Analysis of chemical mediators and cytokines in allergic inflammation models in rats and their advantages for the screening of anti-allergic inflammatory drugs

Kazuo Ohuchi

Department of Pathophysiological Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University, Aoba Aramaki, Aoba-ku, Sendai, Miyagi 980-77, Japan

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We have established an air pouch-type allergic inflammation model in rats [1,2] and a peritoneal eosinophilia model in rats [3]. Employing the two models, chemical mediators and cytokines responsible for the development of inflammation induced by allergic mechanisms are investigated to clarify the usefulness of the two models for the screening of anti-allergic inflammatory drugs.

1. Induction of air pouch-type allergic inflammation in rats

Male rats of the Sprague-Dawley strain, specific pathogen-free and weighing 120-140 g were immunized by intradermal injection of an antigen, azobenzenearsonate-conjugated acetyl bovine serum albumin and Freund's complete adjuvant. Nine days after immunization, 8 ml of air was injected subcutaneously in the dorsum to form an ellipsoid-shape air pouch. Twenty-four hours after the injection of air, 2 mg of the antigen dissolved in 4 ml of a sterilized solution of 0.8% sodium carboxymethylcellulose in saline solution was injected into the air pouch to provoke allergic inflammation. A group of rats that had been injected intradermally with Freund's complete adjuvant emulsion in the absence of the antigen received the antigen solution into the air pouch and served as the 'non-immunized rats' [1,2].

1-1. Vascular permeability increase in the anaphylaxis phase and its suppression by drugs

An aliquot of ^{131}I-labeled human serum albumin (HSA) was injected intravenously and radioactivity leaked into the pouch fluid during the 30 min interval was measured, expressed as percentage of radioactivity injected, and used for the index of vascular permeability. During the first 30 min interval after injection of the antigen solution into the air pouch, vascular permeability increased prominently in parallel with increase of histamine contents in the pouch fluid [4,5]. The strong increase in vascular permeability at this phase (anaphylaxis phase) was induced by IgE-mediated degranulation of subcutaneous mast cells [6]. Therefore, the combined treatment with the histamine H_1 antagonist pyrilamine and the serotonin antagonist methysergide effectively suppressed the vascular permeability increase at this phase [5]. In addition, sodium cromoglycate, an inhibitor

of mast cell degranulation also suppressed the vascular permeability increase at the anaphylaxis phase in parallel with the decrease in the content of histamine in the pouch fluid [6]. In contrast, prior treatment with dexamethasone dose-dependently suppressed the vascular permeability increase at this phase without lowering histamine contents in the pouch fluid, indicating that dexamethasone suppresses the vascular permeability without inhibiting mast cell degranulation [5]. Furthermore, the adrenoceptor beta agonists, such as isoproterenol, salbutamol and procaterol also suppressed the vascular permeability increase at this phase having no correlation with inhibition of mast cell degranulation [7]. At this phase, although peptide-leukotriene contents increased [8,9], AA-861, an inhibitor of 5-lipoxygenase showed no effects on vascular permeability increase [5], suggesting that peptide-leukotrienes play no significant role in vascular permeability increase at the anaphylaxis phase in this model. These results indicate that this model is useful for the *in vivo* screening of histamine H_1 antagonists, and inhibitors of mast cell degranulation.

1-2. Leukocyte infiltration and its suppression by drugs

In this model, leukocyte infiltration into the pouch fluid became prominent 8 h after injection of the antigen solution into the air pouch. Until 4 h, there was no significant difference in the number of leukocytes in the pouch fluid between the immunized rats and the non-immunized rats. When the infiltrated leukocytes collected from the pouch fluid 4 h after injection of the antigen solution were incubated in medium, neutrophil chemotactic activity in the conditioned medium increased time-dependently. The activity in the conditioned medium of leukocytes from the immunized rats was significantly higher than that from the non-immunized rats [10], suggesting that the infiltrated leukocytes from the immunized rats have been much more activated to produce neutrophil chemotactic factors than the leukocytes from the non-immunized rats. The neutrophil chemotactic factors in the conditioned medium were isolated to two factors, leukocyte-derived neutrophil chemotactic factor 1 (LDNCF-1) and LDNCF-2 [10], the latter being a major component. To clarify the activation mechanism of leukocytes to produce neutrophil chemoattractants, effects of inhibitors of protein kinases on the production of neutrophil chemoattractants were examined. Production of LDNCF-1 and LDNCF-2 was inhibited by the protein kinase C inhibitor H-7, and by the tyrosine kinase inhibitor genistein, but not inhibited by the cAMP-dependent protein kinase inhibitor H-89 [11]. These results suggested that the activation of protein kinase C and tyrosine kinase but not cAMP-dependent protein kinase is necessary to produce LDNCF-1 and LDNCF-2. The production of LDNCF-1 and LDNCF-2 was inhibited also by the protein synthesis inhibitor cycloheximide and the glucocorticoid dexamethasone [11]. The major component LDNCF-2 was characterized [12] and identified as macrophage inflamma-

tory protein 2 (MIP-2) [13]. The level of mRNA for MIP-2 in the infiltrated leukocytes of the immunized rats was higher than that of the non-immunized rats [12]. Dexamethasone treatment lowered the level of MIP-2 mRNA and suppressed the production of MIP-2 [14].

In vivo treatment with the PAF antagonists CV-3988, CV-6209, L-652731 and Y-24180 suppressed leukocyte infiltration at 8 h [10]. Although the precise mechanism for the inhibition of leukocyte infiltration by PAF antagonists has not yet been clarified, this model seems to be useful for determining the effects of PAF antagonists. Because these PAF antagonists inhibited histamine- or thrombin-induced neutrophil adherence to vascular endothelial cells *in vitro* [15], the inhibition of leukocyte infiltration by PAF antagonists in this model might partly due to the inhibition of leukocyte adherence to microvascular endothelial cells.

1-3. Histamine production in the post-anaphylaxis phase and its role in leukocyte infiltration

Histamine contents in the pouch fluid increased within 30 min after the antigen challenge, declined thereafter, and again increased reaching a peak at 24 h [17]. The increase of histamine contents in the post-anaphylaxis phase was preceded by the increase of histidine decarboxylase activity in the surrounding tissue [17], and histamine-production increasing activity in the pouch fluid [17]. The latter activity is due to histamine-production-increasing factor 1 (HPIF-1) [17]. Local administration of the histamine H_2 antagonists cimetidine, famotidine and ranitidine enhanced leukocyte infiltration [18]. Treatment with histaminase to lower the histamine contents in the pouch fluid increased leukocyte infiltration. Furthermore, repeated administration of histamine suppressed leukocyte infiltration [16]. These results suggested that histamine at the post-anaphylaxis phase down-regulates leukocyte infiltration. Treatment with the cyclooxygenase inhibitors indomethacin, diclofenac and tiaprofenic acid increased histamine contents in the pouch fluid and inhibited leukocyte infiltration [18]. Therefore, the mechanism for the inhibition of leukocyte infiltration by the cyclooxygenase inhibitors was suggested to be due to the increase of histamine production [18].

1-4. Role of cyclooxygenase-2 in the recurrence of allergic inflammation

Five days after injection of the antigen solution into the air pouch (first-time antigen challenge), although about 8 ml of pouch fluid was accumulated in the granulation tissue capsule formed, allergic inflammatory reactions calmed down as assessed by vascular permeability, number of leukocytes and prostaglandin (PG) E_2 contents in the pouch fluid [19]. At this phase, when 3 mg of the antigen dissolved in 0.5 ml saline was injected into the pouch (second-time antigen challenge), recurrence of allergic inflammation

was induced [19]. Western blot analysis demonstrated that cyclooxygenase 2 (COX-2) was induced in the granulation tissue with two peaks at 4-6 h and 12-24 h after the second-time antigen challenge [20]. The level of COX-1 in the granulation tissue did not change by the second-time antigen challenge [20]. Intrapouch injection of NS-398, an inhibitor of COX-2, at the time of the second-time antigen challenge, and 1 and 2 days after the second-time antigen challenge, effectively inhibited PGE_2 production, suppressed pouch fluid accumulation and granulation tissue formation when examined 3 days after the second-time antigen challenge [20]. These results suggested that the recurrence model of allergic inflammation is suitable for *in vivo* assessment of COX-2 inhibitors as anti-inflammatory drugs.

2. Induction of peritoneal eosinophilia in rats

Male rats of the Sprague-Dawley strain weighing 190-210 g received oral administration of cyclophosphamide (100 mg/kg) on day 0. Four milligrams protein of *Ascaris suum* extract in 0.5 ml saline containing 5 mg aluminum hydroxide as an adjuvant were divided into five portions. On day 2, each 0.1 ml of the antigen solution was injected intradermally on two nuchal and three lumbar sites, and 0.5 ml of the same solution was injected intraperitoneally. On day 12, 5 mg protein of the antigen in 0.5 ml saline containing 5 mg aluminum hydroxide was injected intraperitoneally as a booster injection. Finally, on day 19, 30 ml saline containing 8 mg protein of the antigen was injected intraperitoneally to induce peritoneal eosinophilia [3].

2-1. Preparation of eosinophils from peritoneal cells

Peritoneal cells harvested 48 h after intraperitoneal injection of the antigen solution were incubated in plastic dishes. The non-adherent cells were collected, suspended at a concentration of 1.5×10^7 cells/ml RPMI-1640 medium, layered over Percoll solution, and centrifuged at 1000 x g and 4^oC for 30 min. Cells at the bottom of the centrifuge tube were collected, washed and suspended in RPMI-1640 medium. Purity of eosinophils was more than 90% and viability was more than 98% [3]. Using this method, highly purified eosinophils are able to obtain and use for *in vitro* experiments. We have purified eosinophil cationic protein and major basic protein from rat eosinophils [3]. Furthermore, we have determined the complete nucleotide sequence for the cDNA encoding rat eosinophil major basic protein (MBP) using the rapid amplification of cDNA ends procedure [21]. The deduced amino acid sequence revealed that the rat prepro-MBP has three functional domains, namely the signal peptide, the acidic peptide, and the mature MBP, as human and guinea pig MBP.

2-2. Eosinophil-specific chemotactic factor and eosinophil-survival enhancing factor

Higher chemotactic activity for eosinophils was detected in the peritoneal fluid collected 4 and 8 h after the antigen challenge into the peritoneal cavity. On the other hand, higher chemotactic activity for neutrophils was detected only at 4 h. Therefore, the 8 h peritoneal fluid was suggested to contain eosinophil-specific chemotactic factor [22]. Molecular weight of this factor was estimated to be 13 kD, and the pI value 7-10 [22].

When the eosinophils were incubated in the presence of peritoneal fluid from the immunized or the non-immunized rats, survival of eosinophils was enhanced only when the eosinophils were incubated in the presence of the peritoneal fluid collected 4 and 8 h after the antigen challenge in the immunized rats. This activity decreased to a half at 16 h, and to very low levels at 24 h [22]. These results suggested that eosinophil-specific chemoattractant and eosinophil-survival enhancing factor are produced by the antigen challenge in the peritoneal cavity. The inhibitor of these factors might be useful to suppress allergic disorders such as allergic bronchial asthma.

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CURRICULUM VITAE

Name: Kazuo Ohuchi

Sex: male **Age:** 53 years old

Nationality: Japan

Present Address: Yurigaoka 3-8-14, Natori, Miyagi 981-12
Japan Tel: +81-22-386-1173

Place of Employment: Department of Pathophysiological Biochemistry
Faculty of Pharmaceutical Sciences
Tohoku University
Aoba Aramaki, Aoba-ku, Sendai,
Miyagi 980-77, Japan
Tel: +81-22-217-6860
Fax: +81-22-217-6859
E-mail: j23600@cctu.cc.tohoku.ac.jp

Academic Position: Professor **Title:** Ph.D.
Councilorship of Tohoku University

Academic Career:
1967 March, graduated Tokyo University Faculty of Pharmaceutical Sciences
1972 March, completed graduate course of Tokyo University, got Ph.D. title

Professional Career:
1972 April, Researcher at Central Research Laboratories, Sankyo Co. Japan
1974 April, Assistant Professor, Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University
1976 September, Research Associate, Albert Einstein Medical Center, Philadelphia PA, USA
1977 September, Senior Research Associate, Graduate Department of Biochemistry, Brandeis University, Boston, MA, USA
1978 September, Assistant Professor, Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University
1984 April. Associate Professor, Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University
1989 September, Full Professor,

Department of Pathophysiological
Biochemistry, Faculty of Pharmaceutical
Sciences, Tohoku University

Award: 1988 March, Prize for Research Encouragement from
Japanese Pharmaceutical Society
"Analysis of Chemical Mediators in
Inflammatory Reactions and the Mechanism
of Anti-inflammatory Drugs"

Member of Society: Japanese Inflammation Society
(Councilorship)
Japanese Biochemical Society
(Councilorship)
Japanese Pharmacological Society
(Councilorship)
Japanese Pharmaceutical Society
(Head of North-East Branch)