

## STIMULUS SPECIFICITY OF THE GENERATION OF LEUKOTRIENES BY DOG MASTOCYTOMA CELLS

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Mast cell-derived mediators of hypersensitivity include amines, polypeptides, and proteoglycans that are present preformed in the granules, as well as metabolites of arachidonic acid and other lipids that are generated *de novo* from membrane constituents before release (1, 2). While several types of pharmacological agents and other inhibitors suppress differentially the release of preformed or newly generated mediators from mast cells (3), immunological challenge and IgE-independent activation of mast cells appear to stimulate without preference the release of both classes of mediators. The availability of dog mastocytoma cells of high purity that release histamine and leukotrienes (LT)<sup>1</sup> B<sub>4</sub>, C<sub>4</sub>, and D<sub>4</sub> in response to antigen and ionophore A23187 (4, 5), has permitted an initial comparative analysis of the specificity of activation of the two compartments of mediators by distinct stimuli.

### Materials and Methods

Bovine plasma albumin-fraction V, bovine gamma-globulin, histamine diphosphate, kits for the quantification of lactic acid dehydrogenase activity, penicillin G, streptomycin, compound 48/80 (Sigma Chemical Co., St. Louis, MO), recrystallized ovalbumin (Miles Laboratories, Elkhart, IN), calcium ionophore A23187, which was stored at -20°C as a 3 mM stock solution in dimethylsulfoxide (Calbiochem Corp., La Jolla, CA), polyethylene glycol (mol wt 3,000-3,700) (MCB Manufacturing Chemists, Inc., Cincinnati, OH), O-phthalaldehyde (Dionex Co., Sunnyvale, CA), Joklik-modified minimum essential medium (MEM; Grand Island Biological Co., Grand Island, NY), Hydrofluor scintillation counting fluid (National Diagnostics, Somerville, NJ), short ragweed (*Ambrosia elatior*) pollen extract (Hollister-Steir Co., Spokane, WA), amphotericin B with deoxycholate (Fungazone, E.R. Squibb & Sons, La Miranda, CA), protease-free collagenase (CLSPA, Worthington Biochemical Co., Freehold, NJ), arachidonic acid (Supelco, Inc., Bellefonte, PA), [14,15-<sup>3</sup>H(N)]-leukotriene C<sub>4</sub> (34.0 Ci/mmol) (New England Nuclear, Inc., Boston, MA), and organic solvents that had been redistilled from glass (HPLC grade) (Burdick and

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<sup>1</sup> *Abbreviations used in this paper:* LT, leukotriene; HETE, hydroxy-eicosatetraenoic acid; HPETE, hydroperoxy-eicosatetraenoic acid; HPLC, high performance liquid chromatography; ODS, octadecylsilane; PF4, platelet factor 4.

Jackson Laboratories, Inc., Muskegon, MI) were obtained from the designated suppliers. Synthetic leukotrienes B<sub>4</sub>, C<sub>4</sub>, and D<sub>4</sub> were kindly provided by Dr. J. Rokach of Merck-Frosst Laboratories, Dorval, Canada. 12-hydroxy-eicosatetraenoic acid (12-HETE) and 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) were prepared and characterized as described (6, 7). Synthetic 15-HETE was a gift from Dr. E. J. Corey of Harvard University, Cambridge, MA. [<sup>3</sup>H]-Leukotriene B<sub>4</sub> (specific activity 26.2 ± 5.9 Ci/mmol, mean ± range, n = 5) and [<sup>3</sup>H]-12-HETE (specific activity = 11.7 ± 8.4 Ci/mmol) were generated from [5,6,8,9,11,12,14,15-<sup>3</sup>H(N)] arachidonic acid (specific activity 60–100 Ci/mmol (New England Nuclear, Inc. Boston, MA) and purified as described (8, 9). The carboxy-terminal dodecapeptide of human platelet factor 4 (PF4(59–70)), Leu-Tyr-Lys-Lys-Ile-Ile-Lys-Lys-Leu-Leu-Glu-Ser, was prepared by standard solid-phase synthesis, purified by sequential counter-current distribution and filtration on Sephadex G-25 in 0.5 M NH<sub>4</sub>OH, and characterized by amino acid analysis and sequence determination (10). Histamine in supernates and cell pellets of mastocytoma suspensions was quantified by a manual fluorimetric method, utilizing a standard curve of the values obtained from 5–200 ng/ml of histamine (11).

*Preparation, Sensitization, and Activation of Dog Mastocytoma Cells.* Subcutaneous nodules of mastocytoma tissue were removed from a dog under general anesthesia (acepromazine maleate, Ayerst Laboratories, New York, NY and sodium thiamylal, Bio-Centric, Inc., St. Joseph, MO), freed of connective tissue, and minced with scissors into fragments of ~1-mm diameter (4, 5). The fragments were washed and resuspended in 20 ml of calcium-free MEM with 25 mM Hepes (pH 7.4) without (unsensitized) or with (sensitized) 5 ml of dog IgE-rich hyperimmune anti-short ragweed serum, and incubated in a 10-cm diameter plastic petri dish for 16–18 h at room temperature. It has been demonstrated that incubation with IgE before dispersion of the mastocytoma cells did not provide sufficient sensitization for a maximal response to antigen, but did enhance the responses achieved by resensitization and antigen challenge of isolated cells, presumably by preserving IgE receptors (5). Both sets of fragments were treated for 60 min at 37°C with 80 U/ml of collagenase in order to disperse the mastocytoma cells (4, 5). The residual fragments were filtered through cheesecloth and the filtrate was centrifuged at 400 g for 10 min to recover mastocytoma cells of 93–97% purity, as assessed by staining with toluidine blue, and viability of 94% or greater; minor contaminants consisted of equal numbers of eosinophils, macrophages, and fibroblasts. Replicate aliquots of 10<sup>6</sup> mastocytoma cells from the respective sets were resuspended and incubated for 4 h at room temperature in 1.0 ml of calcium-free Tyrode's buffer without or with a 1/10 dilution of the anti-short ragweed serum, washed twice, and resuspended in 1 ml of complete Tyrode's buffer with 0.1 g/100 ml of ovalbumin before incubation for 2–45 min at 37°C without or with 10 pnu/ml of short ragweed pollen extract. Unsensitized mastocytoma cells were challenged similarly with 0.2–2 μM calcium ionophore A23187, 0.1–1.0 μg/ml of compound 48/80, 0.1–3.0 μg/ml of 15-HETE, and 10<sup>-6</sup> M–10<sup>-4</sup> M PF4(59–70).

*Identification and Quantification of Lipoxygenase Products of Arachidonic Acid.* Each suspension was centrifuged for 1 min at 10,000 g and one-fifth of the supernate was saved in two equal aliquots at -70°C for radioimmunoassays. The lipoxygenase products of arachidonic acid were recovered from the remainder of each suspension by extraction with 2 ml of chloroform/methanol (2:1, vol/vol) twice and 2 ml of ethyl ether after the addition of 20,000 cpm of [<sup>3</sup>H]-12-HETE. The extracts were pooled, dried under nitrogen to ~10 μl, diluted to 150 μl with methanol/water/glacial acetic acid (60:40:0.03, vol/vol), and subjected to reverse-phase high performance liquid chromatography (HPLC) with a 10-μm octadecylsilane (ODS; Ultrasil, Altex Scientific Co., Beckman Instruments, Mountainview, CA) pre-column and a 4.6-mm × 25-cm column of 10 μm ODS that was developed isocratically with methanol/water/glacial acetic acid (70:30:0.03, vol/vol; adjusted to pH 6.0 with concentrated ammonium hydroxide) at a flow rate of 1.7 ml/min (5). The leukotrienes were identified initially by elution at the same times as authentic standards. LT<sub>B</sub> and the C-6 peptide leukotrienes were quantified by the optical density at 270 nm and 280 nm, respectively, relative to that of known amounts of standard compounds (5). The retention times of synthetic standards were: LTC<sub>4</sub> = 4.7–5.3 min

(range,  $n = 8$ ),  $LTD_4 = 8.1$ – $8.8$  min, and  $LTB_4 = 9.8$ – $10.7$  min. The amounts of [ $^3H$ ]-12-HETE in the eluates were measured and utilized to calculate the percentage recovery of the products.

*Radioimmunoassays of  $LTC_4$  and  $LTB_4$ .* The assays were performed as described (12), except that the final dilution of anti- $LTB_4$  plasma was 1/150, whereas that of anti- $LTC_4$  plasma was 1/1,050. Each of triplicate tubes contained 0.2 ml of 0.05 M Tris-HCl (pH 8.0), 0.1 ml of radioligand (10,000 cpm), 0.1 ml of standard compound or unknown in Tris buffer, 0.2 ml of 5 mg/ml bovine gamma-globulin in Tris buffer, and 0.1 ml of diluted antiplasma. One set of tubes received 0.1 ml of additional buffer instead of antiplasma for the determination of total radioactivity. After the mixtures were incubated for 60 min at room temperature and 15 min at 0°C, 0.7 ml of 25% (w/vol) aqueous polyethylene glycol at 0°C was added to each tube. After vigorous mixing, the tubes were centrifuged at 1,400  $g$  for 60 min at 4°C and 1-ml aliquots of the supernates were transferred to vials containing 10 ml of Hydrofluor for quantification of radioactivity. The assays for  $LTB_4$  and  $LTC_4$  were of similar sensitivity and specificity; 50% displacement of the respective radioligands required 0.1 ng of  $LTB_4$  and 0.2 ng of  $LTC_4$ , while the cross-reactivity of either antiplasma with the other class of leukotrienes was <1% (12).

## Results

Dog mastocytoma cells were challenged with a range of structurally distinct stimuli of histamine release in order to examine the concurrent effects on leukotriene generation. Preliminary studies indicated that the time course of generation of  $LTC_4$  by mastocytoma cells was a function of the specific stimulus. HPLC analysis of the  $LTC_4$  produced by mastocytoma cells incubated at 37°C with ragweed antigen, 0.2  $\mu$ M ionophore A23187, 10  $\mu$ M PF4(59–70), 0.3  $\mu$ g/ml of compound 48/80, and buffer alone revealed the release of a mean ( $n = 2$ ) of 18.4, 6.1, 4.7, 2.5, and 1.8 ng/ $10^6$  mastocytoma cells, respectively, at 1 min, 19.2, 11.4, 7.3, 1.9, and 2.2 at 10 min, 19.8, 31.0, 9.5, 2.2, and 1.9 at 30 min, and 18.7, 26.9, 10.1, 2.4, and 2.4 at 45 min. 1  $\mu$ g/ml of 15-HETE, which is known to modulate the activity of 5-lipoxygenases (13, 14) but had not been investigated in relation to histamine release from mast cells, evoked the generation of a mean ( $n = 2$ ) of 6.4, 14.9, 26.1, and 28.2 ng of  $LTC_4$ / $10^6$  mastocytoma cells at 1, 10, 30, and 45 min, respectively, in the same experiments.

The stimulus concentration-dependence of the generation of leukotrienes and of the release of histamine by mastocytoma cells then was defined for each principle with a standard incubation interval of 30 min, that reflected the maximum production of  $LTC_4$  with each stimulus. 15-HETE enhanced significantly the generation of leukotrienes at concentrations of 0.1–3  $\mu$ g/ml, irrespective of whether the isolated mediators were quantified by optical density or whether  $LTB_4$  and  $LTC_4$  were assessed by radioimmunoassay (Fig. 1). 15-HETE failed to evoke the release of histamine in amounts greater than that released spontaneously in buffer. In contrast, compound 48/80 did not stimulate the generation of leukotrienes at concentrations that released significant quantities of histamine (Fig. 2). Neither 15-HETE nor compound 48/80 was cytotoxic for mastocytoma cells, as evidenced by their failure to augment significantly the release of lactic acid dehydrogenase relative to that observed in buffer alone (Figs. 1 and 2). The previously unrecognized selective activation of mast cells by 15-HETE and 48/80, as reflected in the release of only one class of mediators, was not achieved by other IgE-independent stimuli or by antigen. In the same experiments ionophore A23187 (0.2  $\mu$ M), PF4(59–70) (10  $\mu$ M), and ragweed

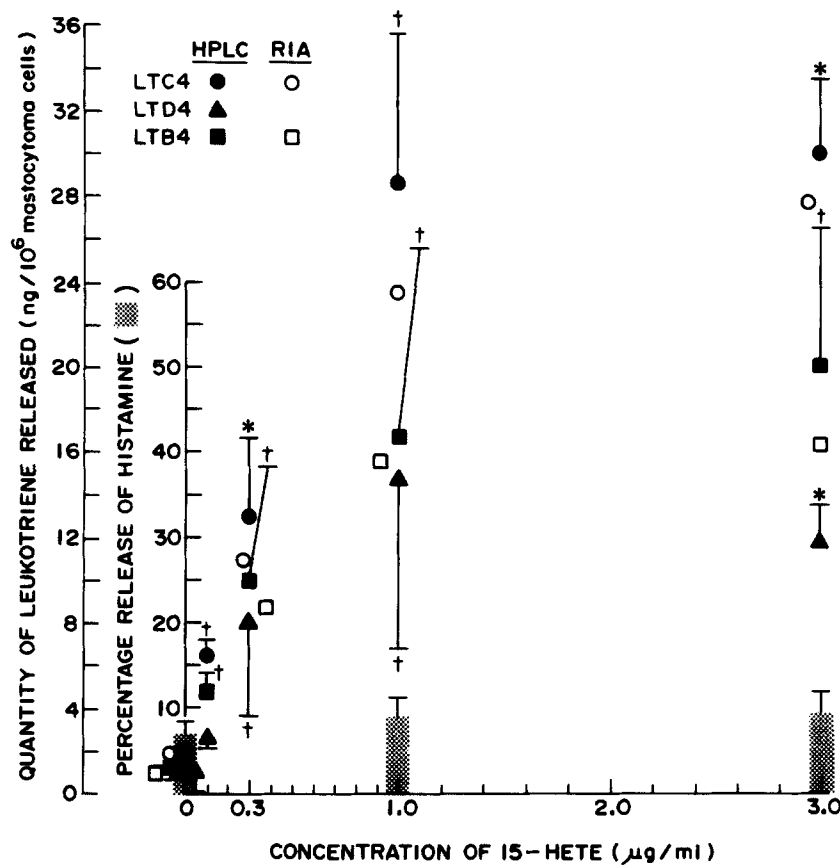


FIGURE 1. Selective generation and release of leukotrienes by mastocytoma cells challenged with 15-HETE. Each point and bracket depicts the mean  $\pm$  SD of the results of two (0.1  $\mu$ g/ml of 15-HETE), three (1 and 3  $\mu$ g/ml), or four (buffer control and 0.3  $\mu$ g/ml) experiments for which LTC<sub>4</sub> (●), LTD<sub>4</sub> (▲), and LTB<sub>4</sub> (■) were purified by HPLC and quantified by optical density. The mean levels of LTC<sub>4</sub> (○) and LTB<sub>4</sub> (□) were determined in two of the experiments by radioimmunoassays. Each bar and bracket shows the mean  $\pm$  SD of the corresponding values for histamine release. The significance of the increases in the amounts of leukotrienes was assessed by a standard Student's *t*-test and is indicated by a + ( $P < 0.05$ ) or an \* ( $P < 0.01$ ). The release of lactic acid dehydrogenase by 0.3, 1, and 3  $\mu$ g/ml of 15-HETE was  $1.5 \pm 0.9\%$  (mean  $\pm$  SD,  $n = 3$ ),  $2.5 \pm 1.0\%$ , and  $3.0 \pm 1.4\%$ , respectively, compared to  $2.1 \pm 0.6\%$  in buffer alone.

antigen resulted in the net release of  $77.5 \pm 3.9\%$  (mean  $\pm$  SD,  $n = 3$ ),  $21.9 \pm 2.9\%$ , and  $18.7 \pm 2.4\%$ , respectively, of the histamine in  $10^6$  mastocytoma cells, and evoked the generation of  $21.2 \pm 5.5$ ,  $9.1 \pm 5.1$ , and  $16.4 \pm 3.4$  ng of LTC<sub>4</sub> (mean  $\pm$  SD,  $n = 3$ ),  $4.8 \pm 0.6$ ,  $3.2 \pm 2.5$ , and  $3.7 \pm 1.6$  ng of LTD<sub>4</sub>, and  $10.6 \pm 2.0$ ,  $6.1 \pm 2.9$ , and  $9.5 \pm 2.3$  ng of LTB<sub>4</sub> by  $10^6$  mastocytoma cells ( $P < 0.05$  for all values relative to buffer controls, except the generation of LTD<sub>4</sub> with PF4(59–70)). The specificity of the stimulation of leukotriene generation by 15-HETE was established by the lack of effect of 0.3–3  $\mu$ g/ml of 15-HPETE and the lesser effect of 12-HETE, 1  $\mu$ g/ml of which evoked the generation of a mean of 1.2, 2.7, and 4.9 ng of LTC<sub>4</sub> at 1, 10, and 30 min ( $n = 2$ ), relative to 1.7, 1.9, and 2.1 ng with buffer alone.

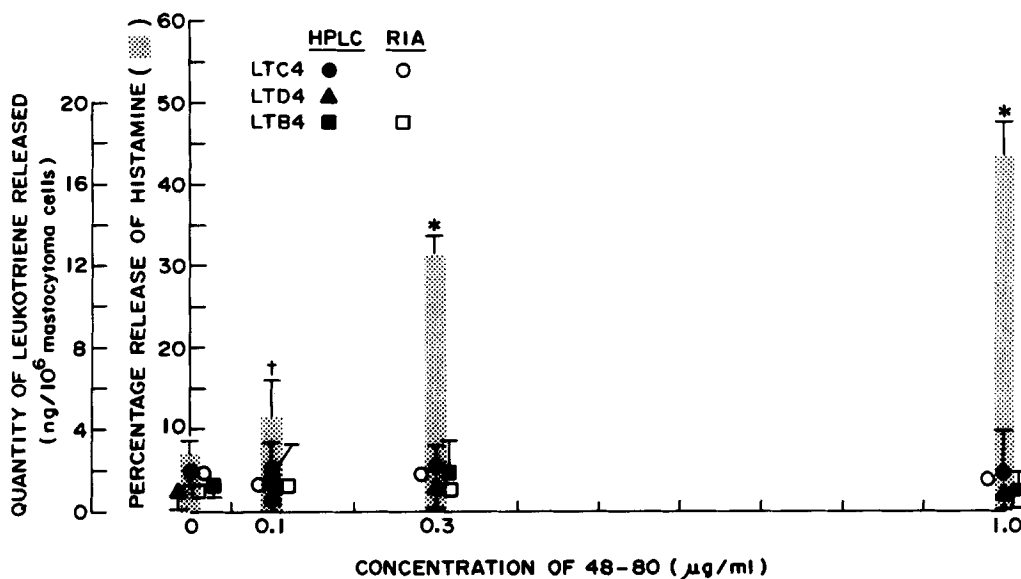


FIGURE 2. Selective release of histamine by mastocytoma cells challenged with 48/80. Each point or bar and bracket depicts the mean  $\pm$  SD of the results of three experiments, in which the analyses were performed with the same techniques as noted in Fig. 1. The symbols for each mediator and for statistical significance also are the same as in Fig. 1. The release of lactic acid dehydrogenase by 0.1, 0.3, and 1  $\mu$ g/ml of 48/80 was  $2.3 \pm 1.4\%$  (mean  $\pm$  S.D.,  $n = 3$ ),  $2.5 \pm 0.9\%$ , and  $3.6 \pm 0.6\%$  respectively, compared to  $2.1 \pm 0.6\%$  in buffer alone.

### Discussion

The exposure of IgE-sensitized dog mastocytoma cells to antigen and of unsensitized mastocytoma cells to ionophore A23187 or PF4(59-70) stimulates both the release of histamine and the generation and release of LTB<sub>4</sub>, LTC<sub>4</sub>, and LTD<sub>4</sub>. In contrast, the interaction of mastocytoma cells with 15-HETE stimulates selectively the generation of leukotrienes, in the absence of histamine release, while compound 48/80 releases histamine without enhancing the generation of leukotrienes. The stimulation of leukotriene generation was significant at concentrations of 15-HETE of 0.1  $\mu$ g/ml or higher and reached a plateau at 1-3  $\mu$ g/ml (Fig. 1). The types and maximal quantities of leukotrienes released by mastocytoma cells exposed to 15-HETE were not substantially different from those released by mastocytoma cells challenged with optimal concentrations of antigen or ionophore A23187 (5). Although the mechanism of enhancement of leukotriene generation by 15-HETE was not investigated, neither of the structurally related principles 15-HPETE nor 12-HETE possessed the same capacity. Compound 48/80 elicited a concentration-dependent release of histamine, that was similar in magnitude to the release evoked by antigen or ionophore A23187, but failed to stimulate the generation of leukotrienes (Fig. 2).

That natural stimuli are capable of activating only one compartment of mediators suggests either that some subsets of mast cell receptors are coupled selectively to a restricted synthetic or secretory response or that such stimuli are incapable of initiating the sequence of biochemical events required for a complete response. Although it may be simpler to postulate a selective stimulation of

mastocytoma cells, the data presented do not exclude the possibility that 15-HETE and compound 48/80 might stimulate both compartments of mediators and concurrently inhibit the release of histamine and the generation or release of leukotrienes, respectively. Nonetheless, effectively selective stimuli may be useful probes in investigations of the cellular prerequisites for mediator generation and release. If mast cell activation *in vivo* is similarly selective, then it will be possible to examine separately the systemic and local organ effects of granule-associated and newly generated mediators, in the absence of the other class of mediators.

### Summary

Isolated dog mastocytoma cells sensitized with dog anti-ragweed IgE and challenged with ragweed antigen or incubated with ionophore A23187 or the carboxy-terminal dodecapeptide of platelet factor 4, PF4(59–70), release histamine and concurrently generate leukotrienes B<sub>4</sub>, C<sub>4</sub>, and D<sub>4</sub>. In contrast, the exposure of mastocytoma cells to 0.1–3 µg/ml of 15-hydroxyeicosatetraenoic acid (15-HETE) stimulates selectively the generation of leukotrienes, in the absence of histamine release, while 0.1–1 µg/ml of compound 48/80 releases histamine without enhancing the generation of leukotrienes. That natural stimuli are capable of selectively activating one synthetic or secretory compartment of mast cells suggests that separate subsets of receptors as well as different biochemical events may serve to mobilize each class of mediators.

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### References

1. Austen, K. F., S. I. Wasserman, and E. J. Goetzl. 1976. Mast-cell derived mediators: structural and functional diversity and regulation of expression. *In* Molecular and Biological Aspects of the Acute Allergic Reaction. S. G. O. Johansson, K. Strandberg, and B. Uvnäs, editors. Plenum Publishing Co., New York. p. 293–320.
2. Goetzl, E. J. 1980. Mediators of immediate hypersensitivity derived from arachidonic acid. *N. Engl. J. Med.* 303:822.
3. Goetzl, E. J. 1977. Mast cell-mediated reactions of host defense and tissue injury: the regulatory role of eosinophil polymorphonuclear leukocytes. *Inflammation* 2:239.
4. Phillips, M. J., L. Calonico, and W. M. Gold. 1982. Morphological and pharmacological characterization of dog mastocytoma cells. *Am. Rev. Respir. Dis.* 125:63A. (Abstr.).
5. Phillips, M. J., W. M. Gold, and E. J. Goetzl. 1983. IgE-dependent and ionophore-induced generation of leukotrienes by dog mastocytoma cells. *J. Immunol.* In press.
6. McGuire, J. C., R. C. Kelley, R. R. Gorman, and F. F. Sun. 1978. Preparation and spectral properties of 12-hydroxy-eicosatetraenoic acid (HETE). *Prep. Biochem.* 8:147.
7. Boeynaems, J. M., J. A. Oates, and W. C. Hubbard. 1980. Preparation and characterization of hydroperoxy-eicosatetraenoic acids (HPETEs). *Prostaglandins* 19:87.
8. Goldman, D. W., and E. J. Goetzl. 1982. Specific binding of leukotriene B<sub>4</sub> to receptors on human polymorphonuclear leukocytes. *J. Immunol.* 129:1600.
9. Goetzl, E. J., C. Derjan, and F. H. Valone. 1980. The extracellular and intracellular roles of hydroxy-eicosatetraenoic acids in the modulation of PMN leukocyte and macrophage function. *J. Reticuloendothel. Soc.* 28:105s.
10. Watt, K. W. K., I. L. Brightman, and E. J. Goetzl. 1983. Isolation of two polypeptides

- comprising the neutrophil-immobilizing factor of human leukocytes. *Immunology*. 48:79.
11. Siraganian, R. P. 1976. Histamine release and assay methods for the study of human allergy. *In* Manual of Clinical Immunology, 2nd ed. N. R. Rose and H. Friedman, editors. American Society for Microbiology, Washington, D.C., p. 603–615.
  12. Lindgren, J. A., S. Hammarström, and E. J. Goetzl. 1983. A sensitive and specific radioimmunoassay for leukotriene C<sub>4</sub>. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 152:83.
  13. Goetzl, E. J. 1981. Selective feed-back inhibition of the 5-lipoxygenation of arachidonic acid in human T-lymphocytes. *Biochem. Biophys. Res. Commun.* 101:344.
  14. Vanderhoek, J. Y., N. S. Tare, M. Bailey, A. L. Goldstein, and D. H. Pluznik. 1982. New role for 15-hydroxy-eicosatetraenoic acid. Activator of leukotriene biosynthesis in PT-18 mast/basophil cells. *J. Biol. Chem.* 257:12191.