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HETEROGENEITY OF HUMAN MAST CELLS AND BASOPHILS

EFFECTS OF A PUTATIVE 5-LIPOXYGENASE INHIBITOR

VICTORIA COHAN, JANE MCKENZIE-WHITE, MASSIMO TRIGGIANI, WILLIAM MASSEY, ANNE KAGEY-SOBOTKA and LAWRENCE M. LICHTENSTEIN*†

Division of Clinical Immunology, Johns Hopkins University School of Medicine at The Good Samaritan Hospital, Baltimore, MD 21239, U.S.A.

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Abstract—The effects of a putatively specific 5-lipoxygenase inhibitor, 2(12-hydroxydodeca-5,10-dinyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861), and its major metabolite, M-I, were assessed using anti-IgE activated human basophils, lung mast cells and skin mast cells. In basophils and lung mast cells, no effects on histamine release were observed, whereas leukotriene C_4 (LTC_4) production was inhibited (IC_{50} values $< 1\ \mu M$). In addition, prostaglandin D_2 (PGD_2) production was inhibited in lung mast cells ($IC_{50} \simeq 5\ \mu M$). In contrast, in skin mast cells both histamine release and PGD_2 production were reduced by AA-861 and M-I, with IC_{50} values of $\simeq 5$ and $0.4\ \mu M$ for histamine and PGD_2, respectively. These data reveal biochemical heterogeneity among human histamine-containing cells and underscore the necessity of assessing a pharmacologic agonist in relevant cell systems.

The activation of the 5-lipoxygenase pathway of arachidonic acid metabolism in human basophils and mast cells, stimulated with anti-IgE or an appropriate allergen, is evidenced by the ability of these cells to generate the pro-inflammatory sulfidopeptide leukotriene C_4 (LTC_4) [1]. We and others have also speculated that the enzymes of the 5-lipoxygenase pathways of basophils are involved in the mechanisms for the release of other mediators [2, 3]. For these and other cogent reasons, the pharmaceutical industry has been engaged in research attempting to develop specific inhibitors of this enzymatic activity. Although many 5-lipoxygenase inhibitors have been described, most have been found to also inhibit the metabolism of arachidonic acid via the cyclooxygenase pathway or to have other activities such as being oxygen radical scavengers [4]. Rokach and colleagues have described a relatively specific inhibitor of 5-lipoxygenases [5], which we have demonstrated to block leukotriene production from human basophils and lung mast cells without affecting histamine release [5, 6]. Takeda Chemical Industries has synthesized and described the activity of another putatively specific 5-lipoxygenase inhibitor, 2(12-hydroxydodeca - 5, 10 - dinyl) - 3, 5, 6 - trimethyl - 1, 4 -benzoquinone (AA-861). This drug has been studied widely in isolated cell systems and in animal models of human disease [7–11]. Its activity has also been explored in human polymorphonuclear leukocytes (PMNs) and platelets, where it inhibited LTC_4 and LTB_4 generation in the former and failed to block cyclooxygenase product generation or the production of 12-lipoxygenase products in the latter [11]. Clinical trials with this agent have begun [12].

The present report describes the results of studying the effects of AA-861 (Abbott-61589) and its major *in vivo* metabolite, M-I, in three systems involving the release of histamine and arachidonic acid products from human lung and skin mast cells and from basophils. The effects of the drugs are somewhat different in each cell type, providing another index of the heterogeneity found in human histamine-containing cells [13].

MATERIALS AND METHODS

Materials. The following reagents were purchased: piperazine-*N*-*N*′-bis [2-ethanesulfonic acid] (PIPES) (Sigma Chemical Co., St Louis, MO); ethylenediamine tetra-acetic acid (EDTA) and glucose (Fisher Scientific, Silver Spring, MD); human serum albumin (HSA) (Calbiochem, San Diego, CA); and dextran (McGaw, Irvine, CA). The 5-lipoxygenase inhibitor AA-861 and its metabolite were provided by Takeda Chemical Industries, Ltd (Osaka, Japan). Goat anti-human IgE (anti-IgE) was prepared as described [14].

Buffers. PIPES (P) contained 25 mM PIPES, 110 mM NaCl, and 5 mM KCl adjusted to pH 7.4; PAG additionally contained 0.003% HSA and 0.1% D-glucose; PAGCM also contained 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

Preparation of peripheral blood basophils. After informed consent, peripheral blood was obtained by venipuncture. Mixed leukocytes containing basophils were prepared by dextran sedimentation as previously described [15]. Briefly, 40 ml of venous blood were mixed with 12.5 ml of 6% clinical dextran and 5 ml of 0.1 M EDTA and allowed to sediment

* Lawrence M. Lichtenstein is recipient of the Pfizer Biomedical Research Award.

† Correspondence: Lawrence M. Lichtenstein, M.D., Johns Hopkins University School of Medicine at The Good Samaritan Hospital, 5601 Loch Raven Boulevard, Baltimore, MD 21239.

for 90 min at room temperature. The leukocyte-containing plasma layer was then removed, and cells were pelleted by centrifugation (150 *g*, 8 min) and were washed twice with PAG.

Preparation of lung mast cells. Lung mast cells were obtained using the methods previously described [16]. Briefly, normal human lung parenchyma obtained at surgery was minced into 5–10 mg fragments with scissors. Fragments were washed overnight in Tyrode's buffer, and then resuspended in Ca^{2+}-free Tyrode's buffer and incubated successively in pronase (2 mg/ml), chymopapain (0.5 mg/ml), elastase type II (10 units/ml), and collagenase (1 mg/ml). For the last incubation and all washings, Tyrode's buffer with deoxyribonuclease type I (15 mg/L) and gelatin (1 g/L) was used. Digested cells were filtered through Nitex cloth with a pore size of 100 μm (Tetko, Elmsford, NY), washed, and brought to a 1–10% purity as determined by Alcian blue staining [17]. Prior to use in the experiments described, cells were washed again and resuspended in PAGCM.

Preparation of skin mast cells. Previously described methods were used [13]. In brief, adult skin obtained from mastectomies or cosmetic surgery procedures was placed in cold calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS) (GIBCO, Grand Island, NY) and used within 1 hr. After removal of fat by blunt dissection, the skin specimen was chopped into 1-mm pieces and washed in PAG, once at 4°, then at 23°. Fragments were incubated in PAG (1 g tissue/10 ml buffer) containing 1.5 mg/ml collagenase type II (Worthington Biochemical Co., Freehold, NJ), 0.4 mg/ml hyaluronidase (Sigma Chemical) and 1000 units deoxyribonuclease 1 (DNase) (Calbiochem) for 4 hr at 37°. The partially digested tissue was filtered through Nitex cloth (150 μm pore size). Dispersed cells were washed three times with PAG at 23°. Mast cell numbers were determined by light microscopy after staining with Alcian blue at pH 1.0 [17].

Short-term culture of skin mast cells. Dispersed cells were resuspended in RPMI 1640 (Whitaker, M.A. Bioproducts, Walkersville, MD) supplemented with 25 mM *N*-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 1% penicillin-streptomycin and 10% heat-inactivated fetal calf serum (GIBCO). Cells were cultured at 2×10^5 mast cells/ml in 25 cm^2 tissue culture flasks (BELLCO Biotechnology, Vineland, NJ) in humidified 95% air, 5% CO_2 at 37° for 12–16 hr. Cells were washed twice in PAG at 23°, counted following Alcian blue staining, and resuspended in an appropriate volume of PAGCM prior to experimental use.

Histamine release assay. Reaction volumes for the histamine release assay were 0.1 ml. Cell preparations were incubated in PAGCM. Cell concentrations were adjusted so that there were approximately 2×10^4 basophils or 1×10^4 mast cells per tube (i.e. a total of ~20–30 ng histamine/tube). The drugs were allowed a 10-min preincubation with the cells at 37° (skin mast cells were preincubated at 30°). Following addition of anti-IgE, an incubation period of 45 min at 37° was chosen for the release reaction (skin mast cells were incubated for 30 min at 30°). At the end of the incubation, 0.4 ml of cold PAG was added to each tube. The tubes were then centrifuged (1000 *g*, 2–5 min, 23°) and aliquots of the cell-free supernatant fractions were retained for LTC_4 and/or PGD_2 radioimmunoassays. The remaining supernatant fraction was brought to 1 ml with cold PAG and assessed for histamine content by the automated fluorometric assay of Siraganian [18]. Histamine results were based on the mean of duplicate determinations. Histamine release was calculated from the percentage of total histamine content, determined by lysis of cells in 2% perchloric acid, corrected for spontaneous release of unstimulated cells (typically 2–5% during the assay).

Measurement of arachidonic acid metabolites. The LTC_4 or PGD_2 produced was measured using previously described radioimmunoassays [19, 20]. While radioimmunoassays were used, we had verified previously the authenticity of PGD_2 and LTC_4 by other techniques [21].

RESULTS

As previously reported by Mita *et al.* [7], AA-861 had no effect on human basophil histamine release over the concentration range of 0.1 to 10 μM (Fig. 1A). Its major metabolite, M-I, was also without effect. The drug, however, was a potent inhibitor of the generation and/or release of LTC_4 from this cell

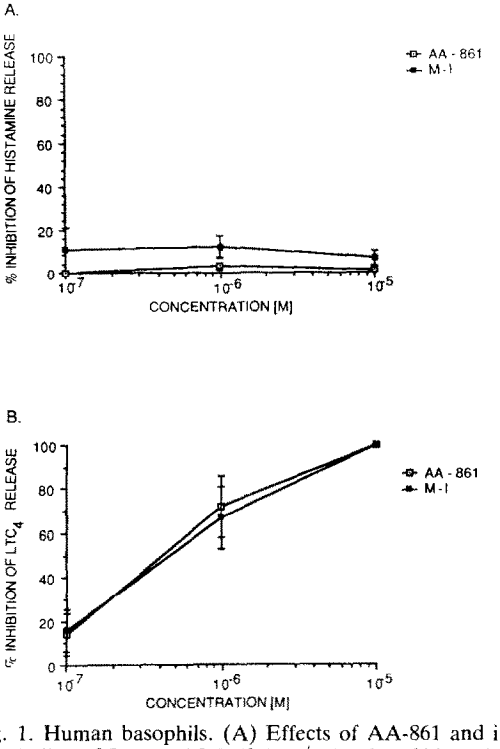

Fig. 1. Human basophils. (A) Effects of AA-861 and its metabolite, M-I, on anti-IgE (0.1 μg/ml) induced histamine release. Control histamine release was 71.6 ± 7.3%. (B) Inhibition of anti-IgE (0.1 μg/ml) induced LTC_4 release by AA-861 and its metabolite, M-I. The IC_{50} for both compounds was ≈0.4 μM. Control LTC_4 production was 83.7 ± 28.7 pg/0.1 ml. Data for both panels are expressed as means ± SE, N = 3.

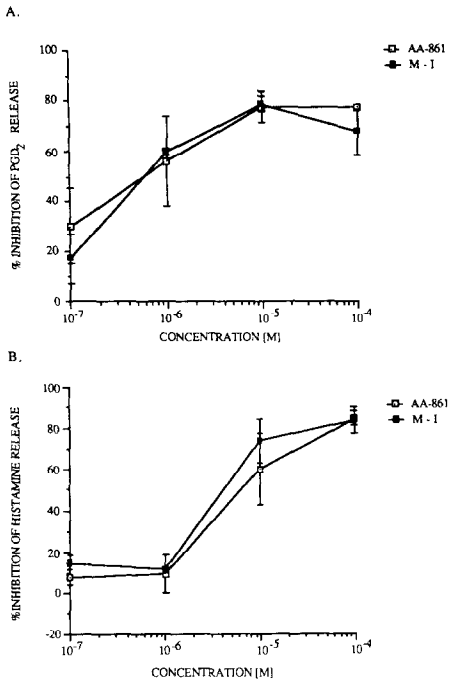

Fig. 2. Human skin mast cells. (A) Inhibition of anti-IgE (3 μg/ml) induced PGD_2 release by AA-861 and its metabolite, M-I. The IC_{50} for both compounds was ≈0.4 μM. Control PGD_2 release was 29 and 41 ng/10^6 mast cells, N = 2. (B) Inhibition of anti-IgE (3 μg/ml) induced histamine release by AA-861 and its metabolite, M-I. The IC_{50} was ≈5 μM for both compounds. Control histamine release was 29.3 ± 7.9% (mean ± SE), N = 3.

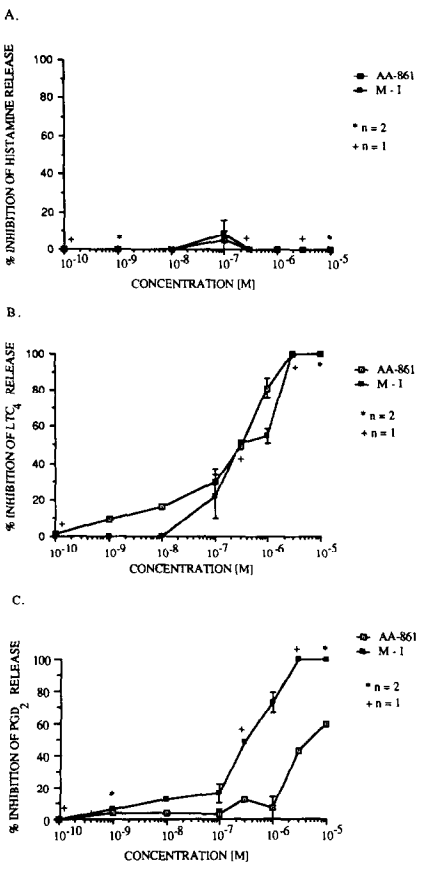

Fig. 3. Human lung mast cells. (A) Effects of AA-861 and its metabolite, M-I on anti-IgE (5 μg/ml) induced histamine release. Control histamine release was 29.5 ± 5.1%. (B) Inhibition of anti-IgE (5 μg/ml) induced LTC_4 release by AA-861 and its metabolite, M-I. The IC_{50} for both compounds was 0.3 μM. Control LTC_4 release was 84.8 ± 30.0 pg/0.1 ml. (C) Inhibition of anti-IgE (5 μg/ml) induced PGD_2 release by AA-861 and its metabolite, M-I. The IC_{50} for the parent compound was ≈5 μM; for the metabolite, ≈0.4 μM. Control PGD_2 release was 72.3 ± 49.8 pg/0.1 ml. Data for the three panels are expressed as means ±SE, N = 3–4 except as indicated.

type (Fig. 1B). The amount of LTC_4 generated in these experiments ranged from 30 to 130 pg/0.1 ml, and the IC_{50} was about 0.4 μM, similar to the concentration found for LTB_4 inhibition in the human PMN (0.3 μM) [11]. At 10 μM concentrations, 100% inhibition of basophil LTC_4 release occurred. The dose–response curve of the metabolite was identical to that of the parent drug. Since human basophils do not generate PGD_2 (or any known prostaglandin), the effect of AA-861 on the basophil cyclooxygenase pathway could not be studied.

The human skin mast cell generates only very small quantities of LTC_4 which are below the sensitivity limits of our assay; greater than 90% of its arachidonic acid metabolism leads to PGD_2 production [13]. In these experiments, 29–40 ng of PGD_2 was produced per 10^6 skin mast cells; both the parent drug and its metabolite were equally effective in blocking the release of the prostanoid with an IC_{50} of about 0.4 μM (Fig. 2A), the same as found to inhibit LTC_4 generation in the basophil. Thus, the drug has potent activity as an inhibitor of cyclooxygenase product formation, although whether this is a direct effect on the cyclooxygenase enzymes or on earlier events such as phospholipase A_2 inhibition is unclear. Other cyclooxygenase inhibitors such as indomethacin have been studied in human cells; they enhance histamine release from human basophils but have no effect on histamine release by skin or lung mast cells [22]. Surprisingly, however, both AA-861 and its metabolite were reasonably potent inhibitors of histamine release from the skin mast cells (Fig. 2B). The IC_{50} for this effect was 3–5 μM; inhibition increased to about 80% at 10 μM, but did not increase further with higher concentrations of drug.

The effects of AA-861 and its metabolite on the lung mast cell were different still. This cell was perhaps the most interesting in the present context since it makes about equal quantities of both LTC_4 and PGD_2. In these experiments, LTC_4 production was 37–148 pg/0.1 ml, while the amount of PGD_2 generated was 13–34 pg/0.1 ml. The effects of AA-861 and M-I were similar to those observed in basophils; there was no significant inhibition of histamine release over a concentration range of 10^{-9} to 10^{-5} M (Fig. 3A) and with respect to LTC_4 production, both the drug and its metabolite were effective, having IC_{50} values in the range of 0.3 μM (Fig. 3B). However, in the lung mast cell AA-861 was 10 to 20-fold less active at inhibiting PGD_2 production than in the skin mast cell, having an IC_{50} of about 5 μM. Its

metabolite, however, was quite active, with an IC_{50} on PGD_2 production of about 0.4 μM and causing 100% inhibition at micromolar concentrations (Fig. 3C).

DISCUSSION

Several points can be made from the data presented in this manuscript. First, they illustrate the difficulty in using a pharmacologic agonist to assess biochemical pathways without simultaneously making parallel biochemical measurements, since even the same cell type at different anatomical locations in the same species may be different with respect to drug effects. The present observations also illustrate how difficult it has been to develop a truly specific inhibitor of the 5-lipoxygenase enzyme. Also, and rather surprisingly, these data further illustrate human mast cell heterogeneity, in this instance at the level of biochemical pathways.

These points are reflected in the following data. AA-861 manifested the expected 5-lipoxygenase inhibitory effect in basophils where it had no effect on histamine release; cyclooxygenase effects could not be assessed. However, in the skin mast cell the drug was as active against PGD_2 production as it was against LTC_4 production in the basophil. Moreover, it inhibited histamine release in this cell type, albeit with an IC_{50} one order of magnitude higher than its 5-lipoxygenase effect. The lung mast cell (and the gut mast cell) is the only human histamine-containing cell (thus far studied) which generates roughly equivalent quantities of products of both the lipoxygenase and cyclooxygenase pathways, LTC_4 and PGD_2. In this cell, AA-861 had effects similar to those in the basophil—inhibition of LTC_4 production at submicromolar concentrations without an effect on histamine release. However, it also blocked PGD_2 production at about a 10-fold higher concentration than that required for effects on LTC_4. The major human metabolite of AA-861, which differs from it only in that the side chain alcohol has been oxidized to the acid, tracked its parent drug in all systems in which it was studied except in the lung mast cell. In this cell, it was 10-fold more active as an inhibitor of cycloxygenase product formation, with an IC_{50} similar to the IC_{50} of the parent compound for LTC_4 production. Thus, with an IC_{50} for PGD_2 inhibition in skin mast cells in the submicromolar range and a similar activity in human lung mast cells at a 10-fold higher concentration than for its activity on LTC_4, AA-861 cannot be called a specific 5-lipoxygenase inhibitor.

With respect to human mast cell heterogeneity, these results are the first to comment upon differences in biochemical pathways, whereas there is abundant evidence of functional differences. With regard to the latter, different mediators made by each cell are illustrated in this manuscript. The gut mast cell is like the lung mast cell in making both LTC_4 and PGD_2, while the mast cell obtained on bronchial alveolar lavage is like the skin mast cell in making predominantly PGD_2 [23, 24]. Other differences relate to optimal temperature, relevant secretagogues and pharmacologic control. The data presented herein are an extension of these findings, with biochemical rather than functional measurements. The major differences are that AA-861 is an inhibitor of histamine release in the skin mast cell and has no such activity in the other two cells; and that, in this cell as well, the drug is an order of magnitude more active against PGD_2 generation than in the lung mast cell.

A future clinical trial will ascertain activities of AA-861 on the same mast cells and basophils *in vivo*, which may help us to understand the implications of these pathways for human disease.

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REFERENCES

1. MacGlashan DW Jr and Lichtenstein LM, Characteristics of human basophil sulfidopeptide leukotriene release: releasability defined as the ability of the basophil to respond to dimeric crosslinks. *J Immunol* **136**: 2231–2239, 1986.
2. Peters SP, Siegel MI, Kagey-Sobotka A and Lichtenstein LM, Lipoxygenase products modulate histamine release in human basophils. *Nature* **292**: 455–457, 1981.
3. Clancy RM, Dahinden CA and Hugli TE, Arachidonate metabolism by human polymorphonuclear leukocytes stimulated by *N*-formyl-Met-Leu-Phe or complement component $C5_a$ is independent of phospholipase activation. *Proc Natl Acad Sci USA* **80**: 7200–7204, 1983.
4. Furst DE, Comments on possible long-term consequences of nonsteroidal anti-inflammatory use. *J Clin Pharmacol* **28**: 550–553, 1988.
5. Guidon Y, Girard Y, Marycock A, Ford-Hutchinson AW, Atkinson JG, Belanger PC, Dallob A, DeSousa D, Dougherty H, Egan R, Goldberg MM, Ham E, Forton R, Hamel P, Hamel R, Lau CK, LeBlanc Y, McFarlane CS, Piechuta H, Therier M, Yoakin C and Rokach J, L651–392: A novel potent and selective 5-lipoxygenase inhibitor. *Adv Prostaglandin, Thromboxane Leukotriene Res,* in press.
6. Warner JA, Lichtensten LM and MacGlashan DW Jr, Effects of a specific inhibitor of the 5-lipoxygenase pathway on mediator release from human basophils and mast cells. *J Pharmacol Exp Ther* **247**: 218–222, 1988.
7. Mita H, Yui Y and Shida T, Effect of AA-861, a 5-lipoxygenase inhibitor, on leukotriene synthesis in human polymorphonuclear leukocytes and on cyclooxygenase and 12-lipoxygenase activities in human platelets. *Allergy* **41**: 493–498, 1986.
8. Morita Y, Suzuki S and Miyamoto T, The role of 5-lipoxygenase pathway activation in basophil histamine release. *Int Arch Allergy Appl Immunol* **78**: 77–80, 1985.
9. Ashida Y, Saijo T, Kuriki H, Makino H, Terao S and Maki Y, Pharmacological profile of AA-861, a 5-lipoxygenase inhibitor. *Prostaglandins* **26**: 955–972, 1983.
10. Ishihara Y, Kitamura S and Takaku F, Effects of AA-861 on thromboxane B_2 and 6-keto prostaglandin $F_{1\alpha}$ values in the blood of dogs during experimental endotoxic shock. *Ensho* **4**: 469–470, 1984.
11. Ohuchi K, Watanabe M, Taniquchi J, Tsurufuji S and Levine L, Inhibition by AA861 of prostaglandin E_2 production in activated peritoneal macrophages of rat. *Prostaglandins Leukotrienes Med* **12**: 175–177, 1983.

12. Fujimara M, Sasaki F, Nakatsumi Y, Takahashi Y, Hifumi S, Taga K, Mifine J-I, Tanaka T and Matsuda T, Effects of a thromboxane synthetase inhibitor (OKY-046) and a lipoxygenase inhibitor (AA-861) on bronchial responsiveness to acetylcholine in asthmatic subjects. *Thorax* **41**: 955–959, 1986.
13. Lawrence ID, Warner JA, Cohan VL, Hubbard WC, Kagey-Sobotka A and Lichtenstein LM, Purification and characterization of human skin mast cells. Evidence for human mast cell heterogeneity. *J Immunol* **139**: 3062–3069, 1987.
14. Adkinson NF Jr, Measurement of total serum immunoglobulin E and allergen-specific immunoglobulin E antibody. In: *Manual of Clinical Laboratory Immunology* (Eds. Rose, NR, Friedman H and Fahey JL), 3rd Ed., pp. 664–674. American Society of Microbiology, Washington, DC, 1986.
15. Lichtenstein LM and Osler AG, Studies on the mechanisms of hypersensitivity phenomena. IX. Histamine release from human leukocytes by ragweed pollen antigen. *J Exp Med* **120**: 507–530, 1964.
16. Schulman ES, MacGlashan DW Jr, Peters SP, Schleimer RP, Newball HH and Lichtenstein LM, Human lung mast cells: purification and characterization. *J Immunol* **129**: 2662–2667, 1982.
17. Gilbert HS and Ornstein L, Basophil counting with a new staining method using Alcian blue. *Blood* **46**: 279–286, 1975.
18. Siraganian RP, An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal Biochem* **57**: 383–394, 1974.
19. Hayes EC, Lombardo DL, Girard Y, Maycock AL, Rokach J, Rosenthal AS, Young RN, Egan RW and Zweerink HJ, Measuring leukotrienes of slow reacting substance of anaphylaxis: development of a specific radioimmunoassay. *J Immunol* **131**: 429–433, 1983.
20. Adkinson NF Jr, Newball HH, Findlay S, Adams GK and Lichtenstein LM, Anaphylactic release of prostaglandins from human lung *in vitro*. *Am Rev Respir Dis* **121**: 911–920, 1980.
21. Peters SP, Schulman ES, Liu MC, Hayes EC and Lichtenstein LM, Separation of major prostaglandins, leukotrienes, and monoHETES by high performance liquid chromatography. *J Immunol Methods* **64**: 335–343, 1983.
22. Marone G, Kagey-Sobotka A and Lichtenstein LM, Effects of arachidonic acid and its metabolites on antigen-induced histamine release from human basophils *in vitro*. *J Immunol* **123**: 1669–1677, 1979.
23. Fox CC, Dvorak AM, Peters SP, Kagey-Sobotka A and Lichtenstein LM, Isolation and characterization of human intestinal mucosal mast cells. *J Immunol* **135**: 483–491, 1985.
24. Bleecker ER, Liu MC, Hubbard WC, Kagey-Sobotka A and Lichtenstein LM, Reactivity of human bronchial mast cells obtained by bronchoalveolar lavage. *Am Rev Respir Dis* **137**: 210, 1988.