

Evaluation of ^3H -Histamine Incorporation into Human Peripheral Blood Leukocytes

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We have examined the use of ^3H -histamine for evaluating *in vitro* basophil responses. Incubation of ^3H -histamine for 4 h with basophil-enriched mononuclear fractions containing autologous plasma, resulted in the best incorporation of the label. Even so, less than 3% of the added label was actually recovered and other leukocytes also bound or incorporated ^3H -histamine. Labeled mononuclear preparations failed to release ^3H -histamine when challenged with zymosan-activated serum or anti-IgE, but endogenous histamine released under the same experimental conditions could be determined fluorometrically. The calcium ionophore, A23187, however, did cause a dose-dependent release of ^3H -histamine. We conclude that ^3H -histamine is not preferentially incorporated into basophil granules, and that this technique cannot be used for assessing allergen and/or IgE-mediated basophil responses.

Key words: histamine release; leukocytes.

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In vitro diagnostic tests of allergic disorders have been largely confined to the measurement of IgE antibodies. The specialized equipment required for histamine analysis or the tedium of measuring degranulation microscopically, often preclude the routine analysis of basophil responses. A simple direct method of measuring ^3H -serotonin release has been used in the rat mast cell model for several years (13, 20), but the incorporation of labeled mediators had not, to our knowledge, been applied to the human basophil. Day and his colleagues (2) reported in an abstract that release of labeled histamine from highly purified human basophils closely parallels histamine release measured fluorometrically, but those findings could not be confirmed in later experiments (J. Bienenstock, personal communication).

While we were beginning the development of

a practical method to assess basophil releasability isotopically, Stahl Skov et al. (19) described a very sensitive method of ^3H -histamine incorporation, whereby 40 samples could be assayed using only 0.5 ml of blood. Such a sensitive technique would be especially useful in the evaluation of pediatric patients or adapted for use in a routine clinical laboratory. We tried to repeat the method of Stahl Skov, and subsequently varied the incubation conditions to optimize the amount of label incorporated into the basophils. We were unable to recover more than 3-4% of the label in our preparations nor could we correlate the release of ^3H -histamine with release measured fluorometrically. We conclude that the label is not incorporated specifically into the basophil granule and that this method is not reliable for the evaluation of allergic patients.

Abbreviations: POPOP: [2, 2'-p-phenylenebis (5-phenyloxazol)]; PPO: (2,5-diphenyloxazol); HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; DMSO: dimethylsulfoxide; EDTA: disodium ethylenediamine tetraacetate.

MATERIAL AND METHODS

Reagents. The following reagents were purchased: Toluol, POPOP, PPO, from Merck; calcium ionophore A23187 from Calbiochem AE; goat anti-IgE Fc specific from Nordic Laboratories; Ficoll 400 from Pharmacia; Ronpacon 440 (Isopaque) from Cilag Chemie AG; Hypaque 45% from Winthrop Laboratories; 2,5-³H-histamine dihydrochloride, specific activity 9–10 Ci/m mol, from Amersham; zymosan from ICN Pharmaceuticals; Biosolve Solubilizer Formula BBS-3 from Beckman.

Buffer and solutions. HCM: 120 mM NaCl, 5 mM KCl, 20 mM HEPES containing 0.6 mM CaCl₂ and 1 mM MgCl₂. The calcium ionophore was prepared as a 2 mM solution in 10% DMSO and stored frozen. The final concentration of DMSO did not interfere with the histamine release reaction. RPMI medium 1640 and Hank's balanced salt solution without added calcium and magnesium were obtained from the Insel Apotheke, Berne.

Preparation of cells for labeling. Blood from healthy volunteers was collected in 6 mM EDTA and immediately centrifuged, 1500 rpm at room temperature for 10 min. For each 10 ml of blood, 2.2 ml of the plasma supernatant was removed and replaced with 2.2 ml Hank's balanced salt solution. The pooled plasma, after centrifugation at 1500 × g for 20 min to remove the platelets, was used as a source of autologous plasma for ³H-histamine incorporation.

The remaining blood was reconstituted with Hank's, mixed with dextran (1.2% final concentration) and allowed to stand at room temperature for 60 min until the bulk of the erythrocytes had settled. Two ml of the supernatant containing leukocytes was applied to a double Ficoll layer, densities 1.119 and 1.077 (3) which separated cells into two fractions: a basophil-rich mononuclear layer, (containing about 85–90% lymphocytes, 5–8% mononuclear cells and 1–3% basophils, with less than 2% contaminating neutrophils) and a basophil-poor granulocyte fraction (over 95% polymorpho-

nuclear leukocytes which also contained eosinophils, but usually had less than 0.2% basophils). The cells from the respective layers were washed 3 times with Hank's and then resuspended in 2.2 ml of this buffer.

In other experiments, the blood was directly diluted with Hank's buffer and leukocytes were separated on a Ficoll gradient according to the method of Böyum (1), yielding a basophil-rich mononuclear fraction, as above.

The basophil-poor mononuclear fraction used in one experiment was obtained following passage of the mononuclear fraction over a glass bead column (15). This preparation contained less than 0.2% basophils.

Incorporation and release of ³H-histamine. Two ml of cell suspension were centrifuged, the supernatant solution discarded, and the pellet resuspended in 2 ml autologous EDTA plasma, or the medium to be tested. Twenty μ Ci of ³H-histamine was incubated with the cells, followed by three washes with calcium and magnesium-free Hank's solution. The cells were resuspended in HCM for histamine release and 20 μ l of cells added to tubes containing 1.5 ml of releasing agents.

After 30 min incubation at 37°C, tubes were centrifuged, the supernatant solutions discarded or saved for fluorometric analysis of histamine, and the pellets lysed with 100 μ l of distilled water. The labeled suspension was transferred quantitatively to a glass vial containing 2.5 ml scintillation fluid (Toluol, containing 10 mg POPOP and 6 g PPO liter, and Biosolve in an 8:1 proportion). In each experiment, two sets of control tubes were incubated with buffer. The first set, designated C, was lysed immediately to determine the total ³H-histamine content and the second set, designated B, was incubated with the rest of the reaction tubes to determine the spontaneous histamine release.

The percent histamine release was calculated from the mean of triplicate samples using the formula: % histamine release = 100 [(C-B)-(E-B))/(C-B)] where E = mean ct/min of the experimental tubes and C and B = mean ct/min of control tubes described above. The

standard deviation of triplicate samples was less than 10%.

Cell counts. Leukocytes were counted in a Fuchs Rosenthal chamber after staining the basophils with alcian blue (6). The actual number of basophils counted in each experiment ranged from 56 to 400, with an average of 137 basophils. The mean coefficient of variation for counting duplicate samples of basophils was 7.3% for this method.

Viability of mononuclear cell fractions before and after ^3H -histamine incorporation was assessed by counting the number of cells, out of 400, which excluded trypan blue.

Histamine release from human leukocytes. In some experiments release of endogenous histamine was also determined by conventional techniques (10). The supernatant solutions from these samples and/or the labeled cells were analyzed for histamine content on a Technicon Auto-analyzer II® (17).

Preparation of zymosan-activated serum. Fresh human serum incubated with zymosan (10 mg/ml) according to the method of Hook et al. (7) served as a crude source of C5a. Control serum, incubated at 56°C before addition of zymosan, did not release histamine from human leukocyte preparations.

RESULTS

Our attempts to incorporate ^3H -histamine into whole blood using the method of Stahl Skov et al. (19) were disappointing. In the best of three experiments we recovered only 660 counts per experimental tube (containing 7.6×10^4 cells, and 0.4% basophils), after overnight incubation with 10 μCi ^3H -histamine in RPMI medium 1640, followed by a Ficoll gradient according to the published protocol. We thus examined other conditions for incorporation.

In our own preliminary experiments using a 4-h incubation, we determined that mononuclear cell preparations containing undiluted autologous plasma incorporated approximately 3–5-fold more counts than did preparations suspended in RPMI or Hank's buffer alone.

Moreover, since rat mast cells incorporate ^3H -serotonin within 30–60 min (12, 13, 20), we examined the time course of ^3H -histamine incorporation into human leukocytes (Fig. 1). In both the mononuclear fraction and the basophil-poor granulocytes, the number of counts recovered increased as a function of the incubation time up to 6 h. By 12 h, the recovered counts decreased markedly. In three other experiments, the amount of label incorporated into the mononuclear fraction was maximal at 4–6 h; 4 h was selected as a convenient incubation time in subsequent experiments.

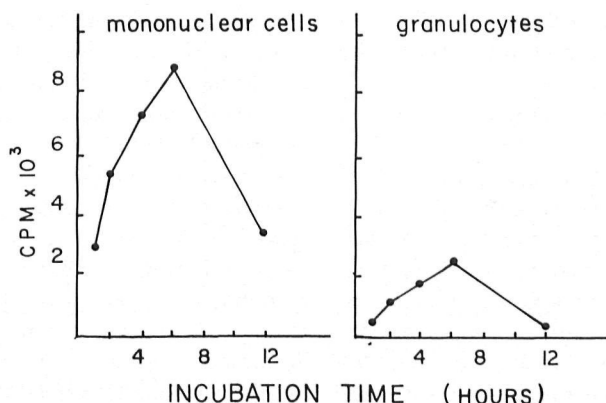


Fig. 1. Time course of ^3H -histamine incorporation into mononuclear cells, left, and basophil-poor granulocytes, right. Cells were incubated at 37°C with 20 μCi ^3H -histamine and aliquots removed as indicated. Mononuclear cell concentration was $1.5 \times 10^7/\text{ml}$, 1.5% basophils; granulocytes were 1.2×10^6 cells/ml, < 0.2% basophils. Original reaction volume was 2.2 ml.

Table 1
Incorporation of ^3H -histamine into leukocyte fractions

Donor	Mononuclear fraction			Granulocyte fraction		
	cells tube	counts min	counts basophil	cells tube	counts min	counts basophil
	$\times 10^4$			$\times 10^4$		
1	35.8	121128	2.3	3.2	3528	57.9
2	84.3	16453	3.5	51.0	19736	322.5
3	57.0	18676	4.0	7.2	2200	34.0
4	20.8	11452	7.3	4.6	1517	23.6
5	29.6	6189	4.0	6.0	1686	28.1

Mononuclear fractions and basophil-poor granulocyte fractions were incubated with 20 μCi ^3H -histamine according to the procedure described in the text. The percent basophils in the mononuclear fraction was 1.5, 0.56, 0.83, 0.75 and 0.53 % for donors 1–5 respectively; in the granulocyte fraction: 0.19, 0.012, 0.09, 0.13 and 0.1 % respectively.

The uptake of ^3H -histamine in the basophil-poor granulocyte fraction (Fig. 1) was unexpected. We performed additional studies to determine whether these counts were due to contaminating basophils or to the binding or uptake of label by the neutrophils. To compare the different preparations, data are expressed as ct/min per basophil. Table 1 summarizes the counts recovered in experiments from five donors. In the mononuclear cell fraction, containing $0.8\text{--}2.5 \times 10^5$ basophils/ml, the number of counts per basophil was relatively constant, ranging from 2.2–7.3, but was as much as 150 times higher in the basophil-poor granulocyte fraction which contained less than 3.4×10^3 basophils/ml.

Since the basophils represented such a small percentage of the total cells, we verified the microscopic counting method by measuring the histamine content of four samples.

Mononuclear cells from donors 1 and 2 in Table 1 contained 302 and 168 ng histamine/ml corresponding to 1.12 and 0.72 pg histamine/basophil respectively, within the range reported in the literature (8). Endogenous histamine in the basophil-poor granulocyte fractions from these two donors was too low to detect, less than 0.5 ng/ml, or less than 500–1000 basophils/ml. We concluded that the counts in the granulocyte fractions must come from the neutrophils and/or eosinophils, since there were so few basophils in these preparations.

To explore the possibility that lymphocytes contributed counts in the mononuclear fraction, we prepared a "basophil-poor" lymphocyte fraction. The 600 ct/min recovered in this preparation, which contained 1.5×10^5 lymphocytes and 0.25×10^3 basophils per experimental tube, could be explained by the contaminating basophils (2.4 counts/basophil) if the data are compared with that in Table 1.

The radioactivity recovered in the mononuclear fraction in these and other experiments represented only about 2% of the total label added (average 1.3% label recovered, range 0.2–3.5%, $n = 41$). We were unable to increase the amount of label taken up by the mononuclear fraction using the various media and autologous plasma for incubation. Nevertheless, we still examined the relationship between the release of ^3H -histamine and the release of endogenous histamine following challenge with anti-IgE, zymosan-activated serum and the calcium ionophore, A23187.

As indicated in Fig. 2, there was a marked discrepancy between anti-IgE induced ^3H -histamine release and the release of histamine measured fluorometrically. In spite of the graded dose response obtained by fluorometric analysis, there was no detectable release of ^3H -histamine. Three additional experiments yielded qualitatively similar results.

Cells from 15 additional donors, known from previous experiments to be "good releasers", were challenged with anti-IgE after incubation

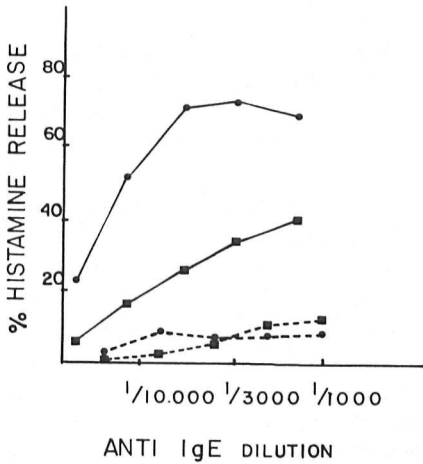


Fig. 2. Comparison of anti-IgE-induced histamine release measured by the fluorometric method (solid lines) and ^3H -histamine release method (broken lines) as described in the text. Two donors are represented: ●, ■.

with ^3H -histamine. There was a dose-dependent release of ^3H -histamine in only two of the experiments, with maximum release of 58 and 44%. In all the other experiments in which positive results were also expected, ^3H -histamine release was marginal. Furthermore, there was usually a lack of dose-dependent response obtained with the labeled cells. This is illustrated

in Fig. 3a and b. Cells from four donors were challenged in the same experiment with zymosan-activated serum, anti-IgE and the calcium ionophore. Only the calcium ionophore consistently released ^3H -histamine in a dose-dependent manner (Fig. 3c). The consistency of the response to ionophore is further illustrated in experiments with cells from 10 additional donors (Fig. 4).

Although the ratio of ^3H -histamine to the total cells in the mononuclear fraction was constant following three washes with Hank's buffer, the spontaneous release of ^3H -histamine, subtracted from experimental values in these experiments, was quite high (average 18%, range 4–28%). Endogenous histamine released spontaneously under the same experimental conditions was less than 6%. Cell viability as assessed by trypan blue exclusion was 92–95% after the 4-h labeling and 95–97% before this incubation.

DISCUSSION

The experiments reported here represent an attempt to develop a simple reliable method to

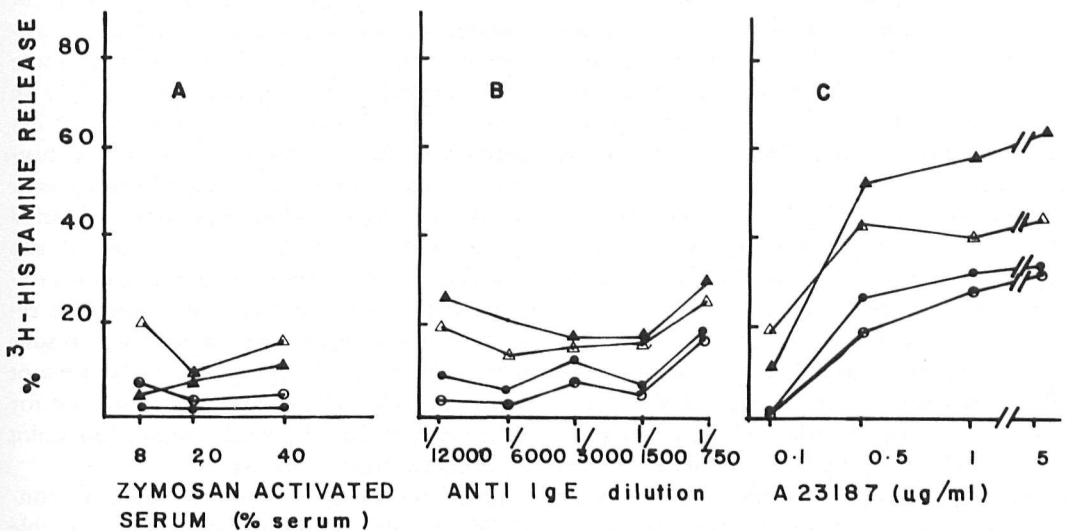


Fig. 3. Release of ^3H -histamine from mononuclear cells by zymosan-activated serum, anti-IgE and the calcium ionophore A23187. The data from the same donor are represented by the same symbol for each stimulus.

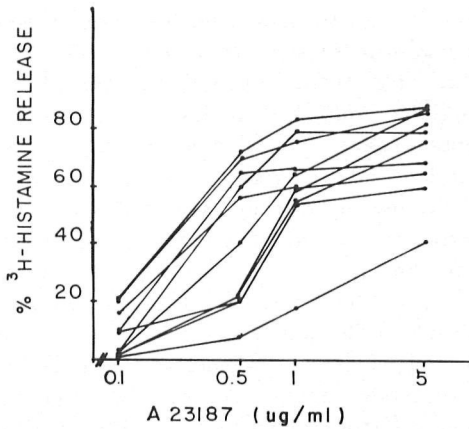


Fig. 4. Release of ^3H -histamine from mononuclear cells by the calcium ionophore A23187. Results from 10 different donors are shown.

assess basophil reactivity *in vitro*. Although the labeling of human basophils has been reported (2, 19), we were unable to obtain consistent results of either ^3H -histamine uptake or release, using the more recently published procedure (19) or our own modifications.

The main difficulties were not only the low number of counts recovered, but the lack of cellular specificity of the ^3H -histamine incorporation. Unlike the rat mast cell which actively takes up amines (4, 16), the human basophil did not incorporate label very efficiently. We found that a 4-h incubation was required for maximum incorporation (only about 2% of the total added label), whereas the rat mast cell incorporated 20% of added labeled serotonin within an hour (20). It is difficult to make further comparisons of labeled amine uptake in these and other cell systems, since quantitative data are not available (2, 12, 13, 19, 20).

The human basophil may resemble the guinea pig basophil when amine uptake is considered. Galli and coworkers (5) and Stewart et al. (21, 22) have shown that short-term cultures of guinea pig basophils do not take up labeled histamine although these cells are able to synthesize this amine from histidine. Stewart et al., who noted that ^{14}C -histamine was detected in

small amounts in all cells in the cultures, concluded that the label passively diffused into the cells. Passive diffusion would explain the low numbers of counts we obtained in our mononuclear and basophil-poor granulocyte fractions (Fig. 1). Whether amine uptake is a fundamental difference between basophils and mast cells, or a species difference, has not been established.

The close correlation between release of labeled and endogenous mediators in rat and platelet models suggest that the label is mixed homogeneously with the mediators already present (11, 12, 13, 20). Our inability to release ^3H -histamine with anti-IgE or zymosan-activated serum challenge, as well as the high spontaneous release of label in control samples, indicated that the ^3H -histamine was not granule associated in the human basophil. Our experiments do not distinguish whether the label was leaking from the cytoplasm or whether it was membrane bound. Release was not related to the dose of challenging agent nor to the release of endogenous histamine measured fluorometrically in the same cell preparations (Figs. 2 and 3).

In this context, the release of ^3H -histamine by the calcium ionophore (Fig. 4) is difficult to interpret. The mechanism of ionophore-induced histamine release is different from that of antigen- or anti-IgE-induced release (9). Although there are several reports that some ionophore effects may be cytotoxic (14, 18), we consider this explanation unlikely. In our experiments, the maximum release of ^3H -histamine usually occurred at 1 $\mu\text{g}/\text{ml}$ and concentrations of 5 $\mu\text{g}/\text{ml}$ did not release more labeled histamine. It is not clear whether the calcium ionophore can release histamine from non-granule sites, such as membrane histamine receptors, which might account for these results seen here. Since the purpose of the present work was the development of a practical test for studying basophil histamine release, this point was not investigated further.

Based on the data presented here, we conclude that the ^3H -histamine is not a reliable method to assess human basophil reactivity *in vitro*.

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