

**RESULTS**

**Effect of *Bulgaria inquinans* Extract on Compound 48/80-Induced Scratching Behavior** At first, we studied the effect of compound 48/80 on scratching behavior. Compound 48/80 caused a significant increase in the number of scratchings at doses of 3 and 10 μg/site. However, the effect of 3 μg/site dose of compound 48/80 was not as potent, therefore the 10 μg/site dose was used in the following study (Fig. 1A). Figure 1B shows the effect of *Bulgaria inquinans* extract on compound 48/80-induced scratching behavior. *Bulgaria inquinans* extract caused a dose-related inhibition of this response and a significant effect was observed at doses of 300 and 600 mg/kg. Tranilast, used as positive control at a dose of 300 mg/kg, also caused an inhibition of this response.

**Effect of *Bulgaria inquinans* Extract on Histamine and Serotonin-Induced Scratching Behavior** As shown in Table 1, *Bulgaria inquinans* extract caused a dose-dependent inhibition of the serotonin-induced scratching behavior and a significant effect was observed at 300 and 600 mg/kg. On the other hand, it caused no significant inhibition of the histamine-induced scratching behavior, even at a dose of 600 mg/kg. Ketotifen, used as positive control, significantly inhibited the histamine-induced scratching behavior at a dose of 10 mg/kg. Cyproheptadine also inhibited significantly the serotonin-induced scratching behavior at a dose of 1 mg/kg.

**Effect of *Bulgaria inquinans* Extract on the Vascular Permeability Induced by Compound 48/80** At first, we studied the effect of compound 48/80 on the skin vascular permeability. Compound 48/80 caused a significant increase in vascular permeability at doses of 0.5 and 1.5 μg/site. In this study, the 0.5 μg/site dose of compound 48/80 was used in the following study (Fig. 2A). *Bulgaria inquinans* extract at a dose of 150 mg/kg caused no significant inhibition of the increased vascular permeability induced by compound 48/80. However, at 300 and 600 mg/kg, it significantly inhibited the increased vascular permeability (Fig. 2B). Tranilast, at a dose of 300 mg/kg, also significantly inhibited the increased vascular permeability induced by compound 48/80.
ability Induced by Compound 48/80 in ICR Mice

The histamine release induced by compound 48/80. The histamine release induced by compound 48/80 (0.5 μg/ml) was 35.3±1.9% (n=10) of the total content. The extract was effective in inhibiting the histamine release induced by compound 48/80, and significant differences were observed at concentrations of 10 and 50 μg/ml.

DISCUSSION

In the present study, we have demonstrated that the ethanol extract of *Bulgaria inquinans* significantly inhibited the scratching behavior induced by compound 48/80 in ICR mice at doses of 300 and 600 mg/kg (Fig. 1B). In a previous paper, we reported that some histamine H1 receptor antagonists potently inhibited the compound 48/80-induced scratching behavior in BALB/c mice39) after injection of histamine produced an itching sensation in humans. At first, therefore, we studied the effect of histamine on scratching behavior. As shown in Table 1, contrary to our expectation, *Bulgaria inquinans* extract caused no inhibition of the scratching behavior induced by histamine, even at a dose of 600 mg/kg. This observation suggests that the inhibition of scratching behavior induced by *Bulgaria inquinans* extract may have occurred through a histamine-releasing mechanism but not the H1-antagonistic activity. The histamine-releasing mechanism was further supported by the fact that the histamine release from rat peritoneal mast cells induced by compound 48/80 was potently inhibited by the extract. That is, *Bulgaria inquinans* extract significantly inhibited the histamine release from rat peritoneal mast cells at concentrations of 10 μg/ml or more (Fig. 3).

Serocon is another important mediator responsible for pruritus in humans and also has been suggested to be involved in some pruritic diseases21,22) There are some reports demonstrated that it elicited a mild but significant scratching behavior in healthy humans23) and rats.24) As shown in the present study, serotonin was injected into the rostral back of mice, it elicited an apparent scratching response in the injected regions, similar to Inagaki et al.25) Thus, we investigated the effect of *Bulgaria inquinans* extract on the scratching induced by serotonin. The result of the study indicated that the ethanol extract of *Bulgaria inquinans* significantly inhibited the scratching behavior induced by serotonin.

**Effect of *Bulgaria inquinans* Extract on Vascular Permeability Induced by the Histamine or Serotonin**

As shown in Table 2, *Bulgaria inquinans* extract caused a dosedependent inhibition of the serotonin-induced vascular permeability, and a significant effect was observed at doses of 300 and 600 mg/kg. On the other hand, it caused no inhibition of the histamine-induced vascular permeability even at a dose of 600 mg/kg. Ketotifen and cyproheptadine have been used as positive controls also caused an inhibition of the vascular permeability induced by histamine or serotonin.

**Fig. 2. (A) Vascular Permeability Induced by Compound 48/80 in ICR Mice and (B) Effect of *Bulgaria inquinans* Extract on the Vascular Permeability Induced by Compound 48/80 in ICR Mice**

*Bulgaria inquinans* extract was administered orally 1 h before compound 48/80 (0.5 μg/site) injection. Each column and vertical bar shows the means±S.E.M. (n=8—10). * * Significantly different from the control group at p<0.05 and p<0.01, respectively.

<table>
<thead>
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<th>Drugs</th>
<th>Dose (mg/kg)</th>
<th>Bluing area (mm²)</th>
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<tbody>
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<td>Control (histamine, 10 nmol/site)</td>
<td>150</td>
<td>51.2±5.2</td>
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<tr>
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</tr>
<tr>
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<td>51.4±2.2</td>
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<tr>
<td>Ketotifen</td>
<td>600</td>
<td>54.7±1.7</td>
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<tr>
<td>Ketotifen</td>
<td>1</td>
<td>17.2±1.1**</td>
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<tr>
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<tr>
<td>Basil</td>
<td>600</td>
<td>30.5±0.9**</td>
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<td>Basil</td>
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<td>25.2±1.5**</td>
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*Bulgaria inquinans* extract was administered orally 1 h before histamine (10 nmol/site) or serotonin (10 nmol/site) injection. Each column and vertical bar shows the means±S.E.M. (n=8—10). ** Significantly different from the control group at p<0.01.

**Fig. 3. Effect of *Bulgaria inquinans* Extract on Histamine Release from Rat Peritoneal Mast Cells Induced by Compound 48/80 (0.5 μg/ml)**

Each column and vertical bar shows the means±S.E.M. (n=10). * * Significantly different from the control group at p<0.05 and p<0.01, respectively.

**Effect of *Bulgaria inquinans* Extract on Histamine Release from Rat Peritoneal Mast Cells Induced by Compound 48/80**

Figure 3 shows the effect of *Bulgaria inquinans* extract on the histamine release from rat peritoneal mast cells induced by compound 48/80. The histamine release induced by compound 48/80 (0.5 μg/ml) was 35.3±1.9% (n=10) of the total histamine release from rat peritoneal mast cells induced by compound 48/80. The histamine release induced by compound 48/80 was 35.3±1.9% (n=10) of the total histamine release from rat peritoneal mast cells induced by compound 48/80. The histamine release induced by compound 48/80 was 35.3±1.9% (n=10) of the total histamine release from rat peritoneal mast cells induced by compound 48/80.
in ICR mice at doses of 300 and 600 mg/kg (Table 1). We also studied the effect of *Bulgaria inquinans* extract on the vascular permeability induced by compound 48/80 (Fig. 2), histamine and serotonin (Table 2). Plasma exudation characterizes the early phase of acute inflammation, and mast cell mediators, such as histamine and serotonin, are known to increase the vascular permeability in various species. It was revealed that *Bulgaria inquinans* extract inhibited not only compound 48/80-induced vascular permeability, but also the serotonin-induced one, which indicated that the extract directly reduced the inflammatory action of serotonin.

In this study, *Bulgaria inquinans* extract was administered orally 1 h before injection of compound 48/80, histamine or serotonin. Preliminary study revealed that peak time of action *Bulgaria inquinans* extract was 1 h. Similar to many previous papers, the effect of *Bulgaria inquinans* extract was performed before injection of pruritogens, the effect after appearance of the disease symptoms should be studied.

Cui et al. reported that *Bulgaria inquinans* contained ergosterol, galactititol and ethanedioic acid. Stadler et al. also isolated three azaphilones, bulgarialactone A, B and C, from the ascomycete *Bulgaria inquinans*. Bulgarialactone A and B possess antimicrobial and cytotoxic activities. On the other hand, Bao et al. reported that *Bulgaria inquinans* contained palmitic, oleic and linoleic acid. In a previous paper, we reported that oleic acid dose-dependently inhibited the histamine release from rat peritoneal mast cells induced by compound 48/80. Therefore, it may be that oleic acid is one of the effective constituents of the ethanol extract of *Bulgaria inquinans*.

In conclusion, the ethanol extract from *Bulgaria inquinans* showed inhibitory effects on the immediate allergic reactions, which were probably mediated by inhibiting the release of histamine from mast cells, and directly antagonizing serotonin. Further work is necessary to clarify the active components and precise mechanism especially for an inhibition of histamine release induced by *Bulgaria inquinans* extract.

**Acknowledgement** The authors would like to thank Professor Zhong-Kai Yan at the Academy of Traditional Chinese Medicine and Materia Medica of Jilin Province for authenticating the ascomycete which used in this study is *Bulgaria inquinans*.

**REFERENCES**