

Full Paper

A Late Cutaneous Response in Actively Sensitized Rats: a New Method for Evaluating the Efficacy of Antiallergic Drugs

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Abstract. We established a new and facile model to investigate allergic mechanism and assess the effect of antiallergic compounds. Male Wistar rats were actively or passively sensitized. Active sensitization was performed by injection of both dinitrophenylated-ovalbumin (DNP-OA) and *Bordetella pertussis*. Nine days later, DNP-OA was injected into the right hind footpad. This antigen challenge induced a biphasic footpad swelling that consisted of an early-phase (EPR) and a late-phase response (LPR). In rats passively sensitized with rat anti-DNP-OA serum, DNP-OA induced only EPR. The EPR was suppressed by disodium cromoglycate, a mast cell stabilizer, but not by cyclosporin A, an immunosuppressant, while the LPR was suppressed by cyclosporin A. Furthermore, to investigate these two allergic responses determined by the interactions between the hapten and the carrier proteins, two distinct haptenated antigens were created. DNP-Ascaris (DNP-As) induced a marked EPR and LPR in DNP-As-sensitized rats. However, DNP-As induced only EPR in DNP-OA-sensitized rats, indicating that the usage of the same carrier protein in both sensitization and challenge was necessary for induction of LPR. These data suggest that this active sensitization model in which EPR and LPR are functionally distinguishable should be useful for evaluating the efficacy of antiallergic compounds.

Keywords: allergy, late-phase response, T cell, early-phase response, mast cell

Introduction

Antigen-induced airway obstruction is characterized by acute bronchoconstriction that occurs within several minutes after antigen challenge in asthmatic patients. This rapid response, or early-phase response (EPR), is followed by a prolonged inflammatory response that occurs at 4 to 8 h after provocation and sometimes persists for 24 h or more. This prolonged response with late onset is called the late-phase response (LPR). It is generally accepted that LPR plays a more important role in the pathogenesis of allergic diseases because of its correlation with the disease severity (1). Auty pointed out that the therapeutic effects of anti-asthmatic drugs correlated with their effects on LPR rather than EPR, based on the results of various clinical trials in asthma (2).

Disodium cromoglycate (DSCG) made the first therapeutic achievement as an antiallergic drug for asthma, rhinitis, and conjunctivitis. DSCG has been used only as a topical administration due to its poor absorption in the gastrointestinal tract. The unique profile and success of DSCG have encouraged many pharmaceutical companies to direct tremendous effort towards the discovery of an oral DSCG-like compound. However, no products have yet been marketed in Europe or the US as an oral DSCG-like compound, except that nedocromil has been used as an inhaler. Since DSCG is thought to inhibit the IgE-mediated activation of mast cells (3), most pharmaceutical companies have evaluated histamine release and passive cutaneous anaphylaxis mediated by IgE antibody as screening procedures. Nevertheless, new mast cell-stabilizing compounds after DSCG have shown no notable efficacy in clinical trials. Auty stated that DSCG inhibited both EPR and LPR, and most mast cell stabilizers inhibited antigen-induced EPR, but failed to

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inhibit LPR (2). In contrast, corticosteroids markedly inhibited LPR with no significant actions on EPR in an antigen provocation study in asthmatic patients (4).

Considering these findings, antiallergic drugs should be evaluated using a model of LPR in addition to EPR. An appropriate animal model, in which a characteristic efficacy of allergic drugs such as DSCG for EPR and LPR can be obviously detected, has been required. In addition, its pathophysiological conditions need to be estimated. In this study, we developed a novel and facile rat model of allergic responses and assessed the underlying mechanism.

Materials and Methods

Animals

Five-week-old male Wistar rats were purchased from SLC Japan (Hamamatsu), and five-week-old male Brown Norway (BN) rats were purchased from Charles River Japan (Yokohama). They were acclimated for at least one week. The rats were housed in wire-mesh cages and provided with water and food ad libitum. The animal room was maintained under the following conditions: temperature of 21°C – 25°C, relative humidity of 50% – 70%, and a 12-h light/dark cycle (light on 7:00 to 19:00). All experimental protocols were approved by the ethical committee for animal experiment in Meiji Seika Pharmaceutical Research Department, and they were conducted in accordance with the guidelines of The Japanese Pharmacological Society.

Reagents and drugs

The following reagents and drugs were obtained from commercial sources.

Reagents: chicken egg albumin (OA) (Sigma Chemical Co., St. Louis, MO, USA), *Bordetella pertussis* (Chibaken Kessei Kenkyujo, Ichikawa), dinitrobenzene sulfonic acid (Tokyo Kasei Kogyo Co., Tokyo), and Evans blue (Nacalai Tesque, Kyoto).

Drugs: disodium cromoglycate (DSCG) and promethazine hydrochloride (Sigma Chemical Co.), cyclosporin A (Novartis Pharmaceuticals Co., Tokyo), tranilast (Kissei Pharmaceutical Co., Matsumoto), repirinast (Mitsubishi Kasei Co., Tokyo), amlexanox (Takeda Pharmaceutical Co., Osaka), and pemirolast potassium (Mitsubishi Pharma Co., Tokyo). These drugs were suspended in 1% methylcellulose (MC) for oral administration or dissolved in saline for subcutaneous administration.

Sensitization and antigen challenge

Dinitrophenylated ovalbumin (DNP-OA) and dinitrophenylated *Ascaris* extract (DNP-As) were prepared by

the method of Eisen et al. (5) and used as antigens. Rat anti-DNP-OA serum was prepared by the method of Tada and Okumura (6). We chose BN rat to obtain high titer IgE antibodies according to Pauwels et al. (7). BN rats were sensitized by injections of 10^{10} killed *Bordetella pertussis* organisms and 1 mg of DNP-OA into all four footpads. Five days after the first sensitization, the rats were boosted by intramuscular injections of 0.5 mg of DNP-OA in the back. Three days after the booster injection, blood was collected by aortic puncture, and the pooled antiserum was stored at –80°C. The antibody titer of this serum was 1:2000 as estimated by 48-h passive cutaneous anaphylaxis (PCA). PCA was performed as follows: Wistar rats were subcutaneously sensitized with serial dilution of serum (0.05 ml/site) in their back skin, and DNP-OA (5 mg/kg) and Evans blue (12.5 mg/kg) were intravenously administered to elicit PCA 48 h after the sensitization. In the case of more than 5-mm-diameter blue spot, PCA was considered as positive. We ascertained that the serum heated at 56°C for 30 min was inactive in PCA.

Wistar rats were passively sensitized by intravenous injections of this antiserum in a volume of 1 or 2 ml. Two days later, the antigen was injected into the footpad of the right hind limb. Wistar rats were also actively sensitized by the injection of 10^{10} killed *Bordetella pertussis* organisms and 1 mg of the antigen into three footpads excluding the right hind limb, according to the method of Tada and Okumura (6). Nine days after the sensitization, rats were intradermally injected with 3 µg of antigen in saline (0.05 ml) into the footpad of the right hind limb, unless otherwise noted.

Assessment of the effects of antiallergic drugs on footpad swelling

The footpad swelling volume was measured with a plethysmometer (Ugo-Basile, Comerio, Italy) before and 20 min, 2, 4, 6, 8, 10, 12, and 24 h after antigen challenge. The swelling volume was calculated by subtracting pre-challenge volumes from each time point level. The inflammatory responses at 20 min and 8 h after antigen challenge were defined as EPR and LPR, respectively. DSCG, promethazine, or cyclosporin A was subcutaneously injected 10 min before antigen challenge. Tranilast, repirinast, amlexanox, or pemirolast was orally administered 15 min before antigen challenge.

Statistical analyses

Results were expressed as the mean ± S.E.M. For comparisons of two groups, a two-tailed Student's *t*-test was used. For the comparisons among several groups, Dunnett's multiple comparison test was used.

Differences were considered significant if the *P* value was less than 0.05.

Results

Comparison of active and passive sensitization in the induction of antigen-induced footpad swelling

Figure 1 shows the time course of changes in footpad swelling volume. DNP-OA challenge induced a marked biphasic footpad swelling that peaked at 20 min (0.45 ± 0.02 ml) and again at 8 h (0.54 ± 0.05 ml) in actively sensitized rats. In contrast, passively sensitized rats showed a monophasic response that peaked at 20 min after antigen challenge, and then this response gradually declined. In actively sensitized rats, the PCA titer elicited by DNP-OA was 1:16–64, whereas that

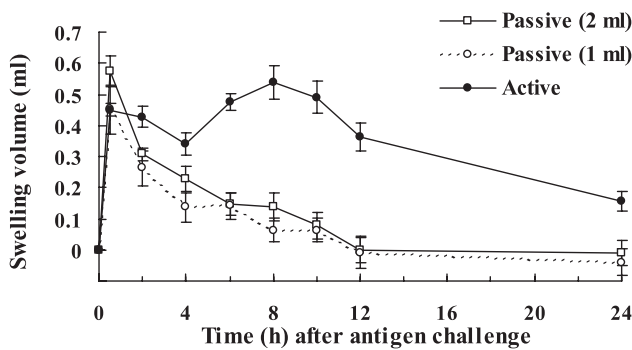


Fig. 1. Comparison of time-dependent swelling volume between passively and actively sensitized rats. Time course of changes in swelling volume were measured in passively and actively sensitized rats. Rats were passively sensitized with anti DNP-OA antiserum in a volume of 1 ml (open circle) or 2 ml (open square) or actively sensitized (closed circle). The footpad swelling was induced by subcutaneous injection of DNP-OA ($3 \mu\text{g}$). Data are expressed as the mean $\pm$ S.E.M. from 5 or 6 rats.

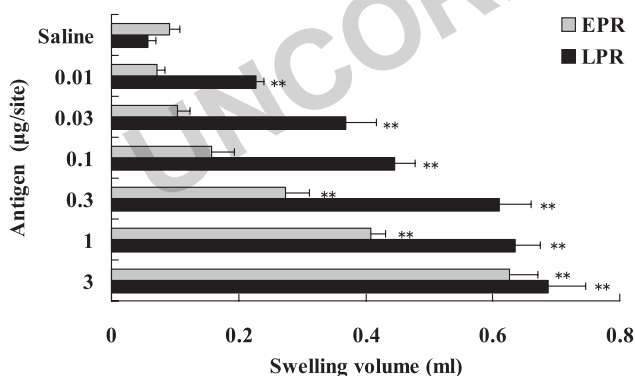


Fig. 2. Antigen dose-response study. Swelling volumes were measured at 20 min (EPR) and 8 h (LPR) after subcutaneous injection of saline or DNP-OA ($0.01 - 3 \mu\text{g}/\text{site}$) in actively sensitized rats. Data are expressed as the mean $\pm$ S.E.M. from 5 rats. $**P < 0.01$, compared to the saline group (Dunnett's multiple comparison test).

elicited by OA was less than 1:4.

Antigen dose-response study

Various doses of DNP-OA were administered to actively sensitized rats. The swelling volumes at 20 min and 8 h after antigen challenge increased in a dose-dependent manner (Fig. 2). Low doses of antigen (0.01 to $0.1 \mu\text{g}/\text{site}$) induced LPR without EPR.

Dependence of the induction of LPR on the carrier protein used in sensitization

This study was designed to examine the specificity of a hapten (DNP) and carrier proteins (OA or As) for the induction of LPR. Rats were divided into four groups based on the two kinds of antigens used for sensitization and challenge. Both EPR and LPR were induced by injection of DNP-OA in DNP-OA-sensitized rats (Fig. 3). Although DNP-As induced a marked EPR in DNP-OA-sensitized rats, LPR was not observed. Likewise, while DNP-As induced both EPR and LPR in DNP-As-sensitized rats, DNP-OA failed to induce LPR in DNP-As-sensitized rats.

Effects of DSCG, promethazine, cyclosporin A, or mast cell stabilizers on antigen-induced footpad swelling

DSCG ($5 \text{ mg}/\text{kg}$), promethazine ($1 \text{ mg}/\text{kg}$), or cyclosporin A ($5 \text{ mg}/\text{kg}$) was subcutaneously administered 10 min before antigen challenge. Both DSCG and promethazine significantly inhibited EPR ($P < 0.01$), but failed to inhibit LPR. In contrast, cyclosporin A inhibited LPR without affecting EPR (Fig. 4). Four kinds of mast cell stabilizers, tranilast ($300 \text{ mg}/\text{kg}$), repirinast ($300 \text{ mg}/\text{kg}$), amlexanox ($150 \text{ mg}/\text{kg}$), and pemirolast ($20 \text{ mg}/\text{kg}$), inhibited EPR, whereas LPR

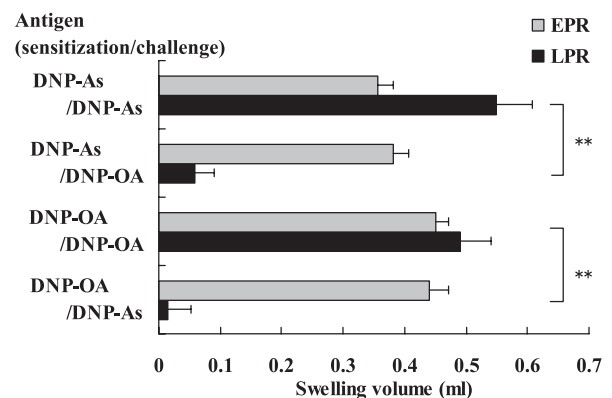


Fig. 3. Dependence of LPR on the carrier proteins used for sensitization and challenge. EPR and LPR were measured in actively sensitized rats. Rats were sensitized with either DNP-OA or DNP-As and then challenged with either of the antigens. Data are expressed as the mean $\pm$ S.E.M. from 5 rats. $**P < 0.01$ (Student's *t*-test).

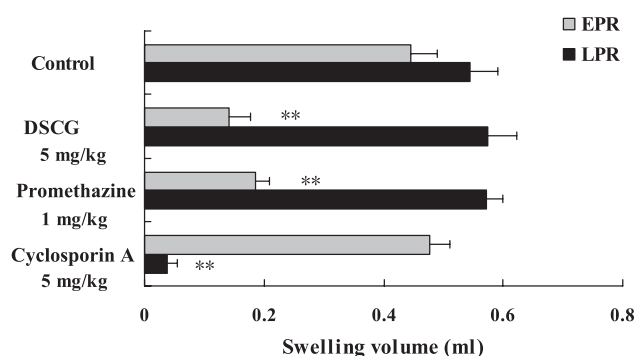


Fig. 4. Effects of DSCG (5 mg/kg), promethazine (1 mg/kg), or cyclosporin A (5 mg/kg) on EPR and LPR in actively sensitized rats. Drugs were subcutaneously administered 10 min before antigen challenge. Data are expressed as the mean $\pm$ S.E.M. from 5 rats. $**P < 0.01$, compared to the control group (Dunnett's multiple comparison test).

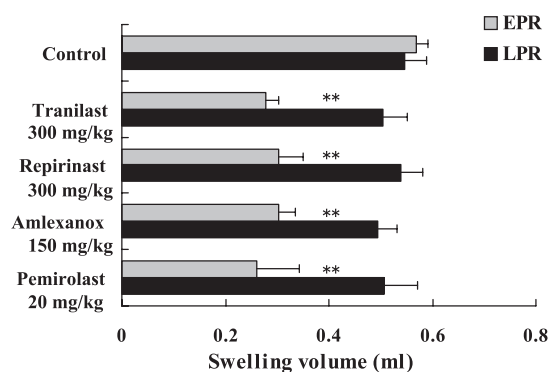


Fig. 5. Effects of mast cell stabilizers, tranilast (300 mg/kg), repirinast (300 mg/kg), amlexanox (150 mg/kg), or pemirolast (20 mg/kg) on EPR and LPR in actively sensitized rats. Drugs were orally administered 15 min before antigen challenge. Data are expressed as the mean $\pm$ S.E.M. from 5 rats. $**P < 0.01$, compared to the control group (Dunnett's multiple comparison test).

was unaffected (Fig. 5). A high dose of DSCG (100 mg/kg) significantly inhibited both EPR and LPR ($P < 0.01$). The effect of DSCG (100 mg/kg) on LPR was weaker than that on EPR (Fig. 6).

Discussion

We developed a new model in which typical allergic responses were shown and the reasonable potency of several drugs was exhibited. Intradermal antigen injection induced a marked biphasic footpad swelling, EPR and LPR, in actively sensitized rats (Fig. 1). The EPR reached its maximum at 20 min and gradually declined until 4 h after antigen challenge. This was significantly inhibited by mast cell stabilizers or H_1 -receptor antagonist (Figs. 4 and 5). Several substances such as

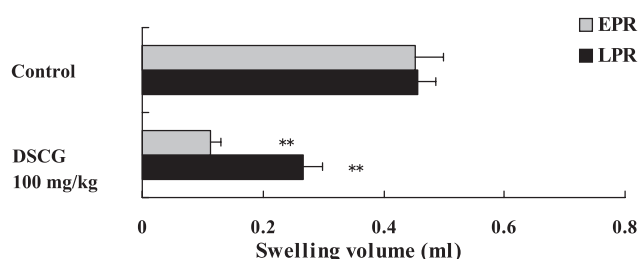


Fig. 6. Effect of a high dose of DSCG (100 mg/kg) on EPR and LPR in actively sensitized rats. DSCG was subcutaneously administered 10 min before antigen challenge. Data are expressed as the mean $\pm$ S.E.M. from 5 rats. $**P < 0.01$, compared to the control group (Student's *t*-test).

histamine, serotonin, and arachidonate metabolites released from mast cells are known to be the primary chemical mediators of type I allergic reaction (8) and, when released from mast cells, cause increased local vascular permeability. We also detected EPR in passively sensitized rats. Our data suggest that the EPR in this model is mediated, at least in part, by histamine released from mast cells. The EPR was followed by LPR that peaked at 8 h. The LPR was markedly suppressed by cyclosporin A, but not by either mast cell stabilizers or H_1 -receptor antagonist (Fig. 4). It is known that cyclosporin A inhibits T cell proliferation, cytotoxicity, and cytokine production (9), but does not inhibit an IgE-mediated PCA reaction (10). Taken together, it is strongly suggested that the EPR and LPR in our model have distinguishable pharmacological features that are similar to those observed in human allergic diseases (11, 12). Moreover, a low dose of antigen induced only LPR without EPR (Fig. 2). There was a distinct difference in a responsivity to the antigen concentration between the induction of EPR and LPR. These results were supported by the evidence that human CD4⁺T cells stimulated with low concentrations of antigen (3–10 ng/ml) released high levels of IL-4 in vitro and that the same T cells with high concentrations of antigen (10–30 μ g/ml) resulted in minimal IL-4 synthesis (13). In addition, a high concentration of antigen (>1 μ g/ml) is generally necessary for mast cell degranulation in vitro. The induction of EPR depended only on the hapten (DNP), while that of LPR depended on the identity of both the hapten and the carrier proteins (OA or As) used in sensitization and challenge (Fig. 3). These data demonstrate that EPR and LPR are regulated by different mechanisms of antigen recognition in this model.

T cells have been recognized to play a central role in the induction of LPR and prolonged inflammation in allergic diseases. This notion is supported by the observation that FK506 and cyclosporin A inhibited

the antigen-induced late asthmatic response, which is mainly regulated by Th2 cytokines such as IL-4, 5, 9, and 13 (14). LPR depended on both the hapten and the carrier proteins (Fig. 3). It is well known that the haptenated protein plays an important role in the production of hapten-specific antibody (15), lymphotoxin (16), and cytokines (17). These phenomena are predominantly mediated by lymphocytes, especially T cells, that recognize the carrier protein (18). Accordingly, LPR in our experiment is presumably modulated by inflammatory mediators like Th2 cytokines and chemokines released from T cells.

LPR is thought to be a more essential component in the pathogenesis of allergic diseases than EPR because of its correlation with the severity of these diseases and also with the results in clinical trials of antiallergic drugs (19, 20). A high (100 mg/kg), but not a low (5 mg/kg), dose of DSCG suppressed both EPR and LPR (Figs. 4 and 6). In addition, as for high dose of DSCG, the effect on LPR was weaker than that on EPR. It was reported that DSCG inhibited both EPR and LPR, but most mast cell stabilizers inhibited only EPR and showed no effect on LPR (2, 21, 22). The concentration of DSCG required to suppress cytokine production from T cells was 100- to 1000-fold higher than that required for mast cell stabilization (23). These reports are entirely consistent with our present data. Kuriyama et al. demonstrated that DSCG inhibited type III (Arthus) allergic reaction that is not related to mast cells (24). These findings confirm that the clinical efficacy of DSCG depends not only on its ability to stabilize mast cells but also on its distinctive anti-inflammatory activity. These unique actions of DSCG on LPR have been thought to be the first reason for its acceptance in the clinical setting for a long time. The LPR described here, in which the effect of DSCG was clearly detected, would be a pharmacologically reasonable process for elucidating predisposing factors and screening therapeutic agents.

Antiallergic drugs should be evaluated with an experimental model of LPR in addition to EPR. We previously established an airway hyperresponsiveness and inflammation model in rats using the same sensitization method as in the present study (25). In that model, both cyclosporin A and a high dose of DSCG attenuated the airway hyperresponsiveness and reduced the number of infiltrated eosinophils and T cells, but several mast cell stabilizers and H₁-receptor antagonists did not affect (Shishikura et al., unpublished data). These patterns of drug efficacy were consistent with the present results in LPR. Thus, our procedure for sensitization and challenge would be applicable to various models of allergic diseases such as conjunctivitis,

rhinitis, dermatitis, and asthma.

In conclusion, we established a new model of allergic responses in actively sensitized rats and pharmacologically characterized the regulatory mechanisms in EPR and LPR. Drugs that target against EPR and LPR, especially prolonged inflammation, may be helpful in the control of inflammatory conditions in allergic diseases (26–28). This model of late cutaneous response should be useful for evaluating antiallergic drugs. Further investigations to determine the precise role of T cells and related mediators in the induction of LPR are currently in progress.

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