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Original Article

Psychosocial stress enhances IgE-mediated triphasic cutaneous reaction in mice: Antagonism by Yokukan-san (a Kampo medicine) and diazepam

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Abstract

Background: In the present study, we investigated the effect of social isolation stress on IgE-mediated triphasic cutaneous reactions after 2,4-dinitrofluorobenzene (DNFB) challenge in male BALB/c mice passively sensitized with anti-dinitrophenol (DNP) IgE antibody, and examined the effects of Yokukan-san (a Kampo medicine with antipsychotic action) and a reference drug (diazepam) on the stress-enhanced cutaneous reaction.

Methods/Results: In response to challenge with 0.01, 0.025 and 0.05% DNFB, triphasic skin reactions, including an immediate-phase response (IPR), a late-phase response (LPR) and a very late-phase response (vLPR) at 1 and 24 h and 8 days after antigen challenge, respectively, were increased in socially isolated mice compared with group-housed mice. Oral administration of Yokukan-san attenuated the isolation stress-exacerbated triphasic skin reactions in a dose-dependent manner, whereas it had no significant effect on cutaneous reactions in the unstressed group-housed mice. In contrast, intraperitoneal administration of diazepam, a classic benzodiazepine receptor agonist, suppressed the enhanced IPR and LPR in socially isolated mice but, surprisingly, stimulated vLPR in both stressed and unstressed mice, showing different efficacy to Yokukan-san. Moreover, the elevated locomotor activity in socially isolated mice was reduced by Yokukan-san and diazepam, while the isolation stress-induced aggressive behavior was normalized only by diazepam and not by Yokukan-san.

Conclusions: The results of the present study indicate that IgE-mediated triphasic cutaneous reactions were exacerbated by social isolation stress and suggest that Yokukan-san and diazepam antagonize isolation stress-induced cutaneous reactions partly through their sedative action on social isolation stress.

Key words: atopic dermatitis, diazepam, IgE-mediated skin reaction, Kampo medicine, psychosocial stress, social isolation stress, Yokukan-san.

Introduction

Increasing evidence suggests an important impact of psychosocial stress, such as the loss of an intimate relationship, divorce, bereavement or other adverse life events, on the subsequent onset or exacerbation of many types of human diseases, including depression, cardiovascular diseases, cancer and dermatologic disorders.[1–4]

Atopic dermatitis (AD) is an inflammatory disease with itch, characterized by chronic and relapsing eczematous

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lesions in the abnormal skin of subjects with a personal or family history of atopy. It was reported that stressful life events may precede the onset of AD in up to 70% of cases.[5] Personal stress and stress from a family environment were shown to become important predictors of the severity of symptoms of AD. Perceived itch was found to be enhanced in atopic patients in response to mental stress. In addition, chronic and intractable atopic eczema in children may be associated with an impaired parent–child relationship.[5–9] Many clinical studies have suggested the relative contribution of psychosocial factors to the development of AD. However, to our knowledge, experimental studies on the effect of psychosocial stress on allergic cutaneous reactions in animals have been only marginally reported.

Social isolation (i.e. individual housing of laboratory animals) is a model of a lack of social interaction among animals involving anxiety and is considered to be relatively comparable with the situation of humans who feel isolated. In rodents, social isolation results in marked behavioral disturbances, such as increased aggressiveness, enhanced locomotor activity and elevated morphine consumption, as well as reduced pentobarbital-induced sleeping time.[10–12] There are also physiological disturbances, including high levels of plasma corticosterone and catecholamines and high corticotropin-releasing factor (CRF) activity and attenuated immune function.[13–16]

Previous studies have revealed that social isolation stress can enhance liver metastasis and angiogenesis of colon carcinoma cells and suppressed immune functions such as natural killer (NK) cell- and macrophage-mediated cytotoxicity in mice.[17,19] Because of its inherent social nature, social isolation is viewed as a more natural and convenient model for constituting psychosocial stress and would be useful for us to investigate the modulatory role of psychosocial stress on allergic inflammatory reactions in mice.

We recently found that passive sensitization with anti-dinitrophenol (DNP) IgE antibody followed by challenge with 2,4-dinitrofluorobenzene (DNFB) to the mouse ear can induce triphasic cutaneous reactions (ear swelling) of an immediate-phase response (IPR), a late-phase response (LPR) and a very late-phase response (vLPR), peaking at 1 and 24 h and 8 days after the challenge, respectively.[20] The IPR was absent in mast cell-deficient mice, but the LPR was sufficiently observed and vLPR was partly attenuated. The LPR is a T cell-independent response, while the vLPR is almost completely absent in T cell-deficient nude mice. Thus, the third-phase response (vLPR), with massive eosinophil infiltration, actually represents an important inflammatory reaction mediated by T cells and partially by mast cells.[20]

In the present study, we investigated the effect of social isolation stress on the IgE-mediated triphasic cutaneous reaction in passively sensitized mice and examined the effects of Yokukan-san, an antipsychosis drug in Kampo medicine,[21] and the reference drug diazepam on triphasic skin reactions affected by isolation stress.

METHODS

Mice

Specific pathogen-free male BALB/c mice, 4 weeks old and weighing 16–19 g, were obtained from Japan SLC (Hamamatsu, Japan). Mice were randomly assigned to be group-housed ($n = 3$ per cage; 24 × 17 × 12 cm) or housed individually in same-sized cages ($n = 1$ per cage; 24 × 17 × 12 cm) for 2 weeks before starting skin testing. Mice were kept in the animal laboratory, which was maintained at a constant temperature (23–25°C), relative humidity (65%) and with a 12 h light–dark cycle (lights on 0800–2000 h). Food and water were available *ad libitum*. This study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

Antigens and chemicals

Yokukan-san (Yi-Gan-San; TJ-54, lot no. 260054010; Tsumura, Tokyo, Japan) is composed of seven crude drugs, the quality of which is controlled by the Japanese Pharmacopeia XIII. Yokukan-san was prepared as follows: a mixture of Atractylodis lanceae rhizoma (Japanese name 'Sojutsu'; 4 g), Hoelen (Bukuryo; 4 g), Cnidii rhizoma (Senkyu; 3 g), Angelicae radix (Toki; 3 g), Bupleuri radix (Saiko; 2 g), Glycyrrhizae radix (Kanzo; 1.5 g) and Uncariae uncis cum ramulus (Chotoko; 3 g) was added to 500 mL water and extracted at 100°C for 40 min. The extract was evaporated and lyophilyzed. The formulation was dissolved in distilled water and administered orally 6–2 days before and 2–6 days after antigen challenge.

High-pressure liquid chromatography (HPLC) pattern analysis, termed the 'fingerprint' method, was performed to assess the homogeneity of the formulation and to prepare batches with a constant formulation, as described previously.[22] Figure 1 shows the HPLC profile

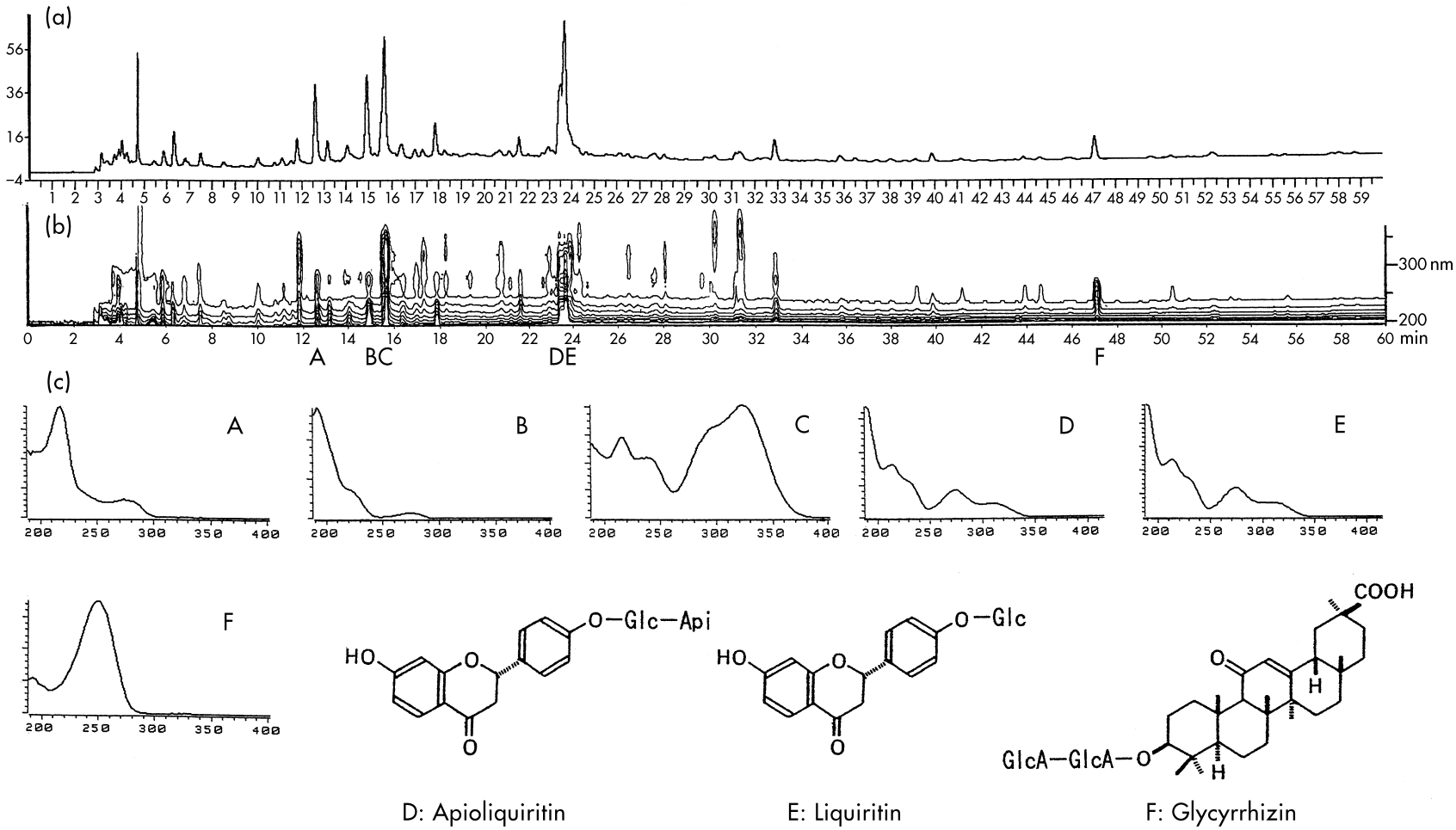

Fig. 1 High-performance liquid chromatography (HPLC) profile of Yokukan-san and ultraviolet (UV) spectra. A dose of Yokukan-san was extracted with H_2O-EtOH (9 : 1; 200 mL), filtered and analyzed by HPLC (HP-1090; Hewlett-Packard, Palo Alto, CA, USA) under the following conditions: column, TS K gel ODS-8OTs (4.6 × 250 mm; Tosoh, Tokyo, Japan); mobile phase, 10 mmol/L phosphoric acid : CH_3CN (linear gradient 95 : 5 to > 35 : 65 for 1 h); flow rate, 0.8 mL/min; oven temperature, 40°C; injection volume, 5 μL. (a) The HPLC pattern analyzed by absorbance at 220 nm; (b) contour plot of the HPLC pattern by UV absorbance (190–420 nm); (c) UV spectra of the main peaks, origins of peaks. A, Atractylodis lanceae rhizoma; B, Glycyrrhizae radix and others; C, mixed peaks of A. lanceae rhizoma, Glycyrrhizae radix, Angelicae radix, Cnidium rhizoma and Uncariae uncis cum ramulus; D, Glycyrrhizae radix (apioliquiritin); E, Glycyrrhizae radix (liquiritin); F, Glycyrrhizae radix (glycyrrhizin).

of Yokukan-san by single monitor (220 nm) and contour plot (190–420 nm) using a photodiode array system as the detector.

2,4-Dinitrofluorobenzene (Nacalai Tesque, Kyoto, Japan) was dissolved in 100% ethanol. Diazepam (Takeda Chemical, Osaka, Japan) was suspended in distilled water and administered intraperitoneally for 5 days before and after skin testing. Prednisolone 21-acetate was purchased from Sigma Chemical Co. (St Louis, MO, USA) and was suspended in 0.5% methylcellulose solution and administered intraperitoneally 2 h before and 4–6 days after challenge.

Preparation of anti-DNP IgE

An anti-DNP monoclonal antibody (mAb)-producing cell line (EC1) was cultured in 10 mL of an equal volume mixture of RPMI-1640 and Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with high glucose, 10% heat-inactivated fetal bovine serum (FBS; Gibco Laboratories, Life Technologies, Grand Island, NY, USA) and 2 mmol/L L-glutamine until reaching a confluent state. The supernatant was harvested, centrifuged at 400 *g* and stored at –80°C until use.[23] The IgE antibody titer was estimated to be 1 : 1024 by heterologous passive cutaneous anaphylaxis in rats injected i.v. with DNP–bovine serum albumin as an antigen.[24]

Induction of skin reaction in mouse ears

BALB/c mice were given an i.v. injection of a 1 mL aliquot of anti-DNP IgE mAb-containing fluid 24 h before DNFB challenge. The skin reaction was elicited by applying 10 μL of 0.01, 0.025, 0.05 and 0.1% DNFB in 100% ethanol to each ear of sensitized mice. The reaction to DNFB was evaluated by measuring ear thickness using a dial thickness gauge (G-1A type; Peacock, Ozaki MFG, Osaka, Japan) immediately before and at appropriate times after challenge. Results are expressed as the mean ear swelling (increase in ear thickness; pm) ±SD in three mice.

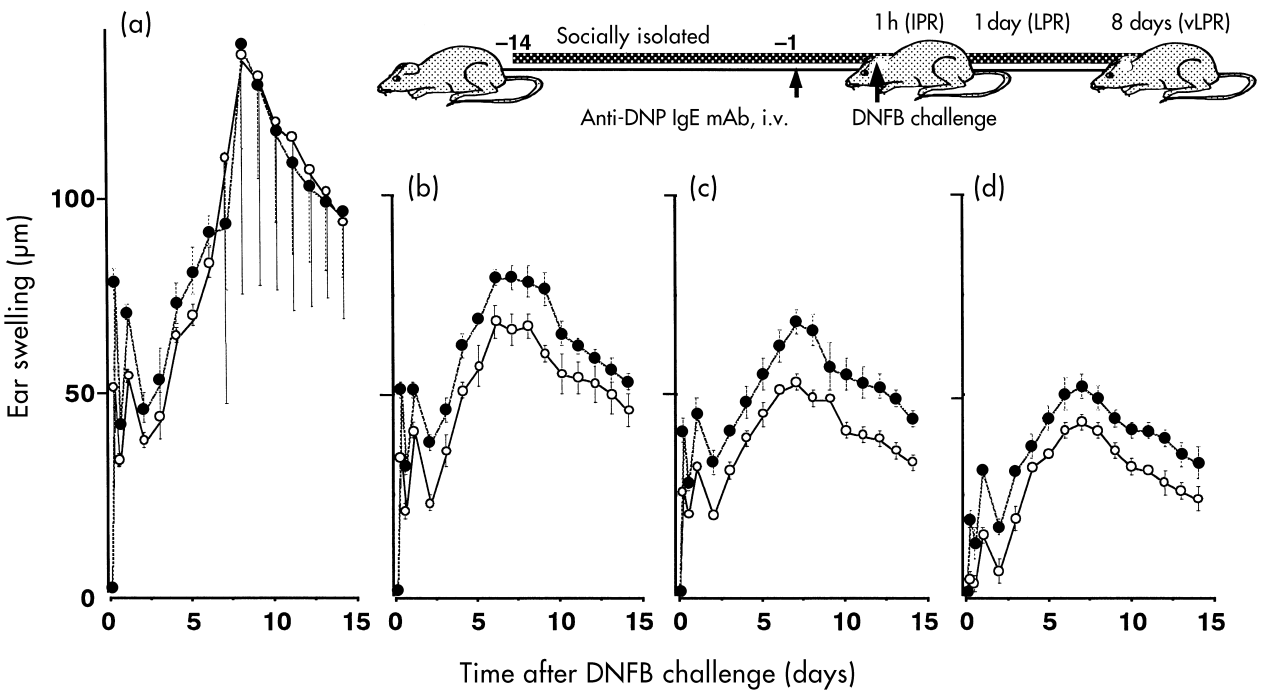

Fig. 2 Effect of social isolation stress on the IgE-mediated triphasic skin reaction in passively sensitized mice. BALB/c mice (4 weeks old; male) were group-housed or socially isolated ($n = 3$) for 2 weeks before skin testing and throughout the experiment. Mice received intravenous injections of 1.0 mL anti-dinitrophenol (DNP) IgE monoclonal antibody (mAb) preparation 24 h before skin testing with different doses of 2,4-dinitrofluorobenzene (DFNB; (a) 0.01%, (b) 0.025%, (c) 0.05%, (d) 0.1%) in 100% ethanol. Ear swelling was measured at 1 and 24 h and 8 days following DNFB challenge to evaluate the immediate-phase reaction (IPR), the late-phase reaction (LPR) and the very late-phase reaction (vLPR), respectively. (○), group-housed; (●), socially isolated.

Measurement of locomotor activity

For the locomotor behavioral experiment, four untreated mice per group were put into an acrylic cage. Two hours after acclimation, locomotor behavior was observed under unmanned conditions with a sensor for measuring small animal locomotor activity (NSAS01; Neuroscience, Tokyo, Japan) located at the top of the cage. The sensor was equipped with a system printer (NSP-008; Neuroscience). Locomotor activity was evaluated by the number of passages by mice under the sensor over a period of 1 h. Each mouse was used for only one experiment.[25]

Determination of aggressive response

When testing aggressive behavior between isolated mice, two isolated mice were placed in a neutral cage the same size as that in which they were housed individually (24 × 17 × 12 cm). Aggressive response was measured in terms of the total duration of biting attacks and/or wrestling over a period of 30 min and was recorded using an 8 mm video camera (CCD-TRV60; Sony, Tokyo, Japan).[26]

Statistical analysis

The statistical significance of differences between groups was determined with the Mann–Whitney's *U*-test for experiments on ear swelling, with two-way repeated-measures ANOVA for locomotor activity and with Student's two-tailed *t*-test for aggressive behavior. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of social isolation stress on triphasic skin reaction in passively sensitized mice

We first investigated whether or not social isolation stress can affect the IgE-mediated triphasic reactions in mice

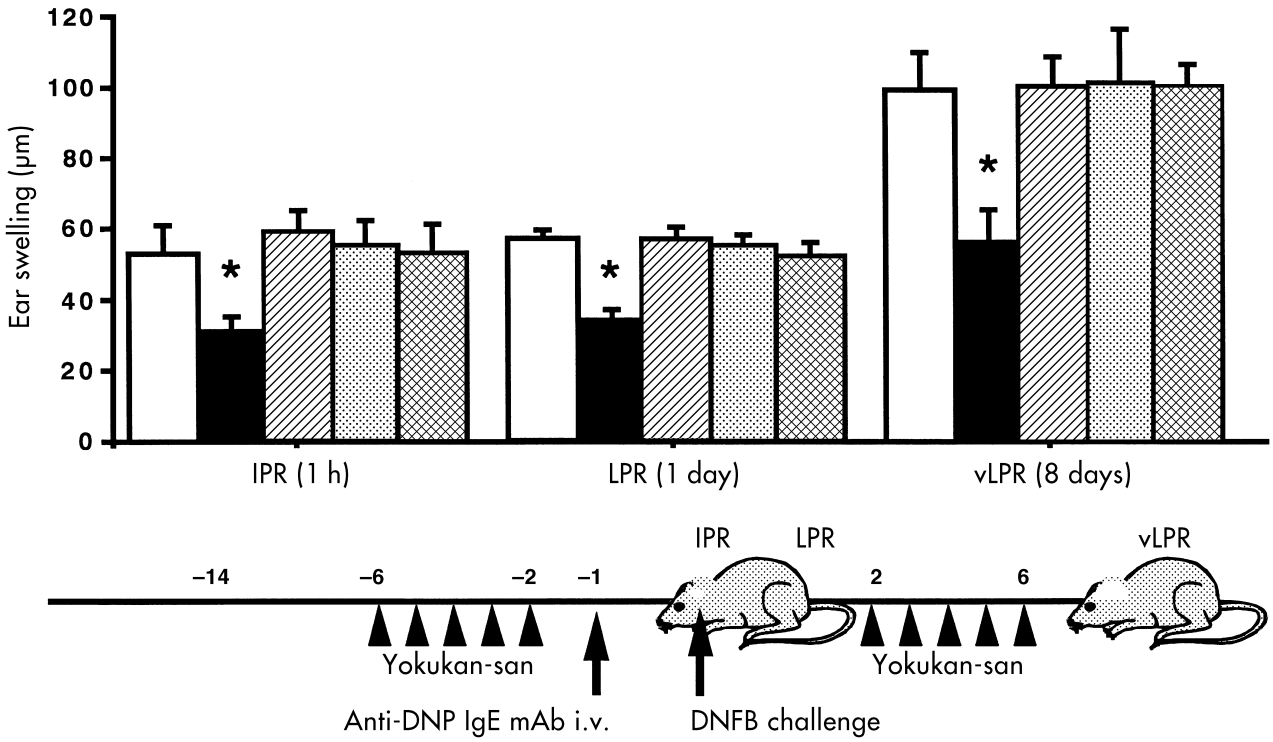

Fig. 3 Effects of Yokukan-san on the IgE-mediated triphasic skin reaction in group-housed mice. Mice received intravenous injections of 1.0 mL anti-dinitrophenol (DNP) IgE monoclonal antibody (mAb) 24 h before skin testing with 0.025% 2,4-dinitrofluorobenzene (DNFB) in 100% ethanol. Yokukan-san was given orally 6–2 days before and 2–6 days after DNFB challenge. Prednisolone (10 mg/kg) was given intraperitoneally 2 h before and 4–6 days after challenge. Data are the mean±SD of three mice. *$P < 0.01$ compared with the respective control group (Mann–Whitney *U*-test). (□), anti-DNP mAb/0.1% DNFB; (■), prednisolone; (▨), 0.5 g/kg Yokukan-san; (▩), 1 g/kg Yokukan-san; (▩), 2 g/kg Yokukan-san. IPR, immediate-phase reaction; LPR, late-phase reaction; vLPR, very late-phase reaction.

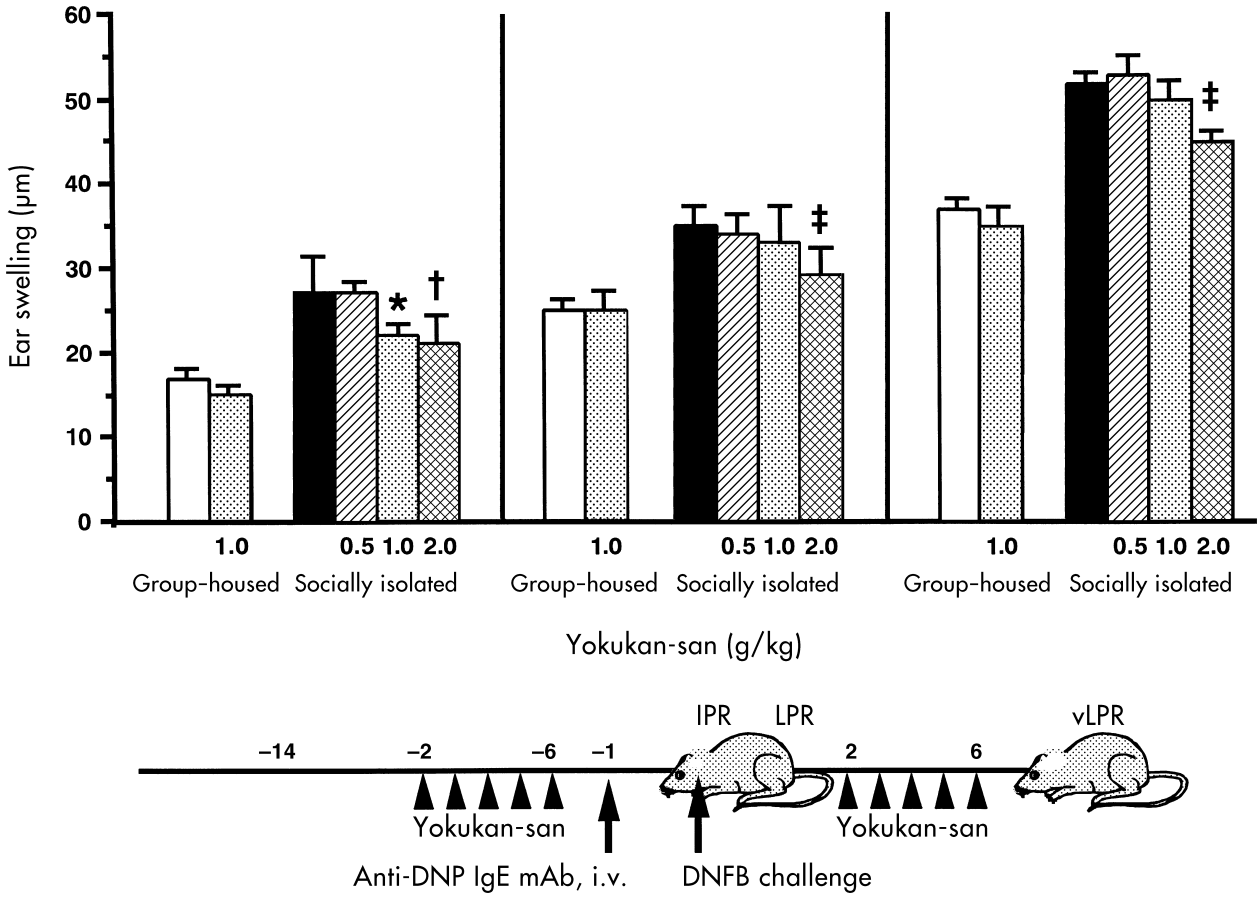

Fig. 4 Effects of Yokukan-san on the IgE-mediated triphasic skin reaction in mice that were either group-housed or socially isolated for 2 weeks before skin testing and throughout the experiment. (a) Immediate-phase reaction (IPR; 1 h); (b) late-phase reaction (LPR; 24 h) and very late-phase reaction (vLPR; 8 days). Mice received intravenous injections of 1.0 mL anti-dinitrophenol (DNP) IgE monoclonal antibody (mAb) 24 h before skin testing with 0.025% 2,4-dinitrofluorobenzene (DNFB) in 100% ethanol. Yokukan-san was given orally 6–2 days before and 2–6 days after DNFB challenge. Data are the mean±SD of three mice. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$ compared with vehicle (control of each group; Mann–Whitney's *U*-test).

passively sensitized with anti-DNP IgE mAb. As shown in Fig. 2, IPR and LPR were significantly enhanced in mice with 2 week social isolation stress after challenge with any dose of DNFB compared with group-housed mice. In contrast, vLPR was markedly elevated in socially isolated mice in response to 0.01–0.05% DNFB, but there was no discernible difference in vLPR after challenge with 0.1% DNFB between group-housed and stressed mice. Thus, social isolation stress could exacerbate the triphasic cutaneous reaction in passively sensitized mice. In subsequent experiments, we used 0.025% DNFB for the challenge, because a marked difference in cutaneous responses after challenge with this dose of DNFB was observed between group-housed and socially isolated mice.

Effect of Yokukan-san and diazepam on triphasic skin reaction in group-housed and socially isolated mice

To further confirm that psychosocial factors do impact on the isolation stress-provoked enhancement of the triphasic cutaneous reaction, the anti-allergic effects of Yokukan-san, a Kampo medicine with an antipsychotic action,[21] and diazepam were examined in this model. Figure 3 illustrates that oral administration of Yokukan-san before and after DNFB challenge at any dose failed to inhibit IPR, LPR and vLPR in group-housed mice. Prednisolone (10 mg/kg) appeared to be effective in inhibiting the triphasic cutaneous reaction. In contrast, the enhancement of the triphasic skin reaction by social

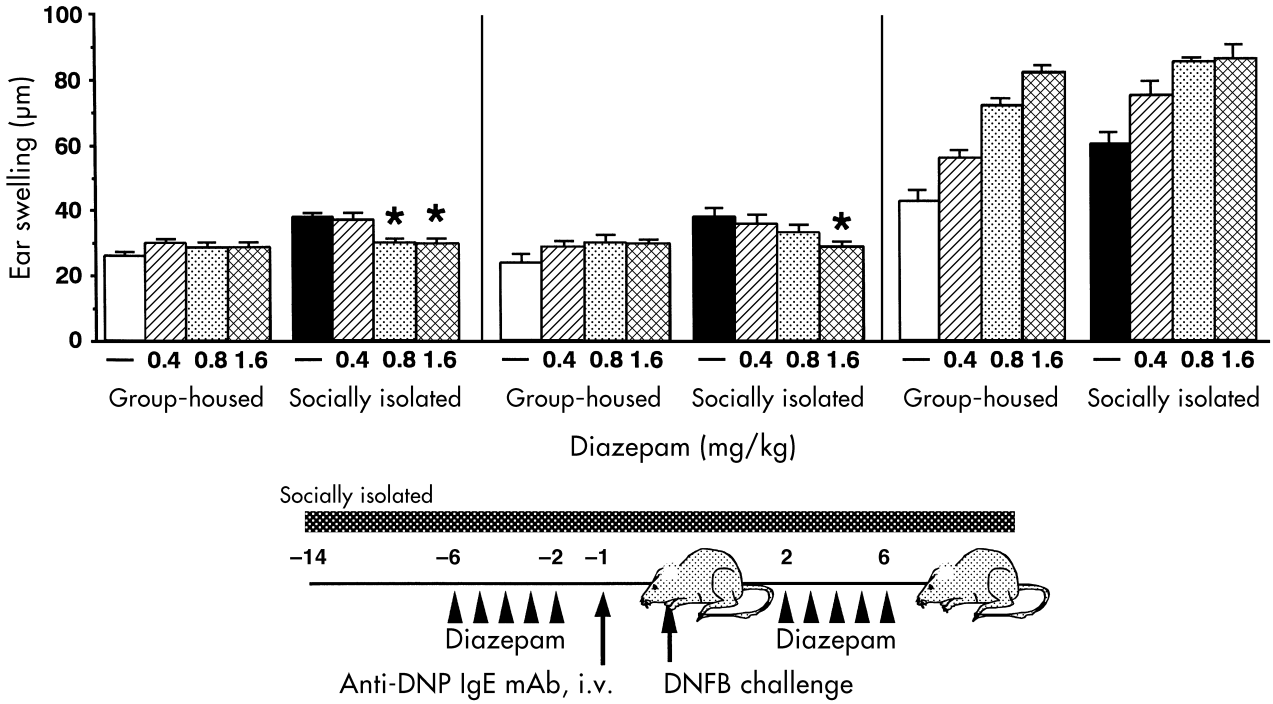

Fig. 5 Effects of diazepam on the IgE-mediated triphasic reaction in group-housed or socially isolated mice that were either group-housed or socially isolated for 2 weeks before skin testing and throughout the experiment. (a) Immediate-phase reaction (IPR; 1 h); (b) late-phase reaction (LPR; 24 h) and very late-phase reaction (vLPR; 8 days). Mice received intravenous injections of 1.0 mL anti-dinitrophenol (DNP) IgE monoclonal antibody (mAb) 24 h before skin testing with 0.025% 2,4-dinitrofluorobenzene (DNFB) in 100% ethanol. Diazepam was given intraperitoneally 6–2 days before and 2–6 days after DNFB challenge. Data are the mean±SD of three mice. *$P < 0.05$ compared with vehicle (control of each group; Mann–Whitney's *U*-test).

isolation stress was dose-dependently inhibited by the administration of Yokukan-san (Fig. 4). Intraperitoneal administration of diazepam dose-dependently inhibited the enhancement of IPR and LPR in socially isolated mice, although it did not show any effect in group-housed mice (Fig. 5). In contrast, surprisingly, diazepam markedly exacerbated the vLPR in both group-housed and socially isolated mice in a dose-dependent manner, quite different from the action of Yokukan-san.

Effects of Yokukan-san and diazepam on locomotor and aggressive behaviors in group-housed and socially isolated mice

Previous studies have reported that behavioral disturbances, including enhanced aggressive and locomotor activities, were frequently observed in socially isolated mice.[26] In addition, Yokukan-san and diazepam have sedative activities (anxiolytic and antidepressive).[21,27,28] To examine whether social isolation stress-induced behavioral disturbances are associated with stress-triggered enhancement of triphasic skin reactions, we investigated the effect of both drugs on locomotor and aggressive activities in mice under isolation stress. Figure 6 shows that locomotor activity was obviously increased in socially isolated mice compared with group-housed control mice. Both Yokukan-san and diazepam significantly inhibited the isolation stress-enhanced locomotor behavior. Moreover, as illustrated in Fig. 7, the administration of diazepam markedly inhibited the induction of aggressive behavior by isolation stress, while administration of Yokukan-san did not.

Discussion

Although a recent increase in the incidence of chronic allergic diseases, including AD, has been reported, the pathogenesis of these conditions is still obscure. In addition to the genetic factors underlying the development of the diseases, environmental influences, including psychosocial factors, have also been suggested to provoke and exacerbate the diseases. Evidence in support of this

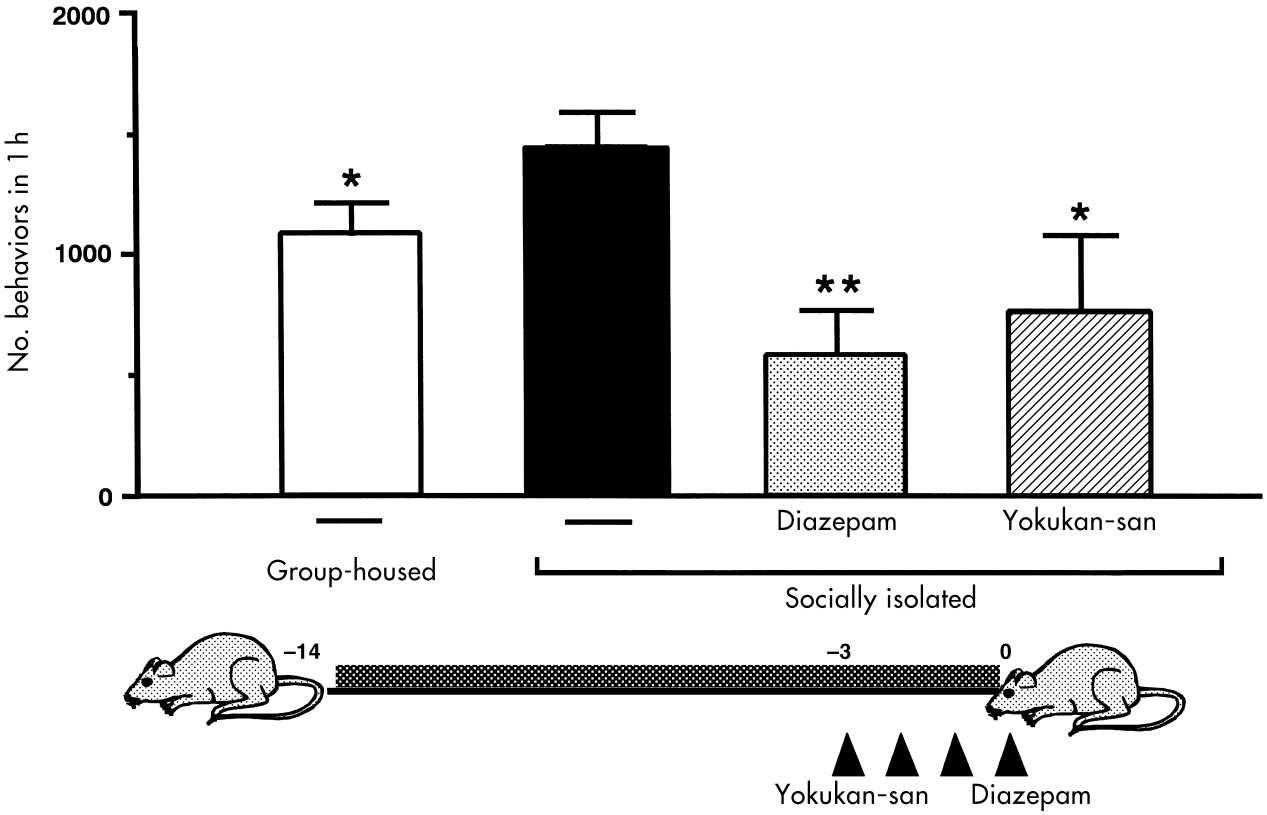

Fig. 6 Effect of Yokukan-san (▨) and diazepam (▩) on isolation stress-enhanced locomotion of mice. Locomoter activity was measured in mice group-housed (□) or socially isolated (■; $n = 5$) for 2 weeks by video camera under unmanned conditions. Mice were given Yokukan-san (2.0 g/kg, p.o.) or diazepam (1.6 mg/kg, i.p.) for 4 days before the measurement. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle (control of the same group; two-way repeated-measures ANOVA).

hypothesis comes from clinical studies, including stressful life events often preceding the exacerbation of AD, and daily emotional stress (such as a rigid family structure or negative communication with significant others) predicting symptom severity in children and adults with AD.[4–9] Experimental studies also support these findings. For example, fundamental cutaneous reactions, such as epidermal cell proliferative activity and lipogenesis in sebaceous glands, were reduced in male Syrian hamsters exposed to immobilization-induced stress.[29,30]

It is well known that complex alterations of the autonomic nerve, immune and endocrine systems are modulated by psychosocial stress. Psychological or physiological stresses can stimulate the hypothalamic–pituitary–adrenal (HPA) axis, sympatho–adrenomedullary system and sympathetic nervous system, but inhibit the hypothalamic–pituitary–testicular axis.[13–15,31] Corticotropin-releasing hormone (CRH), which could be activated by isolation stress, was observed to induce skin mast cell degranulation and increase vascular permeability.[32,33] It is known that mast cells are responsible for skin reactions and, in particular, IPR and vLPR in the triphasic cutaneous reaction model. Therefore, the isolation stress-induced exacerbation of the skin reaction may be associated with an increased production of glucocorticoid and catecholamines, decreased testosterone levels, overactivity of CRH or a combination of these changes.

There is now convincing evidence that eosinophilia is dependent on T cells, particularly T helper (h) 2-type cells, which selectively secrete interleukin (IL)-5 and IL-4.[34] A system shift in cytokine balance towards Th2 proliferation is regarded as a main dysfunction in AD pathology. Previous studies have indicated that an increase in serum levels of glucocorticoid through the HPA axis by isolation stress supported a Th2-type cytokine profile.[35] For example, oversecretion of IL-4 was observed in socially isolated mice.[36] Considering our previous finding that a third-phase cutaneous response

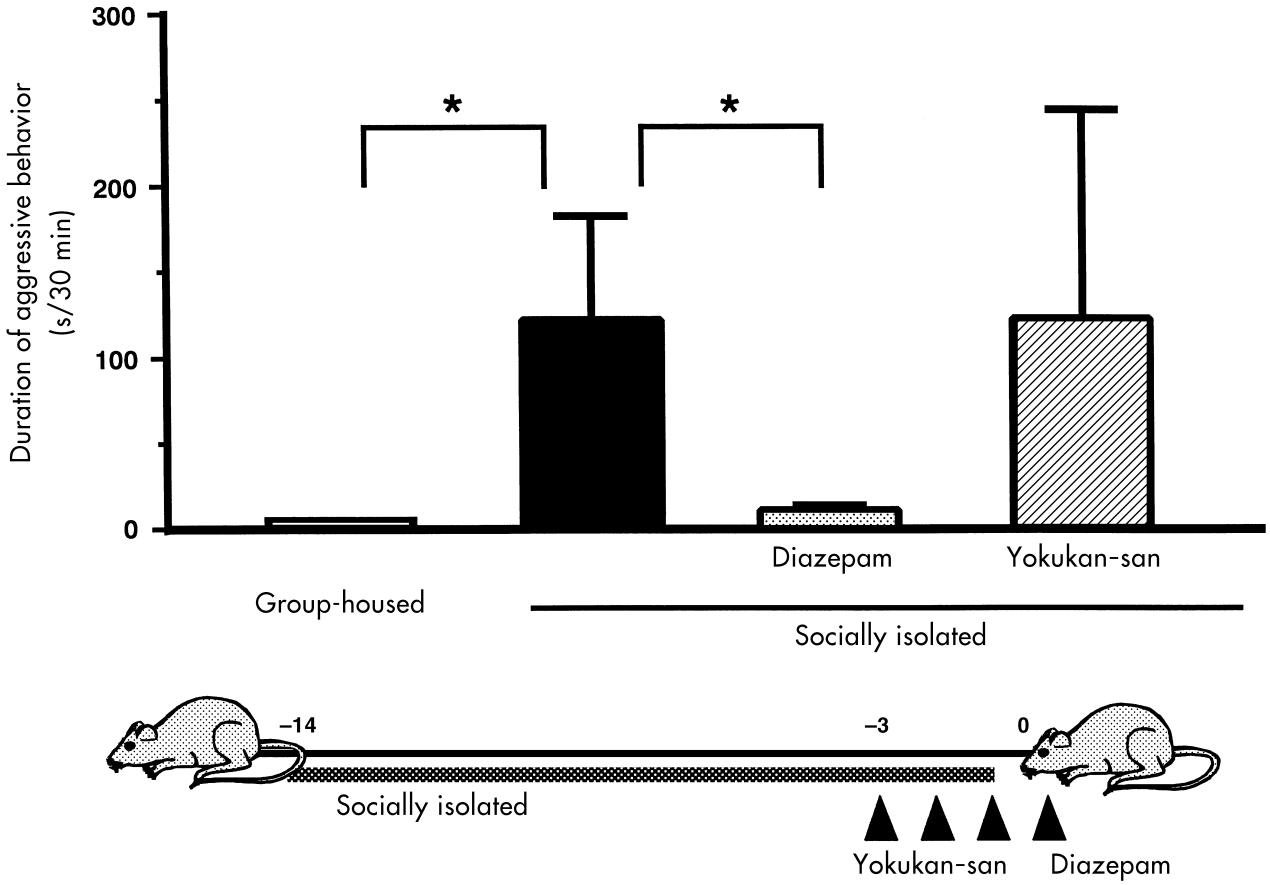

Fig. 7 Effect of Yokukan-san (▨) and diazepam (▩) on isolation stress-increased aggressive behavior. Aggressive behavior was determined in mice group-housed (□) or socially isolated (■; $n = 5$) for 2 weeks by video camera under unmanned conditions. Mice were given Yokukan-san (2.0 g/kg, p.o.) or diazepam (1.6 mg/kg, i.p.) for 4 days before the measurement. *$P < 0.01$ compared with vehicle (control of same group; Student's two-tailed *t*-test).

(vLPR) with massive infiltration of eosinophils was mainly mediated by T cells, isolation stress-enhanced vLPR may be partly involved in a shift to a Th2-type cytokine profile facilitated by stress.

Recently, because of the close anatomic relationship between mast cells and nerve endings, a potential role of innervation and neuropeptides in atopic dermatitis has been proposed. Increased levels of vasoactive intestinal polypeptide (VIP) and substance P (SP) in AD patients are known to induce mast cell degranulation.[37–39] Glucocorticoids have also been shown to upregulate leukocyte VIP receptors on human mononuclear leukocytes.[40] Furthermore, increased levels of VIP and SP were found to be evoked by stress.[41,42] Thus, understanding the role of stress-induced neuropeptides, such as VIP and SP, may provide one more basis for analyzing the mechanism for isolation stress-enhanced triphasic cutaneous reactions. However, considering the possible links between stress and skin functions, the model for the IgE-mediated triphasic cutaneous reaction in socially isolated mice appears to be useful for investigating the relationship between the allergic inflammatory reaction and psychosocial stress and the underlying mechanisms involving the central nervous, endocrine and immune systems.

Yokukan-san formulation has anticonvulsive, sedative and analgesic properties, including preservation of emotional balance.[21,28] It is currently administered to small children with crying fits during the night, patients with convulsions due to fever, twitching and jerking of muscles, mania, insomnia and neurological symptoms. As shown in Figs 3,4, oral administration of Yokukan-san dose-dependently inhibited the enhancement of IPR, LPR and vLPR in socially isolated mice, although it did not show any such inhibition in group-housed mice. In

contrast, the typical tranquilizer diazepam, which acts on both central and peripheral benzodiazepine receptors, reduces anxiety and inhibits the stress-induced increase in anterior pituitary hormone secretion, including adrenocorticotropic hormone, corticosterone, as well as behavior-associated epinephrine.[27,43,44] Intraperitoneal administration of diazepam dose-dependently inhibited the enhancement of IPR and LPR in socially isolated mice, but markedly exacerbated vLPR of both group-housed and socially isolated mice, which differs from the inhibitory effects of Yokukan-san on vLPR.

In addition to the anti-allergic efficacy of Yokukan-san and diazepam in socially isolated mice, diazepam also showed its inhibitory effect on both locomotor and aggressive behavior stimulated by stress, while Yokukan-san exhibited a suppressive effect on the former but not the latter. Thus, Yokukan-san and diazepam exhibited different patterns of inhibitory effects on isolation stress-enhanced triphasic cutaneous reactions and stress-evoked behavioral disturbances. The mechanism underlying the inhibitory effects of Yokukan-san and diazepam on the triphasic cutaneous reaction in socially isolated mice will still need to be examined in detail.

In conclusion, the results of the present study clearly demonstrate that social isolation stress may exacerbate IgE-mediated triphasic skin reactions, including IPR, LPR and vLPR. Yukukan-san and diazepam, antipsychotic medicines, can attenuate the stress-enhanced cutaneous reaction partly due to their sedative action via different mechanisms of action. It is suggested that the model of IgE-mediated triphasic cutaneous reaction in socially isolated mice presents a useful tool for investigating the effect of antistress agents on stress-exacerbated cutaneous reactions, as well as for examining the underlying mechanisms.

Acknowledgments

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