

Human neutrophil-derived histamine-releasing activity (HRA-N) causes the release of serotonin but not arachidonic acid metabolites from rat basophilic leukemia cells

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The effects of neutrophil-derived histamine-releasing activity (HRA-N) on arachidonic acid (AA) metabolism is unknown. Human basophils exposed to HRA-N released 25% of total histamine but no leukotriene C₄ (LTC₄). To confirm this phenomenon, rat basophilic leukemia (RBL) cells were exposed to HRA-N as well as anti-IgE, or calcium ionophore A23187. RBL cells incubated with A23187 released 44% of available serotonin and 59 and 124 pmol/10⁶ cells of prostaglandin D₂ (PGD₂) and LTC₄, respectively. Anti-IgE stimulation resulted in 34% serotonin release and the generation of 34 pmol PGD₂ per 10⁶ cells and 72 pmol LTC₄ per 10⁶ cells. In contrast, HRA-N (2 U/ml) induced 20% serotonin release, 4 pmol PGD₂ per 10⁶ cells, and 0.6 pmol LTC₄ per 10⁶ cells. Neither increasing the dose nor the incubation time of HRA-N enhanced the generation of AA metabolite. Additionally, the spectrum of AA metabolites generated by RBL cells in response to those agents was examined by reverse-phase high-performance liquid chromatography. RBL cells stimulated with A23187 released PGD₂, LTB₄, and its isomers, LTC₄, and 5-hydroxyeicosatetraenoic acid. In contrast, HRA-N stimulation resulted in only minimal PGD₂ generation and no other discernable AA metabolites. Thus, HRA-N causes selective release of serotonin without inducing AA metabolites. These data suggest that HRA-N activates mast cells through a unique pathway. (J ALLERGY CLIN IMMUNOL 1992;89:1085-97.)

Key words: Neutrophil, histamine-releasing factor, basophil, rat basophilic leukemia cell, serotonin, arachidonic acid, prostaglandin, leukotriene

Oxidized products of AA produce various inflammatory actions, such as smooth muscle contraction,^{1, 2} mucus secretion,^{3, 4} increased vascular permeability, vasodilation,⁵ and infiltration of inflammatory cells.⁶ Some AA metabolites can be released during allergic reactions after antigen challenge in vivo.⁷⁻⁹ Antigen stimulation of mast cells and ba-

sophils cause the release of histamine as well as the generation of the 5-lipoxygenase products, LTC₄,¹⁰⁻¹³ and LTB₄,^{11, 14, 15} and, in addition, the cyclooxygenase product, PGD₂,^{10, 11, 16} from mast cells but not basophils. Thus, it is possible that these AA metabolites participate in the pathophysiology of allergic diseases.

The question of whether mast cell/basophil secretagogues other than IgE are important in human diseases is a current topic of investigational interest. A number of inflammatory cells generate HRFs,¹⁷⁻²⁵ and it has been hypothesized that these factors may recruit mast cell and/or basophil participation in events, such as the LPR as well as nonallergic inflammatory conditions. It has been noted in several models that HR occurs during early stages of LPR in which neutrophil infiltration predominates.²⁶⁻²⁹ Furthermore, it has been observed that histamine, but not PGD₂, is recovered in lavages during human LPR.⁹ It was therefore of interest to determine whether HRA-N²⁵ causes both HR and the metabolism of AA.

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MATERIAL AND METHODS

Material

EMEM without L-glutamine, heat-inactivated fetal calf serum, L-glutamine, trypsin-versine mix (Biofluids, Inc., Rockville, Md.) β -nicotinamide adenine dinucleotide pyruvic acid (sodium salt), Histopaque 1077, calcium ionophore A23187, trifluoroacetic acid (Sigma Chemical Co., St. Louis, Mo.), the standards for PGB₂, leukotrienes, mono-HETEs, di-HETEs (Calbiochem Co., San Diego, Calif.), human anti-IgE (goat) (Kirkegaard & Perry Laboratory, Gaithersburg, Md.), [³H]serotonin, [14,15-³H(N)]-LTB₄, [14,15-³H(N)]-LTC₄ (New England Nuclear, Boston, Mass.), [1-¹⁴C]AA, PGD₂ [³H] assay system (Amersham Co., Arlington Heights, Ill.), LTB₄ and LTC₄/LTD₄ antibodies (Advanced Magnetics, Inc., Cambridge, Mass.), and histamine RIA kit (AMAC, Inc., Westbrook, Maine) were purchased from the manufacturers. RBL cells were a generous gift from Dr. Reuben Siraganian, National Institute of Dental Research.

Generation of HRA-N

HRA-N was generated as previously described.²⁴ Human PMNs were isolated from venous apheresis collections by Ficoll-Hypaque (density, 1.077) centrifugation³⁰ followed by hypotonic lysis. Isolated PMNs were resuspended in HBSS with 3 mmol/L of calcium, 2 mmol/L of magnesium, and 20 mmol/L of HEPES at 5×10^7 cells per milliliter, and incubated in a shaker bath, 60 rpm for 1 hour at 37° C. Cells were then centrifuged at 400 g for 5 minutes, and the supernatants were boiled for 10 minutes, followed by centrifugation at 45000 g for 5 minutes. The supernatants were concentrated at 4° C under pressure over a YM2 (Amicon, Danvers, Mass.) (1000 molecular weight cutoff) membrane. The retentates were stored at -70° C until they were assayed for mediator release. The relative concentration of individual HRA-N preparations was determined functionally by measuring the relationship between HRA-N concentration to RBL cell serotonin release. One unit of HRA-N is arbitrarily defined as that concentration of HRA-N that causes 10% serotonin release from RBL cells. The dose-response curve is linear between 0.5 and 4 U/ml of HRA-N and then plateaus at 4 U/ml (data not presented).

Partial purification of HRA-N

Pooled crude neutrophil supernatants were precipitated overnight at 4° C in 75% ammonium sulfate. After centrifugation, the active materials, all of which remained soluble in 75% ammonium sulfate, was applied to a phenyl-Sepharose column (4 × 4.5 cm), equilibrated in 75% ammonium sulfate, and eluted with deionized water at 4° C. Nine milliliter fractions were collected, dialyzed overnight in 4° C deionized water, lyophilized, and reconstituted in HBSS with Ca⁺⁺, Mg⁺⁺, and HEPES. The reconstituted fractions were then tested for HRA-N activity.

Human basophil mediator-release assay

Basophils were obtained from peripheral blood by dextran sedimentation as described.³¹ After a wash with phosphate-buffered saline mixed leukocytes, 0.03 ml (3×10^7

Abbreviations used

HRA-N:	Neutrophil-derived histamine-releasing activity
RBL:	Rat basophilic leukemia
AA:	Arachidonic acid
PGD ₂ :	Prostaglandin D ₂
LTB ₄ , LTC ₄ , LTD ₄ :	Leukotrienes B ₄ , C ₄ , and D ₄
HETE:	Hydroxycicosatetraenoic acid
RP-HPLC:	Reverse-phase high-performance liquid chromatography
ODS:	Octadecylsilane-silica
UV:	Ultraviolet
PMN:	Polymorphonuclear cells (neutrophils)
LPR:	Late-phase reaction
EMEM:	Eagle's minimal essential media with Earle's balanced salt
HBSS:	Hanks' balanced salt solution
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
LDH:	Lactate dehydrogenase
HRF:	Histamine-releasing factor
HR:	Histamine release
[³ H]serotonin:	5-[1,2- ³ H(N)]-hydroxytryptamine binoxalate

cells per tube), were added to 0.17 ml of secretagogue and incubated at 37° C for 45 minutes. The reaction was terminated by the addition of 4° C phosphate-buffered saline and centrifugation, and the histamine in the cells and supernatants was assayed with RIA.³² LTC₄/LTD₄ was measured in the supernatants by RIA as described below.

RBL cell culture

The 2H3 subline of RBL cells was maintained in 175 cm² tissue-culture flasks in EMEM supplemented with 4 mmol/L of glutamine and 15% heat-inactivated fetal calf serum.³³ The cells were treated with trypsin-versine mix, resuspended in media, passaged every 3 to 7 days, and maintained in a 5% CO₂ humidified atmosphere at 37° C.

Radiolabeling of RBL cells

For serotonin-release experiments, 2×10^6 RBL cells per milliliter were radiolabeled by incorporating [³H]serotonin (1 μ Ci/ml) in the culture medium overnight. For AA metabolite determination by HPLC, the same concentration of RBL cells was radiolabeled with [¹⁴C]AA (0.05 μ Ci/ml) overnight. Unlabeled cells were used for the RIA of AA metabolites.

RBL cell mediator-release assay

RBL cells, 5×10^4 cells per well, were placed in a 48-well culture plate and allowed to adhere overnight. The wells were then washed three times with 4° C assay buffer that consists of HBSS with 3 mmol/L of calcium, 2 mmol/L of magnesium, 20 mmol/L of HEPES, and 0.1% gelatin, pH 7.4. These concentrations of Ca⁺⁺ and Mg⁺⁺ were

determined experimentally to support maximal HRA-N-induced serotonin release when concentrations up to 10 mmol/L each were tested (data not presented). Unless it is otherwise specified, RBL cells were incubated with 90 μ l of secretagogues for 30 minutes at 37° C. In experiments with anti-IgE, cells were sensitized with IgE IR-162 in culture medium for 1 hour before the release assay. Reactions were stopped by the addition of 200 μ l 4° C assay buffer to each well. The supernatants were aspirated, centrifuged at 9000 g for 2 minutes to pellet any detached cells, and assayed for mediator release. Adherent cells were lysed by exposure at 37° C to HBSS containing 1% Triton X-100, and the lysate was used for total cell [^{3} H]serotonin determination. In experiments for HPLC analysis, 1×10^7 RBL cells were incubated overnight in a 175 cm² tissue-culture flask and, after a wash, were exposed to 10 ml of secretagogues, and the supernatants were assayed by HPLC. Samples were assayed immediately or stored at -70° C.

Measurement of serotonin release

Release of serotonin was measured by isotope counting. Net percent [^{3} H]serotonin release was calculated as the ratio of radioactivity in the supernatants to those in both cells and supernatants minus control release caused by buffer alone.

RIA measurement for AA metabolites

PGD₂ and LTC₄/LTD₄ released into the supernatants were measured by a competitive RIA, and concentrations were calculated from a standard curve.³⁴ The PGD₂ antibody had 7% cross-reactivity with PGJ₂ and <1% cross-reactivity with other major prostaglandins or thromboxane. Because the LTC₄/LTD₄ antibody has comparable cross-reactivity with LTC₄ and LTD₄ (64% for LTC₄ and 100% for LTD₄), the amount of LTC₄/LTD₄ will be expressed as net LTC₄ mole equivalents. Neither secretagogues themselves nor their solvents affected the RIA.

LDH assay

LDH in the supernatants and cells was assayed by measuring the consumption of reduced nicotinamide adenine dinucleotide during the reduction of pyruvate to lactate.³⁵ The results, measured as the change in optical density₃₄₀ per minute, were expressed as the percent of total cell LDH released.

Sample preparation for HPLC

Before being assayed by HPLC, samples were filtered over Sep-Pak-C₁₈ (Waters Associates, Milford, Mass.) cartridges as modified from the method of Verhagen.³⁶ After cartridges were washed with 15 ml of methanol, 5 ml of 5 mmol/L ethylenediaminetetraacetic acid, and 10 ml of distilled water, samples were passaged through the cartridges, washed with 10 ml of distilled water, and then extracted with 3 ml of methanol. The methanol was evaporated with nitrogen, and samples were resuspended in 150 μ l of Solvent A (75% [vol/vol] distilled water, 25% [vol/vol] acetonitrile, and 2.2 mmol/L of phosphoric acid). The efficiency of this extraction was evaluated by use of

[^{3} H]labeled standards. Recovery of PGD₂, LTB₄, and LTC₄ was 89%, 69%, and 53%, respectively.

HPLC analysis for AA metabolites

RP-HPLC analysis was performed with a modification of the method described by Powell.³⁷ An ODS column (Ultrasphere, 5 μ m particle diameter, 0.46 $\times$ 25 cm) and HPLC apparatus, including two pumps, system controller, autosampler, variable wavelength UV detector (full scale was set at 0.200), and plotter-integrator (Beckman Instruments, Inc., Fullerton, Calif.) were used. After sample application, the column was washed with a mixture of Solvent A and Solvent B (60% [vol/vol] methanol, 40% [vol/vol] acetonitrile, and 0.21 mmol/L of trifluoroacetic acid) at a flow rate of 1.5 ml/min with a combination of linear gradients (Fig. 3, A), and fractions were collected every 30 seconds. HPLC grade reagents were used for all experiments. When samples from [^{14} C]AA-prelabeled RBL cells were applied, the radioactivity in each fraction was counted with a liquid scintillation counter. Recovery of AA metabolites in this HPLC fractionation, measured by use of radiolabeled standards was about 53%, 68%, and 65% for PGD₂, LTB₄, and LTC₄, respectively. When samples from unlabeled RBL cells stimulated with 0.3 μ g/ml A23187 were applied to the column, 30-second fractions were also collected. Fractions 1 to 10, 11 to 20, 21 to 30, and 31 to 40 were combined. These four combined fractions and all subsequent fractions were passaged over Sep-Pak-C₁₈ cartridges and assayed by RIA for PGD₂, LTC₄, LTD₄, and LTB₄.

Statistics

Results were analyzed by the Student's paired t test. Competitive RIAs were analyzed by logit transformation and linear regression analysis.³⁴

RESULTS

Partial purification of HRA-N

Pooled crude neutrophil supernatants were subjected to 10%, 25%, 50%, and 75% ammonium sulfate precipitation. The precipitated and soluble material was dialyzed overnight in deionized water, lyophilized and reconstitution in buffer, and then tested HRA-N activity on RBL cells. All the HRA was found in the fraction soluble in 75% ammonium sulfate (data not presented). Subsequently, 360 ml of pooled crude neutrophil supernatants (10 donors) were exposed to 75% ammonium sulfate. Unprecipitated material was then applied to phenyl-Sepharose column, and 9 ml fractions were collected. All the activity eluted in a broad peak between fractions 2 and 10 (data not presented). Control solutions of 75% ammonium sulfate processed in parallel caused no more serotonin release than did the HBSS control (<5%).

Mediator release from human basophils

The effect of inflammatory cell-derived histamine releasing factors on AA metabolism is unknown. To

TABLE I. Mediator release from stimulated RBL cells*

	n	Serotonin (%)	PGD ₂ (pmol/10 ⁶ cells)	LTC ₄ /LTD ₄ † (pmol/10 ⁶ cells)
Buffer	47	6.0 ± 0.3	9.3 ± 0.9	<0.25‡
Secretagogues§				
Anti-IgE (1:1000)	42	34.2 ± 1.3	33.5 ± 3.1	72.0 ± 9.0
A23187 (0.3 µg/ml)	20	44.1 ± 2.5	58.9 ± 4.7	123.6 ± 18.5
HRA-N (2 U/ml)	8	20.4 ± 1.1	4.0 ± 0.6	0.6 ± 0.6

*RBL cells (5×10^4) were incubated in 48-well tissue-culture plates with secretagogues for 30 minutes at 37° C. Percent serotonin release was measured by counting [³H]serotonin, and PGD₂ and LTC₄/LTD₄ were measured by RIA.

†Release of LTC₄/LTD₄ is expressed as LTC₄ equivalents.

‡Lower limit of assay.

§Results were corrected by subtracting control release and are presented as net release. All results are expressed as mean ± SEM.

||Significant difference of $p < 0.001$ from control release.

address this question, mediator release from human basophils was examined. Mixed leukocytes exposed to buffer spontaneously released $3\% \pm 0.2\%$ of total histamine and no detectable LTC₄/LTD₄. (Lower limit of assay was 6.4 fmol/10⁶ cells.) Anti-IgE (200 µg/ml) induced $55\% \pm 3.5\%$ HR and the generation of 400 ± 0.5 fmol of LTC₄/LTD₄ per 10⁶ cells. In contrast, leukocytes incubated with HRA-N (3 U/ml) released $25\% \pm 0.3\%$ of total histamine but no detectable LTC₄/LTD₄ ($n = 3$).

Mediator release from stimulated RBL cells

To confirm that HRA-N causes the selective release of granular products in the absence of AA metabolism, these data were extended to the culture cell line, RBL cells. RBL cells were incubated with anti-IgE, A23187, or HRA-N, after which serotonin release was measured by isotope counting, and PGD₂ and LTC₄/LTD₄ released into the supernatants were quantitated by RIA. When RBL cells were stimulated with anti-IgE (1:1000) or A23187 (0.3 µg/ml), significant increases in the net release of all three mediators were observed (Table I). Eight different preparations of HRA-N (2 U/ml) caused significant serotonin release in the virtual absence of PGD₂ and LTC₄/LTD₄ generation (Table I). Although net PGD₂ release induced by HRA-N was statistically significant, the magnitude was minimal compared with that induced by the other secretagogues.

Net percent serotonin release induced by anti-IgE and A23187 were higher than that induced by HRA-N. To rule out the possibility that the absence of AA metabolism by HRA-N stimulated RBL cells was a dose-related phenomenon, RBL cells were exposed to various concentrations of anti-IgE, A23187, or HRA-N, and mediator release was measured (Fig. 1). Anti-IgE and A23187 caused the dose-related release of

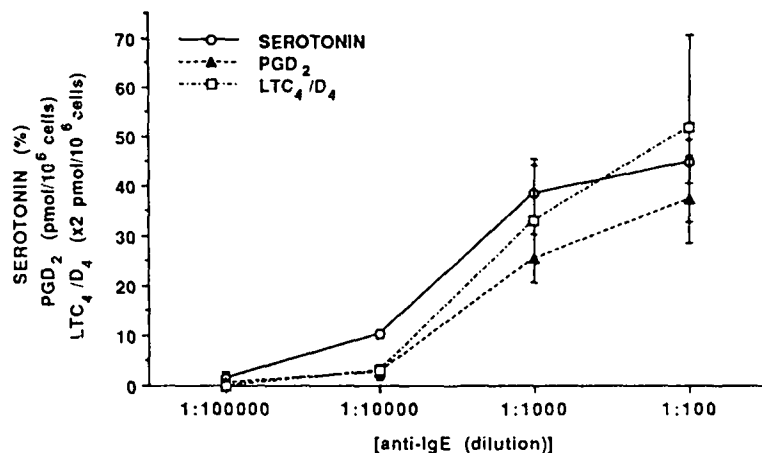
serotonin, PGD₂, and LTC₄/LTD₄. HRA-N, however, caused dose-related serotonin release, minimal PGD₂ generation, and no detectable LTC₄/LTD₄ generation. When the dose of anti-IgE and A23187 was adjusted to induce 40% serotonin release, PGD₂ generation was >25 pmol/10⁶ cells, and LTC₄/LTD₄ generation was >70 pmol/10⁶ cells. In contrast, when the dose of HRA-N was adjusted to cause 40% net serotonin release, LTC₄ generation was not detectable, and PGD₂ generation was only 3 pmol/10⁶ cells. In all of these experiments, LDH release was $<2\%$, except at the highest dose of A23187 (3.0 µg/ml) when LDH release reached 13%.

The time course of mediator release by RBL cells stimulated with anti-IgE, A23187, or HRA-N was next examined (Fig. 2). Release of both LTC₄/LTD₄ and serotonin stimulated by anti-IgE or A23187 was 50% complete at 8 to 10 minutes and essentially completed by 30 to 60 minutes. Release of PGD₂ was more rapid, 50% and 100% complete by 5 and 30 minutes, respectively, for anti-IgE-stimulated RBL cells, and almost complete by 5 minutes for A23187-stimulated RBL cells. In contrast, serotonin release induced by HRA-N is complete within 5 to 10 minutes. In these experiments, RBL cells were exposed to HRA-N for only 30 minutes. To rule out the possibility that HRA-N induced delayed AA metabolism, additional experiments were performed. When the exposure time of RBL cells to HRA-N was extended to 60 minutes, RBL cells released 15% of available serotonin but did not generate any PGD₂ or LTC₄/LTD₄ (data not presented).

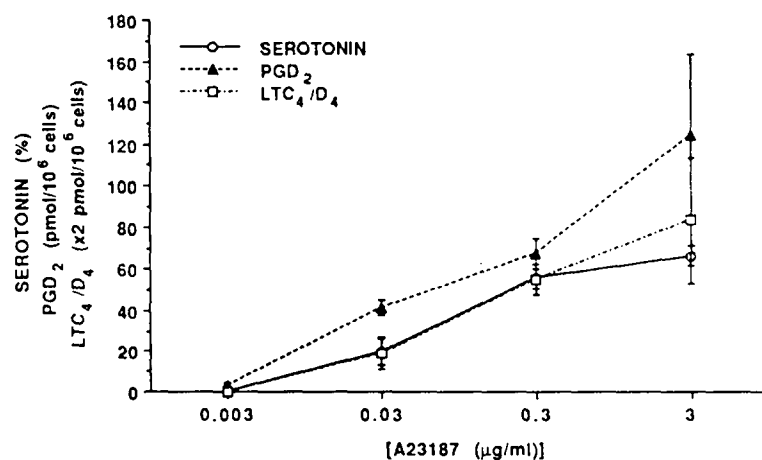
Analysis of AA metabolites by HPLC

To determine whether HRA-N induces a different spectrum of AA metabolites, the release of AA metabolites was analyzed by RP-HPLC. To determine

A anti-IgE



B A23187



C HRA-N

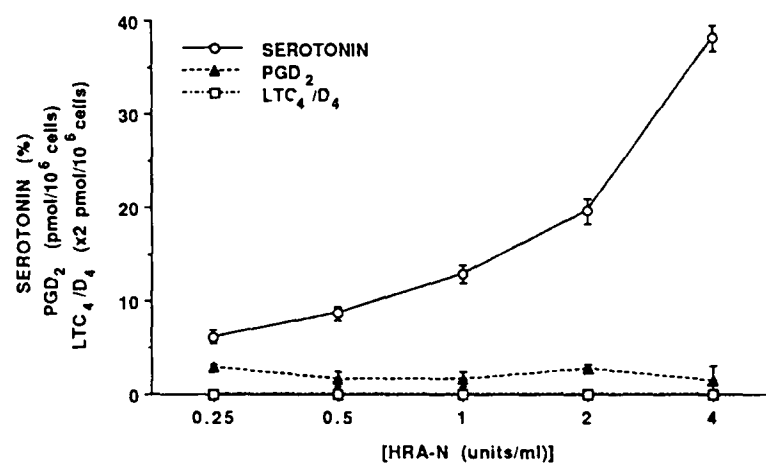


FIG. 1. Dose response of RBL cell mediator release (*net*). RBL cells (5×10^4) were incubated with various concentrations of **A**, anti-IgE; **B**, calcium ionophore A23187; or **C**, HRA-N for 30 minutes. Percent serotonin release measured by [^{3} H]serotonin counting; PGD₂ and LTC₄/LTD₄, measured by RIA. Data, mean $\pm$ SEM of three different experiments performed in duplicate; asterisk, significant differences from control release; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

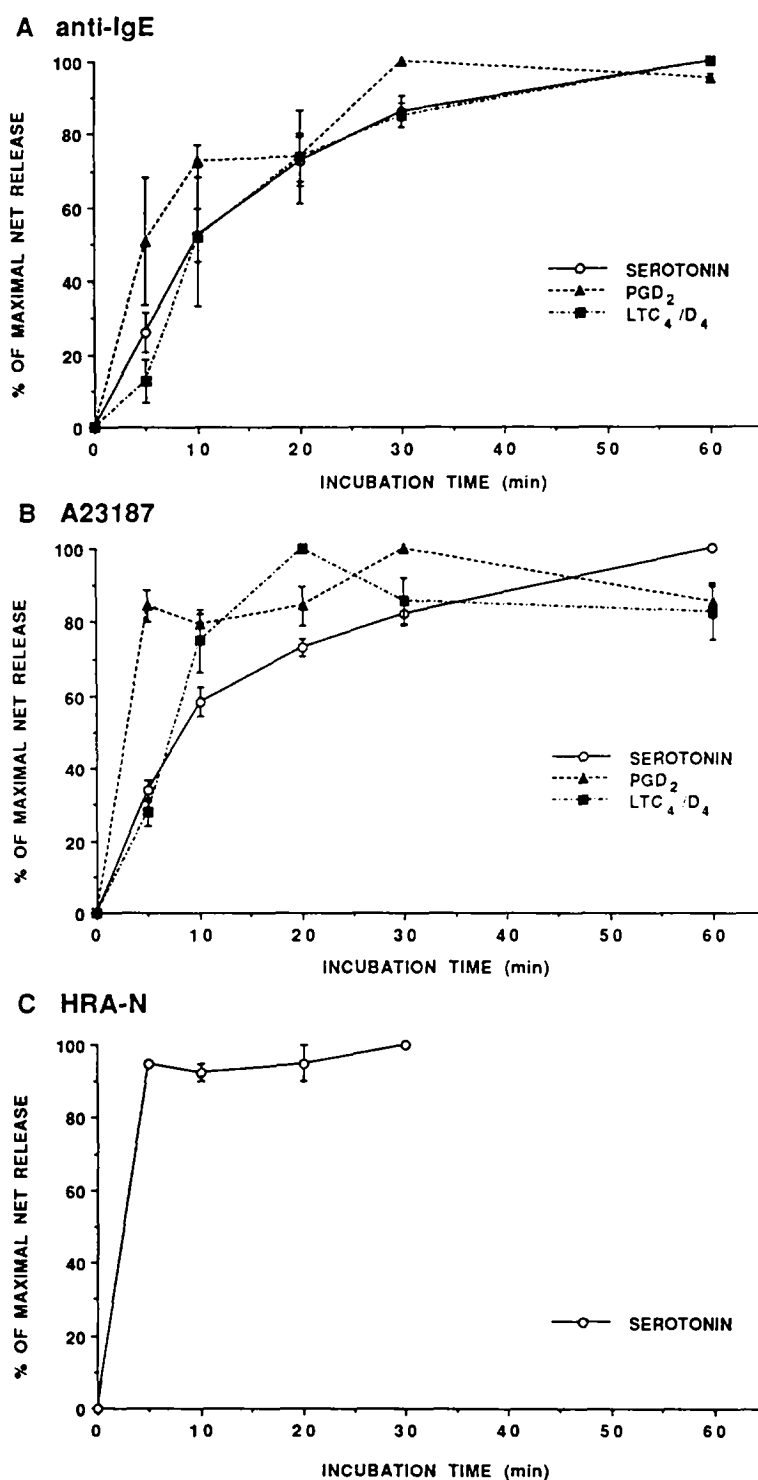


FIG. 2. Time course of RBL cell mediator release. RBL cells (5×10^4) were incubated with **A**, 1:000 anti-IgE; **B**, 0.3 $\mu\text{g}/\text{ml}$ A23187; or **C**, 2 U/ml HRA-N for indicated amount of time; mediators released were assayed. *Results*, percent of maximal net release; *data*, means $\pm$ SEM of three different experiments performed in duplicate; *maximal release*, 47.8% $\pm$ 3.0% serotonin, 80.0 $\pm$ 20.3 pmol/ 10^6 cells PGD₂; 249.7 $\pm$ 70.0 pmol/ 10^6 cells LTC₄/LTD₄ induced by anti-IgE, 49% $\pm$ 1.7% serotonin; 78.0 $\pm$ 1.3 pmol/ 10^6 cells PGD₂; and 209.9 $\pm$ 16.5 pmol/ 10^6 cells LTC₄/LTD₄ induced by A23187; 20% $\pm$ 0% serotonin induced by HRA-N.

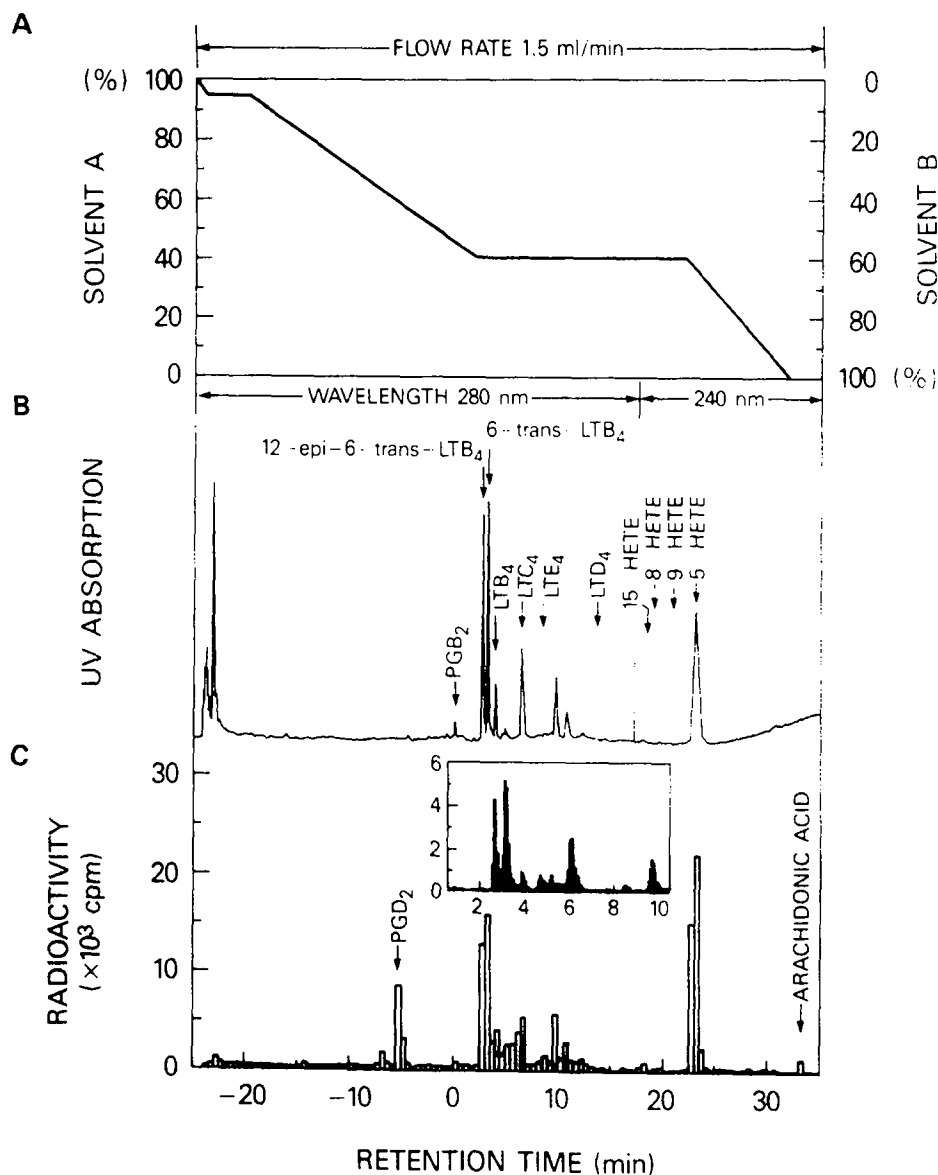


FIG. 3. **A**, Program of gradient elution used in RP-HPLC separations. Graph depicts ratio of Solvent A and B at each elution time. **B**, Analysis of AA metabolites by UV absorption. [^{14}C]AA-prelabeled RBL cells (1×10^7) were incubated with $0.3 \mu\text{g/ml}$ of A23187 at 37°C for 30 minutes. Supernatants filtered over an ODS RP-HPLC column as described in **A**; arrows, retention times at which standards elute, expressed relative to elution of PGB_2 , assigned 0 time. Wavelength changed from 280 to 240 nm at 17 minutes; baseline immediately reset to 0. **C**, Analysis of AA metabolites by [^{14}C]radioactivity in 30-second fractions from same RP-HPLC run as described in **B**; arrows, fractions in which radiolabeled PGD_2 and AA elute; identical elution patterns obtained in 12 separate fractionations. *Insert*, radioactivity in 5-second collections from separate, similar fractionation, resulted in resolution of two peaks between $2\frac{1}{2}$ and $3\frac{1}{2}$ minutes.

the scope of AA metabolites produced by RBL cells. [^{14}C]AA-labeled RBL cells were stimulated with $0.3 \mu\text{g/ml}$ of A23187, and the supernatants were analyzed by both UV absorption and radioactivity in the fractions from HPLC (Fig. 3, B and C). PGB_2 was added

to samples as an internal standard, against which the retention times of standards and samples were compared (Fig. 3, B). Since the baseline of UV absorption was highly variable at 205 nm when many prostaglandins display maximal absorption (data not pre-

sented), prostaglandins were monitored as peaks of radioactivity that eluted at times determined by radiolabeled standards. Leukotrienes and HETEs were readily detectable with 280 and 240 nm UV absorption, respectively. The results of one representative fractionation out of 12 are presented in Fig. 3, *B* and *C*. Fractionation of the supernatant of ionophore-stimulated RBL cells yielded UV absorption peaks that coeluted with standards for LTB₄ (3.8 minutes), 12-*epi*-6-*trans*-LTB₄ (2.6 minutes), 6-*trans*-LTB₄ (3.1 minutes), LTC₄ (6.3 minutes), and 5-HETE (22.9 minutes) (Fig. 3, *B*). Several unknown peaks were observed as well. Assessment of radioactivity revealed peaks corresponding to AA metabolites detected by UV absorbance (Fig. 3, *C*). In addition, another radioactive peak coeluting with [³H]PGD₂ was detected at -5.5 to -4.5 minutes (relative to PGB₂). An assessment of 5-second fractions permitted the resolution of two peaks of radioactivity between 2½ and 3½ minutes, which corresponds to the two isomers of LTB₄ detected by UV absorption (Fig. 3, *C*, *insert*). Thus, assessment of AA metabolites by UV absorption and by radiotracer detection resulted in similar patterns of detection.

The HPLC analysis of AA metabolites was then compared with RIA for several major AA metabolites. In these experiments, unlabeled RBL cells were stimulated with 0.3 µg/ml of A23187 in a parallel experiment with [¹⁴C]labeled RBL cells, the supernatants were fractionated as above, and the individual fractions were extracted by Sep-Pak, and then assayed by RIA for PGD₂, LTC₄/LTD₄, and LTB₄. The preincubation of RBL cells with [¹⁴C]AA did not affect the pattern of AA metabolites generated since eluted supernatants of labeled cells or unlabeled cells yielded identical UV absorption patterns (data not presented). Most immunoreactive PGD₂ coeluted with the [³H]PGD₂ standard, with little (<15% of total) activity detected in other fractions (Fig. 4, *B*). Similarly, almost all the immunologically active LTC₄/LTD₄ coeluted with the LTC₄ standard (Fig. 4, *C*). LTD₄ could not be detected by either radioactivity or by RIA. Thus, it appears that under these conditions, activated RBL cells generate LTC₄ but not LTD₄. The LTB₄ RIA recognized the fraction coeluting with the LTB₄ standard as well as fractions that coeluted with two isomers of LTB₄ (Fig. 4, *D*). Thus, RIA and HPLC analysis yield similar qualitative results. Although the RIAs for PGD₂ and LTC₄ are specific, the RIA for LTB₄ also recognizes isomers of LTB₄.

After RBL cells were incubated with buffer, they spontaneously generated a small amount of PGD₂, but no 5-lipoxygenase metabolites (Fig. 5, *A*). After stimulation with 3 U/ml of partially purified HRA-N,

supernatants of RBL cells contained only a small amount of radioactivity coeluting with PGD₂ and no other AA metabolites (Fig. 5, *B*). The elution pattern was quite similar with that of control stimulation that was induced by buffer alone. Although partially purified HRA-N induced more PGD₂ generation than did the control, the magnitude was minimum compared with that induced by A23187. Thus, although anti-IgE and A23187 stimulation cause both serotonin release and AA metabolism, HRA-N induces selective serotonin release from RBL cells in the virtual absence of AA metabolism.

DISCUSSION

This is the first article of the selective release of granular products in the absence of AA metabolism by mast cells/basophils stimulated with an inflammatory cell-derived HRF. The mechanism by which the various HRFs stimulate mast cells/basophils is unknown. However, it has previously been demonstrated that HRA-N causes equivalent amounts of serotonin release from RBL cells lacking surface IgE compared with that of IgE-sensitized RBL cells. Moreover, the removal of IgE from human basophils has no consistent effect on HRA-N-induced HR. Furthermore, replenishment of IgE-depleted basophils with IgE plus does not reverse any observed effects of IgE removal on HRA-N-induced basophil degranulation.³⁸ Therefore, determining whether HRA-N induces AA metabolism as well as degranulation is important in defining the site of action of HRA-N on mast cells/basophils.

We first examined HRA-N-induced AA metabolism in human basophils. HRA-N-stimulated human basophils were found to release histamine but no detectable LTC₄/LTD₄. In contrast, basophils stimulated with anti-IgE released both histamine and LTC₄/LTD₄. Although the experiments were performed with mixed leukocytes, it was clear that HRA-N did not induce LTC₄/LTD₄ generation from basophils or other bystander cells. This observation is interesting, since basophils are presumed to play a major role in LPR,^{9, 39} whereas HRFs are possibly participating.

To examine in more detail the effect of HRA-N on mast cell/basophil AA metabolism, RBL cells, a cultured cell line of presumed mucosal mast cell lineage,⁴⁰ were next used. The advantage of the use of a cultured cell line is that damage occurring during cell-purification processes can be avoided and that large scale experiments can be performed easily. As expected, stimulation of RBL cells by anti-IgE or A23187 resulted in significant release of both serotonin and the AA metabolites, PGD₂, and LTC₄/LTD₄. In contrast, HRA-N induced significant serotonin re-

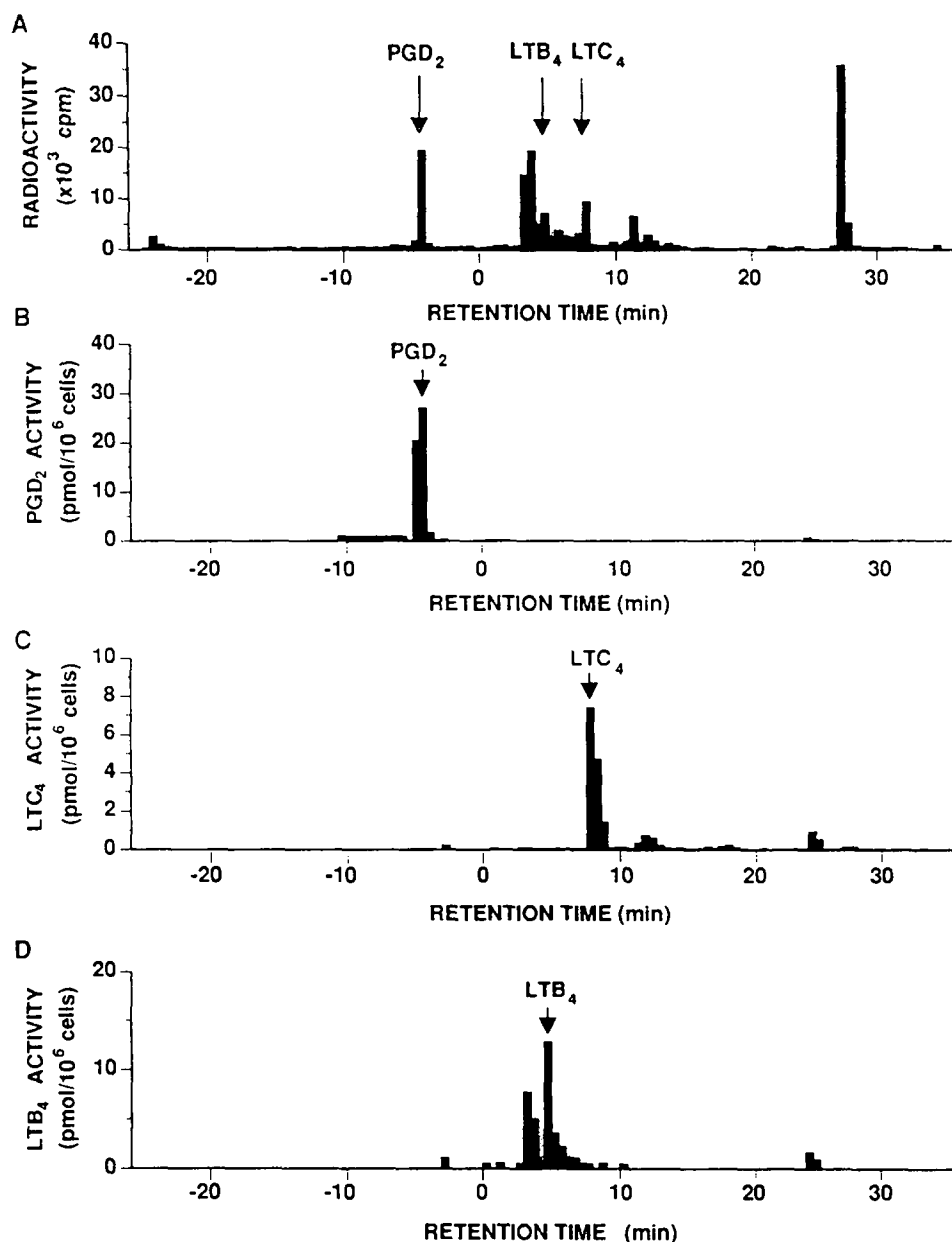


FIG. 4. A, $[^{14}\text{C}]$ radioactivity in HPLC fractions. $[^{14}\text{C}]$ AA-labeled RBL cells (1×10^7) incubated with $0.3 \mu\text{g/ml}$ of A23187 for 30 minutes; supernatant filtered over ODS RP-HPLC column as described in Fig. 3, A; eluate collected every 30 seconds; arrows, retention time at which standards elute, expressed relative to elution of PGB₂, assigned 0 time. B, C, and D, RIA analysis of HPLC fractions. Unlabeled RBL cells (1×10^7) incubated with $0.3 \mu\text{g/ml}$ of A23187 for 30 minutes; supernatants chromatographed as above. Fractions were aliquoted and assayed by RIA for B, PGD₂; C, LTC₄/LTD₄; D, LTB₄. Eluates collected first 20 minutes, combined into four pools coeluted over 5-minute periods; assay results expressed as average of 10 original fractions.

lease, minimal generation of PGD₂, and no generation of LTC₄. It appeared possible that the inability of HRA-N to induce AA metabolism could be due to an inadequate dose of HRA-N. However, even when a maximal dose of HRA-N was used to cause the same magnitude of serotonin release as anti-IgE and

A23187, the magnitude of PGD₂ release induced by HRA-N was remarkably smaller than that induced by anti-IgE and A23187, and LTC₄/LTD₄ was undetectable. Thus, at doses causing comparable serotonin release, anti-IgE and A23187 induce significant PGD₂ and LTC₄/LTD₄ generation, whereas HRA-N induces

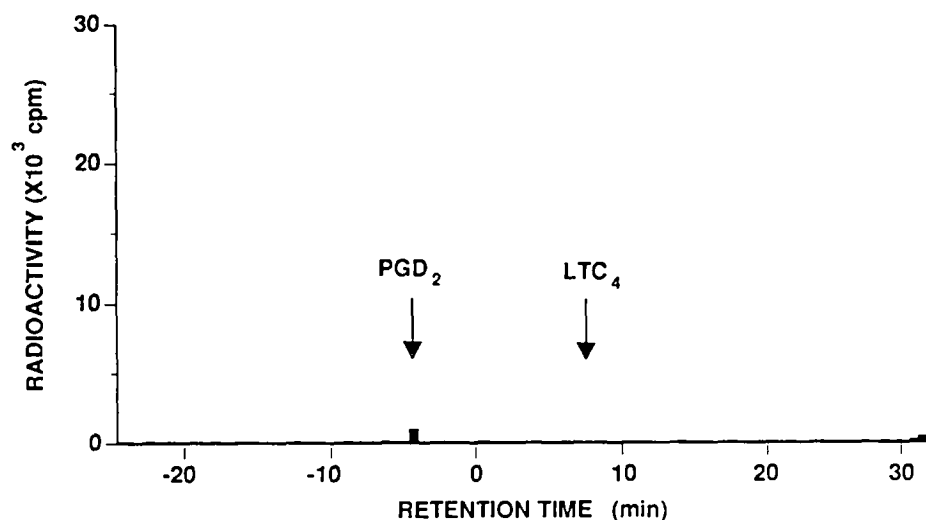
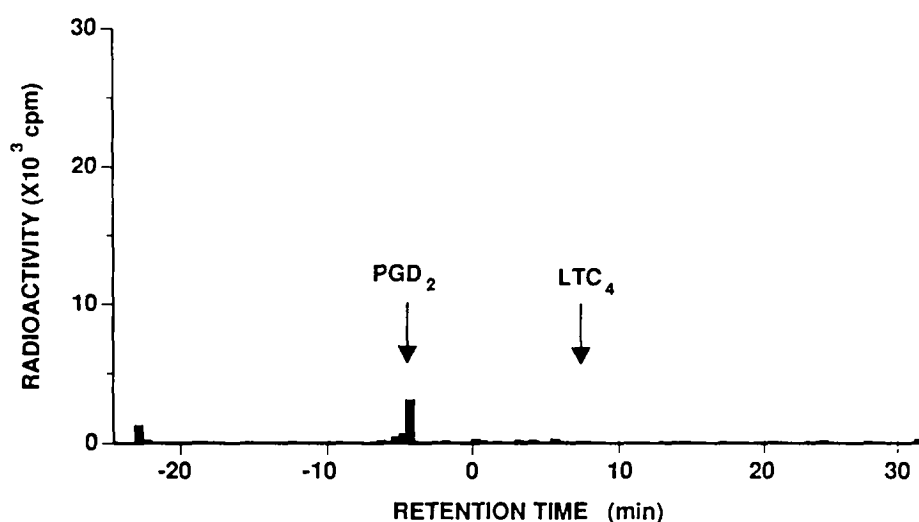
A CONTROL**B HRA-N**

FIG. 5. [^{14}C]radioactivity in HPLC fractions. [^{14}C]AA-labeled RBL cells (1×10^7) incubated with **A**, assay buffer or **B**, 3 U/ml partially purified HRA-N for 30 minutes; supernatant filtered over ODS RP-HPLC column as described in Fig. 3. **A**, eluate collected every 30 seconds; arrows, retention time at which standards elute, expressed relative to elution of PGB_2 , assigned 0 time. Serotonin release performed in parallel was 6.7% and 29.2% (*net*) for control and HRA-N, respectively.

the selective release of serotonin in the virtual absence of AA metabolism. To rule out the possibility that HRA-N-induced AA metabolism requires a longer incubation time. RBL cells were incubated with HRA-N for various periods. HRA-N-induced serotonin release is complete by 5 to 10 minutes. However, even after 60 minutes, HRA-N induced virtually no AA metabolism.

We then examined another possibility that HRA-N induced a different spectrum of AA metabolites from those induced by other secretagogues. In buffer-control experiment, generation of some PGD_2 was observed, but no detectable 5-lipoxygenase metabolites. PGD_2 may be spontaneously metabolized from intracellular-free AA, whereas 5-lipoxygenase activity may require stimulation. Stimulation by A23187,

however, leads to the release of serotonin as well as the generation of significant quantities of PGD₂, LTC₄, LTB₄, and 5-HETE. HPLC analysis of HRA-N-induced AA metabolites in RBL cells demonstrated only minimal generation of PGD₂ and no other metabolites. These results were essentially the same as those obtained with the buffer control. Thus, HRA-N induces the selective degranulation of mast cell/basophil in the absence of the generation of not only PGD₂ and LTC₄ but virtually all AA metabolism. We cannot rule out the possibility, however, that AA metabolites may have been produced but not released.

The RP-HPLC method used in this study allowed for the separation and identification of multiple AA metabolites, such as prostaglandins, leukotrienes, mono-HETEs, and di-HETEs in a single chromatograph. Furthermore, in experiments assayed by RP-HPLC, RBL cells were preincubated overnight with AA labeled with [¹⁴C] in the one position to ensure radioactive labeling of all metabolites. Although the extent of incorporation of the [¹⁴C]AA into various intracellular and membrane pools of AA is unknown, analysis of AA metabolites by detection of [¹⁴C] correlated closely with results obtained by analysis of UV absorption, except for analysis of prostaglandins, which are difficult to detect by UV absorption because of excessive variability in baseline readings at 205 nm, the optimal wavelength for prostaglandin detection. Thus, it is unlikely that an unlabeled pool of AA was metabolized differently from the labeled pool after RBL cell stimulation with A23187, anti-IgE, or HRA-N.

The RIA for PGD₂ and LTC₄ were highly specific, whereas the RIA for LTB₄ detected two isomers of LTB₄, which eluted at 2.6 and 3.1 minutes, immediately before LTB₄. These two isomers, 6-*trans*-LTB₄ and 12-*epi*-6-*trans*-LTB₄, can be formed by nonenzymatic metabolism of LTA₄^{41, 42} or by oxidation of LTC₄.⁴⁴ LTD₄ could not be detected by either radioactivity or by RIA in our experiments. LTC₄ is further metabolized into LTD₄ and LTE₄ by cleavage of the peptide chain.⁴⁴⁻⁴⁶ However, a previous study demonstrated that highly purified mast cells generated only LTC₄.¹¹ Conversion from LTC₄ to LTD₄ probably requires the action of cells other than mast cells. It was previously reported that RBL cells generate PGD₂ and LTC₄.¹⁷⁻¹⁸ The AA metabolites generated by RBL cells are similar to those observed in other experiments with mast cells derived from a variety of sources.¹⁰⁻¹⁶ Without HPLC separation, the magnitude of LTC₄ generated by A23187-stimulated RBL cells was twice as much as that of PGD₂ on average, although more PGD₂ than LTC₄ was generated in some experiments. In contrast, after HPLC separation, we occasionally

observed more PGD₂ produced than LTC₄. This discrepancy would not be fully explained by the difference in recovery after Sep-Pak extraction of eicosanoids. Values of recovery were all based on processing of small quantities of radiolabeled standards. We suspect that recovery of LTC₄ may be lower when much larger quantities of LTC₄ are passed through Sep-Paks, as in our experiments. Nevertheless, with appropriate stimulation, RBL cells are capable of generating PGD₂, LTC₄, LTB₄, 6-*trans*-LTB₄, 12-*epi*-6-*trans*-LTB₄, and 5-HETE, whereas HRA-N induces no AA metabolism. These data provide additional evidence that the mechanisms by which HRA-N-mediated and IgE-mediated stimulations activate mast cells/basophils are different.

The relationship between HRA-N, as well as other HRFs, and mast cell/basophil activation *in vivo* in humans is unclear. In several models of human LPRs (skin, nasal, and lung), HR has been observed,^{9, 30, 38} and in the skin model, this event was closely associated with the influx of PMNs.²⁶ The release of histamine and LTC₄ in the absence of PGD₂ during LPR in the nasal and skin models has been interpreted as evidence for basophil but not mast cell participation in LPRs^{9, 30} since mast cells release both LTC₄ and PGD₂, whereas basophils release LTC₄ but not PGD₂.⁵⁰ An alternative explanation is that mast cells and/or basophils are activated during LPR by HRA-N to release histamine but not AA metabolites. LTC₄ generated during LPR could be derived from other inflammatory cells, such as neutrophils or eosinophils. The ability of other inflammatory cell-derived HRFs to induce AA metabolism is unknown. However, other nonimmunologic mast cell activators (substance P, bradykinin, neurotensin, C5a, 12-O-tetradecanoylphorbol 13-acetate, and neuropeptide Y) have also been noted to cause histamine, but not AA metabolite release.⁵¹⁻⁵⁴ Although the mechanism by which HRA-N stimulates mast cells/basophils is still unclear, it appears clear that HRA-N, as well as other non-IgE-mediated secretagogues, activate mast cells/basophils by mechanisms that do not involve AA metabolism.

REFERENCES

1. Holgate ST, Burns GB, Robinson C. Anaphylactic- and calcium-dependent generation of prostaglandin D₂ (PGD₂), thromboxane B₂, and other cyclooxygenase products of arachidonic acid by dispersed human lung cells and relationship to histamine. *J Immunol* 1984;133:2138-44.
2. Samuelsson B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* 1983; 220:568-75.
3. Marom Z, Shelhamer JH, Bach MK, Morton DR, Kalner M. Slow-reacting substances, leukotrienes C₄ and D₄, increase the release of mucus from human airways *in vitro*. *Am Rev Respir Dis* 1982;126:449-51.

4. Marom Z, Shelhamer JH, Sun F, Kaliner M. Human airway monohydroxyeicosatetraenoic acid generation and mucus release. *J Clin Invest* 1983;72:122-7.
5. Dahlén SE, Hedqvist P, Hammarström S, Samuelsson B. Leukotrienes are potent constrictors of human bronchi. *Nature* 1980;288:484-6.
6. Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME, Smith MJH. Leukotriene B₄, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* 1980;286:264-5.
7. Murray JJ, Tonnel AB, Brash AR, et al. Release of prostaglandin D₂ into human airways during acute antigen challenge. *N Engl J Med* 1986;315:800-4.
8. Creticos PS, Peters SP, Adkinson NF Jr, et al. Peptide leukotriene release after antigen challenge in patients sensitive to ragweed. *N Engl J Med* 1984;310:1626-30.
9. Naclerio RM, Meier HH, Kagey-Sobotka A, et al. Inflammatory mediators in late antigen-induced rhinitis. *N Engl J Med* 1985;313:65-70.
10. Peters SP, MacGlashan DW Jr, Schulman ES, et al. Arachidonic acid metabolism in purified human lung mast cells. *J Immunol* 1984;132:1972-9.
11. Heavey DJ, Ernst PB, Stevens RL, Befus AD, Bienenstock J, Austen KF. Generation of leukotriene C₄, leukotriene B₄, and prostaglandin D₂ by immunologically activated rat intestinal mucosal mast cells. *J Immunol* 1988;140:1953-7.
12. Razin E, Mencia-Huerta JM, Levis RA, Corey EJ, Austen KF. Generation of leukotriene C₄ from a subclass of mast cells differentiated in vitro from mouse bone marrow. *Proc Natl Acad Sci USA* 1982;79:4665-7.
13. MacGlashan DW Jr, Schleimer RP, Peters SP, et al. Generation of leukotrienes by purified human lung mast cells. *J Clin Invest* 1982;70:747-51.
14. Mencia-Huerta JM, Razin E, Ringel EW, et al. Immunologic and ionophore-induced generation of leukotriene B₄ from mouse bone marrow-derived mast cells. *J Immunol* 1983;130:1885-90.
15. Freeland HS, Schleimer RP, Schulman ES, Lichtenstein LM, Peters SP. Generation of leukotriene B₄ by human lung fragments and purified human lung mast cells. *Am Rev Respir Dis* 1988;138:389-94.
16. Lewis RA, Soter NA, Diamond PT, Austen KF, Oates JA, Roberts LJ II. Prostaglandin D₂ generation after activation of rat and human mast cells with anti-IgE. *J Immunol* 1982;129:1627-31.
17. Zheutlin LM, Ackerman SJ, Gleich GJ, Thomas LL. Stimulation of basophil and rat mast cell histamine release by eosinophil granule-derived cationic proteins. *J Immunol* 1984;133:2180-5.
18. Thuesen DO, Speck LS, Lett-Brown MA, Grant JA. Histamine-releasing activity (HRA). I. Production by mitogen- or antigen-stimulated human mononuclear cells. *J Immunol* 1979;123:626-32.
19. Orchard MA, Kagey-Sobotka A, Proud D, Lichtenstein LM. Basophil histamine release induced by a substance from stimulated human platelets. *J Immunol* 1986;136:2240-4.
20. Liu MC, Proud D, Lichtenstein LM, et al. Human lung macrophage-derived histamine-releasing activity is due to IgE-dependent factors. *J Immunol* 1986;136:2588-95.
21. Schleimer RP, Derse C, Landy S, Lichtenstein LM. Vascular endothelial cell supernatants cause histamine release from human basophils [Abstract]. *Fed Proc* 1986;45:1105.
22. Kaplan AP, Haak-Frendscho M, Fauci A, Dinarello C, Halbert E. A histamine-releasing factor from activated human mononuclear cells. *J Immunol* 1985;135:2027-32.
23. Goetzl EJ, Foster DW, Payan DG. A basophil-activating factor from human T lymphocytes. *Immunology* 1984;53:227-34.
24. Sedgwick JD, Holt PG, Turner JJ. Production of a histamine-releasing lymphokine by antigen- or mitogen-stimulated human peripheral T cells. *Clin Exp Immunol* 1981;45:409-18.
25. White MV, Kaliner MA. Neutrophils and mast cells. I. Human neutrophil-derived histamine-releasing activity. *J Immunol* 1987;139:1624-30.
26. Lemanske RF, Kaliner M. Mast cell-dependent late-phase reactions. *Clin Immunol Rev* 1982;1:547-80.
27. Solley G, Gleich G, Jordon R, Schroester A. The late phase of the immediate wheal and flare skin reaction: its dependence on IgE antibodies. *J Clin Invest* 1976;58:408-20.
28. Tannenbaum S, Oertel H, Henderson W, Kaliner M. The biologic activity of mast cell granules. I. Elicitation of inflammatory responses in rat skin. *J Immunol* 1980;125:325-35.
29. Allensmith MR, Baird RS, Greiner JV, Block KJ. Late-phase reactions in ocular anaphylaxis in the rats. *J ALLERGY CLIN IMMUNOL* 1984;73:49-55.
30. Böyum A. Isolation of mononuclear cells and granulocytes from human blood: isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest* 1968;21(suppl 97):77-89.
31. White MV, Baer H, Kubota Y, Kaliner M. Neutrophils and mast cells: characterization of cells responsive to neutrophil-derived histamine-releasing activity (HRA-N). *J ALLERGY CLIN IMMUNOL* 1989;84:773-80.
32. McBride P, Bradley D, Kaliner M. Evaluation of a radioimmunoassay for histamine measurement in biologic fluids. *J ALLERGY CLIN IMMUNOL* 1988;82:638-46.
33. Barsumian EL, Iersky C, Petrino MG, Siraganian RP. IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. *Eur J Immunol* 1981;11:317-23.
34. Granström E, Kindahl H. Radioimmunoassay of prostaglandins and thromboxanes. In: Frölich JC, ed. *Advances in prostaglandin and thromboxane research*, vol 5. New York: Raven Press, 1978:119-210.
35. Vassault A. Lactate dehydrogenase: UV method with pyruvate and NADH. In: Bergmeyer HU, Bergmeyer J, Grassl M, eds. *Methods of enzymatic analysis*, vol III. 3rd ed. Weinheim: Verlag Chemie, 1983:118-26.
36. Verhagen J. Rapid, simple and efficient extraction of arachidonic acid metabolites, including the sulphidopeptide leukotrienes LTC₄ and LTD₄, using octadecyl reversed-phase extraction columns. *J Chromatogr* 1986;378:208-14.
37. Powell WS. Reversed-phase high-pressure liquid chromatography of arachidonic acid metabolites formed by cyclooxygenase and lipoxygenases. *Anal Biochem* 1985;148:59-69.
38. White MV, Kaplan AP, Haak-Frendscho M, Kaliner M. Neutrophils and mast cells: comparison of neutrophil-derived histamine-releasing activity with other histamine-releasing factors. *J Immunol* 1988;141:3575-83.
39. Charlesworth EN, Hood AF, Soter NA, Kagey-Sobotka A, Norman PS, Lichtenstein LM. Cutaneous late-phase response to antigen. *J Clin Invest* 1989;83:1519-26.
40. Seldin DC, Adelman S, Austen KF, Stevens RL, Hein A, Caulfield JP, Woodbury RG. Homology of the rat basophilic leukemia cell and the rat mucosal mast cell. *Proc Natl Acad Sci USA* 1985;82:3871-5.
41. Rådmark O, Malmsten C, Samuelsson B, Goto G, Marfat A, Corey EJ. Leukotriene A: isolation from human polymorphonuclear leukocytes. *J Biol Chem* 1980;255:11828-31.

42. Fitzpatrick FA, Morton DR, Wynalda MA. Albumin stabilizes leukotriene A₄. *J Biol Chem* 1982;257:4680-3.
43. Lee CW, Lewis RA, Corey EJ, Barton A, Oh H, Tauber AI, Austen KF. Oxidative inactivation of leukotriene C₄ by stimulated human polymorphonuclear leukocytes. *Proc Natl Acad Sci USA* 1982;79:4166-70.
44. Örning L, Hammarström S, Samuelsson B. Leukotriene D₅: a slow-reacting substance from rat basophilic leukemia cells. *Proc Natl Acad Sci USA* 1980;77:2014-7.
45. Lewis RA, Drazen JM, Austen KF, Clark DA, Corey EJ. Identification of the C(6)-S-conjugate of leukotriene A with cysteine as a naturally occurring slow-reacting substance of anaphylaxis (SRS-A): importance of the 11-*cis*-geometry for biological activity. *Biochem Biophys Res Commun* 1980;96:271-7.
46. Bernström K, Hammarström S. Metabolism of leukotriene D₅ by porcine kidney. *J Biol Chem* 1981;256:9579-82.
47. Jakschik BA, Lee LH, Shuffer G, Parker CW. Arachidonic acid metabolism in rat basophilic leukemia (RBL-1) cells. *Prostaglandins* 1978;16:733-48.
48. Ortega E, Hazan B, Zor U, Pecht I. Mast cell stimulation by monoclonal antibodies specific for the Fc_ε receptor yields distinct responses of arachidonic acid and leukotriene C₄ secretion. *Eur J Immunol* 1989;19:2251-6.
49. Durham SR, Lee TH, Cromwell O, et al. Immunologic studies in allergen-induced late-phase asthmatic reactions. *J ALLERGY CLIN IMMUNOL* 1984;74:49-60.
50. MacGlashan DW Jr, Schleimer RP, Peters SP, et al. Comparative studies of human basophils and mast cells. *Fed Proc* 1983;42:2504-9.
51. Levi-Schaffer F, Shalit M. Differential release of histamine and prostaglandin D₂ in rat peritoneal mast cells activated with peptides. *Int Arch Allergy Appl Immunol* 1989;90:352-7.
52. Warner JA, Peters SP, Lichtenstein LM, et al. Differential release of mediators from human basophils: differences in arachidonic acid metabolism following activation by unrelated stimuli. *J Leukocyte Biol* 1989;45:558-71.
53. Cohan VL, MacGlashan DW Jr, Warner JA, Lichtenstein LM, Proud D. Mechanisms of release from human skin mast cells upon stimulation by the bradykinin analog, [D-Arg¹-Hyp³-⁵-Phe⁷]bradykinin. *Biochem Pharmacol* 1991;41:293-300.
54. Arzubiaga C, Morrow J, Roberts LJ II, Biaggioli I. Neuropeptide Y, a putative cotransmitter in noradrenergic neurons, induces mast cell degranulation but not prostaglandin D₂ release. *J ALLERGY CLIN IMMUNOL* 1991;87:88-93.

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