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Loxoprofen sodium induces the production of complement C5a in human serum

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ABSTRACT

Basophil activation test (BAT) is an in vitro allergy test that is useful to identify allergens that cause IgE-dependent allergies. The test has been used to detect not only food allergies and allergies caused by environmental factors but also to detect drug hypersensitivity, which has been known to include IgE-independent reactions. In our preliminary studies in which BAT was applied to detect hypersensitivity of loxoprofen, a non-steroidal anti-inflammatory drug (NSAID), conventional BAT with incubation for 30 min did not show basophil activation by means of increased CD203c expression. In this study, we extended the incubation time to 24 h on the basis of the hypothesis that loxoprofen indirectly activates basophils. Basophils from healthy control donors as well as allergic patients showed up-regulation of CD203c after incubation with loxoprofen for 24 h. Activation was induced using loxoprofen-treated serum. Proteomic and pharmacologic analyses revealed that serum incubation with loxoprofen generated an active complement component C5a, which induced CD203c expression via binding to the C5a receptor on basophils. Because C3a production was also detected after incubation for 24 h, loxoprofen is likely to stimulate the complement classical pathway. Our findings suggest that the complement activation is involved in drug hypersensitivity and the suppression of this activation may contribute to the elimination of false positive of BAT for drug allergies.

1. Introduction

The allergen-specific IgE test is a common diagnostic tool for identification of allergens in IgE-dependent allergies [1,2]. The test has been improved in terms of the panel of allergens that can be tested [3,4], but it would be difficult to cover all allergens in this test. Allergens include many kinds of drugs, which, under specific conditions, can cause severe and fatal diseases such as Stevens–Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug-induced hypersensitivity syndrome (DIHS) [5–8]. One of the reasons for the test not being comprehensive is the rapid increase in types of drugs available. Another limitation of the IgE test with regard to drug allergies is low sensitivity [9]. Low sensitivity may result from two main factors: antigenicity of small compounds on a solid phase of an immunoassay and complex phenotypes of drug allergy involved in IgE-dependent and -independent reactions [7,9–11]. Small compounds exert their antigenicity presumably through association with high-molecular-weight molecules in vivo. The in vitro test has reflected, in part, such association conditions.

Recently, basophil activation test (BAT), a flow cytometry-based allergy test, has been introduced to diagnose allergies and to identify the causal allergen [12–14]. The principal of BAT is that basophils express activation markers, CD203c and CD63, in response to cross-linking of allergen and allergen-specific IgE on their surface FcεRI [12–17]. In BAT, expression of CD203c and/or CD63 is measured after incubation of the patient's whole blood with a test allergen for 15 min to 1 h. BAT has an advantage in terms of the range of allergens has been applied to detect drug allergies induced by NSAIDs, β-lactams, and myorelaxants [18–22].

We have studied the applicability of BAT to a drug allergy caused by an NSAID, loxoprofen sodium 2-{4-[(2-oxocyclopentyl)methyl]phenyl} propanoate dehydrate. Loxoprofen is highly prescribed in Japan because it causes lesser gastric mucosa damage than that caused by other NSAIDs [23–25]; however, it is reported to frequently cause adverse reactions among loxoprofen-prescribed patients with postoperative pain [26]. During the course of our study, we found that the conventional procedure of BAT was inadequate for examination of loxoprofen-mediated hypersensitivity. No case was detected by the conventional BAT with specimens that were determined to be positive for loxoprofen-mediated hypersensitivity by the lymphocyte stimulation test. In the lymphocyte stimulation test, proliferation of T cells was

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measured after cell incubation with drugs for a couple of days. We thus wondered whether long-time incubation of basophils with allergens in BAT may significantly alter the measurement of IgE-independent responses of basophils. In our experiments, long-term exposure to loxoprofen induced the expression of CD203c on basophils even from healthy donors. In this study, we addressed why and how loxoprofen activates basophils in BAT after changing the incubation period.

2. Materials and methods

2.1. Reagents and blood samples

C5a and C3a were quantitated using the Human ELISA kit for C3a and C5a (Hycult Biotech, Uden, The Netherland) according to the manufacturer's instructions. Formyl-methionyl-leucyl-phenylalanine (fMLP), C5a, C5a desArg, and a C5a receptor antagonist W-54011 were purchased from Sigma-Aldrich (St. Louis, MO, USA), R&D systems (Minneapolis, MN, USA), Hycult Biotech, and Merck Millipore (Billerica, MA, USA), respectively. Whole blood and sera from healthy volunteers were obtained with written informed consent.

2.2. Flow-cytometric analysis of BAT

BAT was performed using the Allergenicity kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. Briefly, the test allergen (50 μL), heparinized whole blood (50 μL), and antibody cocktail (anti-CD3 PE-Cy7, anti-CD294 [CRTH2] FITC, and anti-CD203c PE antibodies (5 μL)) were incubated at 37 °C for 30 min unless otherwise indicated. In cases in which incubation was performed for more than 1 h, the antibody cocktail was added for the last 30 min. The reactions were stopped by the addition of the stop buffer in the kit (EDTA solution). After hemolysis, the samples were analyzed on a FACSCalibur or FACSVerse flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using CellQuest, FACSuite (BD Biosciences), or FCS Express (De novo software, Glendale, CA, USA) software. At least 500 basophils were analyzed in each assay.

2.3. Preparation of drug-treated serum

One tablet of Loxonin® (Daiichi Sankyo, Tokyo, Japan) (containing 60 mg of loxoprofen) or Caronal® (Showa Yakuhin Kako, Tokyo, Japan) (containing 200 mg of acetaminophen) was dissolved in 40 mL of PBS by sonication for 60 min. After filtration using a 0.22 μm pore filter (Merck Millipore) to remove insoluble compounds, the drug solution (40 mL) was mixed with 40 mL of PBS and 20 mL of human serum and incubated at 37 °C for indicated periods. PBS-treated serum without drugs was used as a control treatment.

2.4. Fractionation of peripheral blood mononuclear cells and red blood cells

Peripheral blood mononuclear cells (PBMCs) and red blood cells (RBCs) were isolated from whole blood by density gradient centrifugation using Ficoll-paque PLUS (GE Healthcare UK, Buckinghamshire, England) and LeucoSep tube (Greiner-bio-one, Frickenhausen, Germany).

2.5. Preparation of a basophil-activating factor from loxoprofen-treated serum

Loxoprofen-treated serum (5 mL) was diluted 10-fold with 5 mM phosphate buffer (pH 7.0), prior to the addition of 5 mL of a strong anion exchange medium UNOsphere Q (BIO-RAD, Hercules, CA, USA). After rotation for 90 min at 4 °C and centrifugation at 2000 rpm for 3 min, the supernatant was applied to a Protein A chromatographic column (5 mL), UNOsphere SUPrA (BIO-RAD), to remove immunoglobulins. The flow-through fraction was subjected to a strong cation exchange medium UNOsphere S (0.5 mL) (BIO-RAD) for 90 min with rotation at 4 °C. After washing away unbound proteins with PBS, a fraction was eluted with 0.5 mL of elution buffer (300 mM NaCl, 5 mM phosphate, pH 7.0) and centrifuged at 2000 rpm for 3 min.

2.6. Two-dimensional electrophoresis (2-DE) and mass spectrometry

The eluted fraction was desalted with Zepa spin desalting columns (Life Technologies Japan, Tokyo, Japan) and subjected to 2-DE as described [27]. Briefly, 10 μg of protein was loaded onto a 7 cm ReadyStrip IPG strip, pH 3–10 nonlinear (BIO-RAD). Isoelectric focusing (IEF) was performed using Ettan IPGphor (GE Healthcare) according to the manufacturer's instructions. Proteins on the IPG strip were transferred onto 15–25% tricine gels, electrophoresed, and stained with the Silver Stain MS kit (Wako Pure Chemical Industries, Osaka, Japan).

Candidate protein spots were excised from the gels and subjected to LC–MS/MS outsourcing analysis, which was carried out by Japan Bio Services (Saitama, Japan).

2.7. Inhibition of C5a receptor

Whole blood (50 μL) was treated with W-54011 at a final concentration of 1 μM for 1 h at 37 °C and then subjected to BAT with the antibody cocktail (5 μL) and anti-IgE antibody (20 μg/mL), fMLP (1 μM), recombinant C5a (300 ng/mL), or loxoprofen-treated serum.

3. Results

3.1. Basophil activation by loxoprofen

In order to evaluate BAT ability to detect loxoprofen-related hypersensitivity, blood from patients with allergy-like symptoms, presumably induced by loxoprofen administration, was incubated with loxoprofen for 30 min in vitro and examined for expression of the basophil activation marker CD203c. No appreciable increase in CD203c expression was observed even at high doses of loxoprofen, implying that the assay conditions of BAT were not effective (data not shown). We speculated if the incubation period for basophils and loxoprofen was inadequate, because of the possibility that loxoprofen may not directly activate basophils and rather loxoprofen-mediated alternation in the blood components might be necessary for basophil activation. To test this possibility, whole blood was incubated with loxoprofen for 24 h in addition to 30 min. Basophils from patients showed up-regulation of CD203c after incubation for 24 h (data not shown). Interestingly BAT with 24 h, but not 30 min, incubation of basophils from some healthy control donors also gave positive results (Fig. 1A). Acetaminophen and surpirin, which are analgesic and antipyretic drugs, were examined for their ability to activate basophils under the same conditions. Both drugs were not found to increase CD203c expression on basophils (Fig. 1B and data not shown).

3.2. Generation of basophil activator by loxoprofen in serum

We assumed that incubation of loxoprofen with blood for 24 h led to the production of a mediator(s) that activated basophils. To verify this hypothesis, a supernatant of healthy heparinized whole blood incubated with loxoprofen for 24 h was tested for its ability to activate basophils. The supernatant was incubated for 30 min with plasma-free auto-leukocytes and CD203c expression was determined. CD203c expression on basophils was significantly enhanced by incubation with the supernatant from culture with loxoprofen for 24 h in comparison to that on basophils incubated with supernatant from the culture without loxoprofen (Fig. 2A). Consistent with the results shown in Fig. 1, supernatant from the basophils treated with loxoprofen for 30 min did not induce an appreciable change in CD203c expression (data not shown).