Development of an Assay Method to Search for Compounds Inhibiting Stress-Enhanced Allergy
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Stress exacerbates allergic disorders such as atopic dermatitis and asthma. It is also an important factor affecting blood flow (BF). Allergic reactions also affect blood flow. For example, we observed that mice sensitized with hen egg-white lysozyme (HEL) have decreased BF during the allergy induction phase. Based on this finding, we established a model for evaluating chronic restraint stress-enhanced allergies. Mice were sensitized with 12.5 µg/head of HEL on day 0, then restrained for 90 min daily on days 1–3, 5, and 6 in a modified 50 mL polystyrene conical centrifuge tube with multiple air holes for ventilation. We used the decrease in BF during that time as a guide for developing an in vivo assay for substances that can inhibit stress-enhanced allergies. Finally, we demonstrated the utility of the new method by testing crude drugs that are used solely or in combination with other crude drugs to treat stress-related illness and neuropsychiatric symptoms. Our model should be useful for identifying potential anti-stress-enhanced allergy drugs.

Key words allergy; chronic restraint stress; blood flow; crude drug; egg-white lysozyme

Allergic diseases, such as atopic dermatitis and asthma, become severe or relapse due to stress. However, there have been no systems that can evaluate allergy enforced by stress.

As stress is an important factor affecting blood flow (BF), we have developed a new assay system based upon our findings1–3 on BF decrease in the induction phase of allergy in the tail vein of mice sensitized by use of hen egg-white lysozyme (HEL). We previously developed an in vivo assay to search natural resources for allergy-preventive substances. We used it to assess BF during the allergy induction phase in the tail veins of mice that have been sensitized to HEL. We used this assay to analyze the mechanism of stress-enhanced allergies and will contribute to the search for new treatments.

MATERIALS AND METHODS

Materials Complete Freund’s adjuvant (CFA), HEL, disodium cromoglycate (DSCG), and crude drugs were purchased from DIFCO (MI, U.S.A.), Sigma Co., Ltd. (St. Louis, MO, U.S.A.), Funakoshi Co., Ltd. (Tokyo, Japan), and Tochimoto Tenkaido Co., Ltd. (Osaka, Japan), respectively.

Animals Male ddY mice (SPF grade), 5 weeks old, were obtained from Japan SLC, Inc. (Shizuoka, Japan) and housed at 26±2°C. Food and water were available ad libitum. All animal experiments were performed in accordance with the Guidelines for Animal Experiments of Mukogawa Women’s University.

HEL Sensitization Sensitization with HEL was performed as previously described10 with slight modifications. On day 0, male ddY mice were injected intraperitoneally with 12.5, 25, and 50 µg of HEL in 50% CFA.

BF Measurement Subcutaneous BF in mice tail veins was monitored using a laser Doppler blood flow meter (FLO-C1; Neuroscience, Tokyo, Japan) as previously reported.2 Each mouse was warmed for 15 min at 36°C prior to the experiment and then placed on a holder (Muromachi Kikai Co., Ltd., Tokyo, Japan) in a measuring chamber kept at 36°C throughout the measurement. The systolic microcirculatory BF in the tail veins of unanesthetized mice was measured (n=5). Normal blood flow was measured for 10 min 1d before studies began. The BF of the sensitized mice was measured for 10 min every other day up to 7–10 d after sensitization. The results were expressed as a relative percent of normal BF for each mouse.

Protocol and Schedules of Chronic Restraint Stress The stress load was applied using a modification of the method described by Castilla-Ortega et al.14 Mice in the stressed groups were restrained in a modified 50 mL clear polystyrene conical centrifuge with multiple air holes for ventilation. Three schedules of restraint were used. Schedule I: Mice were restrained 90 min daily for 10 consecutive days. Schedule...
II: Mice were restrained 90 min daily on days 1–3 and 5–8. Schedule III: Mice were restrained 60 min daily on days 1–3 and 5–9. Unstressed mice remained undisturbed in their home cages.

Measurement of Serum Corticosterone Levels After the final day of stress, blood from the eyebground venous plexus of each mouse was collected and centrifuged (3000 rpm, 15 min, 20°C). The corticosterone level (pg/mL) in the supernatant serum was measured using a Cortisol Express EIA Kit (Cayman Chemical Co., MI, U.S.A.).

Crude Drugs Crude drugs (20 g each), Poria sclerotium (sclerotium of *Poria cocos*; PS, lot No. 009507001), cinnamon bark (bark of *Cinnamomum cassia*; CB, lot No. 002814004), bupleurum root (root of *Bupleurum falcatum*; BR, lot No. 004210011), magnolia bark (bark of *Magnolia obovate*; MB, lot No. 003608005), Japanese valerian (root and rhizome of *Valeriana fauriei*; JV, lot No. 001807001), and Japanese peppermint (herb of *Mentha arvensis* var. *piperascens*; JP, lot No. 00130504) were extracted overnight with 30% EtOH (60 mL) at room temperature, then filtered. The filtrate was evaporated *in vacuo* to yield crude drug extracts (0.41, 0.66, 0.73, 3.39, 6.33, and 6.41 g, respectively). These extracts were suspended in distilled water and used for bioassays.

Effects of Crude Drugs and an Anti-allergic Agent All of the crude drugs were administered orally (200 mg/kg) at 0 (1 h before sensitization), 3, and 6 d as previously reported. An anti-allergic agent (DSCG, 10 mg/kg) was injected on days 0, 3, and 6 as previously reported. Water was administered to the control group on the same schedule.

Statistical Analysis Statistical analysis was performed using Dunnett’s multiple range test coupled with Bonferroni inequality for significant differences between each test group and the control group. For the Bonferroni test, 5 points were used 4 d after the HEL sensitization, because a significance difference was observed between the BF of untreated and sensitized mice after 4 d.

RESULTS AND DISCUSSION

Effects of Chronic Restraint Stress on BF Decrease after Sensitization with HEL (50 µg/Head) Mice sensitized with HEL (50 µg/head) and restrained according to schedule I showed a decrease in BF, but the effect was not significantly different from BF decrease in unrestrained sensitized mice (Fig. 1). The BF decrease in the unrestrained sensitized group was too drastic to evaluate the effect of stress on allergy. Then, we experimented to find an amount of dose of antigen for the induction of a milder decrease of BF to evaluate effects of the stress in the BF.

Dose of HEL and BF Decrease The BF decrease for mice sensitized with HEL 12.5, 25, and 50 µg/head is shown in Fig. 2. While BF decrease was small but partial in the

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**Fig. 1. Effect of Chronic Restraint Stress on BF in HEL (50 µg/Head)-Sensitized Mice**

Each point with bars represents the mean ± S.E. of BF of five mice. ○, only HEL-sensitized mice (sens.); ■, chronic restraint stress enhanced HEL-sensitized mice (sens.+stress).

**Fig. 2. Effect of Concentration of HEL on BF Decrease in HEL-Sensitized Mice**

Each point with bars represents the mean ± S.E. of BF of five mice. HEL concentration is 0 (○, normal), 12.5 (●), 25 (△), and 50 (▲) µg/head. *p < 0.05 compared with normal (Dunnett’s test with Bonferroni).

**Fig. 3. Effect of Chronic Restraint Stress on BF in HEL (12.5 µg/Head)-Sensitized Mice**

Each point with bars represents the mean ± S.E. of BF of five mice. ○, only stress enhanced mice (normal); ▲, only stress enhanced mice; △, only HEL-sensitized mice; ●, stress enhanced HEL-sensitized mice. *p < 0.05 compared with the normal (Dunnett’s test with Bonferroni).
untreated group, it increased as the dose of HEL was raised, demonstrating that BF was dependent on the dose of HEL. We concluded that stress-enhanced allergy could be evaluated by reducing the dose of HEL to 12.5 µg/head.

**Effects of Chronic Restrained Stress on BF after Sensitization with HEL (12.5 µg/Head)** As shown in Fig. 3, the BF of mice that were sensitized with HEL (12.5 µg/head) and restrained (schedule I) gradually decreased. By day 10, it was significantly decreased compared to the BF in the untreated group. The BF in mice that were only sensitized or only stressed (schedule I) was the same as that of the untreated group. These results suggest that we can evaluate stress-enhanced allergy using the decrease in BF as a guide.

**Modification of Stress Schedule** We studied the stress schedule, looking for ways to provide relief for the mice and expedite the method. Schedule II reduced the number of days of stress, and schedule III reduced the number of days of stress and shortened the daily duration of the tests. For both schedules II and III, mice did not receive restraint stress on day 4. If restraint stress was canceled on days 2 or 3, we were concerned about the possibility of BF recovering from chronic stress as enough decrease of BF was not yet established. We decided to cancel days 4 and 5 (the early stage in the second half) when enough decrease of BF was established. For both schedules, the BF of mice in the stressed groups was significantly decreased compared with the untreated group (Fig. 4). Mice subjected to schedule II experienced a significant decrease in BF on day 5. For mice on schedule III, a significant decrease in BF on day 5. For mice on schedule III, a significant decrease in BF on day 5.

![Graph showing the effect of schedules II and III for chronic restraint stress on BF decrease](image)

Each point with bars represents the mean±S.E. of BF of five mice. △, untreated mice (normal); ○, schedule II stress enhanced HEL-sensitized mice; ●, schedule III stress enhanced HEL-sensitized mice. *p<0.05 compared with the normal (Dunnett’s test with Bonferroni).

![Graph showing the effect of stress-schedules II and III on serum corticosterone levels](image)

Data represent the mean±S.E. of five mice. Untreated (normal), schedule II stress enhanced HEL-sensitized (II), and schedule III stress enhanced HEL-sensitized mice (III) were serum measurement corticosterone levels at day 10. *p<0.05 (Dunnett’s test).

![Graph showing the effect of stress-schedules II and III on body weight](image)

Data represent the mean±S.E. of five mice. Body weight of untreated (normal), schedule II stress enhanced HEL-sensitized (II), and schedule III stress enhanced HEL-sensitized mice (III) on days 5 and 7. *p<0.05 compared with the normal (Dunnett’s test).

![Graph showing the assessment of reproducibility](image)

Data represent the mean±S.E. of five mice. The reproducibility could be confirmed, since significant BF decrease was found on day 5 (a) or day 7 (b) for all experiments. a, b: *p<0.05 compared with the normal BF of each mouse (Dunnett’s test with Bonferroni).
CONCLUSION

We could develop a new in vivo assay system for evaluation of the effect of the stress on allergy. Crude drugs that used solely or in combination with other crude drugs as remedy for the disease caused by stress did not show an appreciable effect on the BF decrease by the assay method based only on the sensitization established previously, but the present assay method showed a significant improvement of the BF decrease. Therefore, it indicates that the present system is applicable to evaluate the complex form of allergy, stress, and inflammation.

Thus, this system would contribute to the analysis of the mechanism of allergy enhanced by stress and the search for new substances effective to stress-enhanced allergy. Furthermore, it would be applicable to the analysis of the mechanisms of the enhancement of allergy by stress.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES


