

Original Research Papers

Histamine dilutions modulate basophil activation

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Abstract. *Background:* In order to demonstrate that high dilutions of histamine are able to inhibit basophil activation in a reproducible fashion, several techniques were used in different research laboratories.

Objective: The aim of the study was to investigate the action of histamine dilutions on basophil activation.

Methods: Basophil activation was assessed by alcian blue staining, measurement of histamine release and CD63 expression. Study 1 used a blinded multi-centre approach in 4 centres. Study 2, related to the confirmation of the multi-centre study by flow cytometry, was performed independently in 3 laboratories. Study 3 examined the histamine release (one laboratory) and the activity of H₂ receptor antagonists and structural analogues (two laboratories).

Results: High dilutions of histamine (10⁻³⁰–10⁻³⁸ M) influence the activation of human basophils measured by alcian blue staining. The degree of inhibition depends on the initial level of anti-IgE induced stimulation, with the greatest inhibitory effects seen at lower levels of stimulation. This multicentre study was confirmed in the three laboratories by using flow cytometry and in one laboratory by histamine release. Inhibition of CD63 expression by histamine high dilutions was reversed by cimetidine (effect observed in two laboratories) and not by ranitidine (one laboratory). Histidine tested in parallel with histamine showed no activity on this model.

Conclusion: In 3 different types of experiment, it has been shown that high dilutions of histamine may indeed exert an effect on basophil activity. This activity observed by staining basophils with alcian blue was confirmed by flow cytometry. Inhibition by histamine was reversed by anti-H₂ and was not observed with histidine these results being in favour of the

specificity of this effect. We are however unable to explain our findings and are reporting them to encourage others to investigate this phenomenon.

Key words: Basophils – histamine – high dilutions – cimetidine – ranitidine – histidine

Introduction

Human basophils play a key role in allergic diseases. Activation of basophils via cross-linking of membrane bound IgE induces fusion of the cytoplasmic granules with the plasma membrane and the subsequent release of potent mediators including histamine. Histamine itself can inhibit the further degranulation of the basophil by acting on H₂ receptors [1, 2]. A series of investigations, mainly by one group, has demonstrated that high dilutions of histamine are also capable of inhibiting basophil degranulation [3, 4].

Although a biological action of ultra high dilutions has been shown, this is extremely controversial [5–8]. Indeed the paper published by Benveniste and colleagues in 1988 sparked a series of letters and investigations of various degrees of seriousness [7–15]. This group suggested that high dilutions of anti-IgE were able to stimulate human basophils. Ovelgönne et al. and Hirst et al. attempted to repeat these experiments but found no evidence for any periodic or polynomial change of degranulation as a function of anti-IgE dilution [7, 8]. The results did, however, contain a source of variation that could not be explained [8].

Our experiments began in the early 80's and were initially based on examination of the activity of histamine dilutions

on basophil activation measured by alcian blue staining. The first series of studies used specific allergen and leukocytes taken from allergic subjects and thereafter we employed anti-IgE. These data obtained between 1981–1994 [16–20] demonstrated inhibition of basophil activation by histamine dilutions which were always in the same dilution range (between 10^{-6} and 10^{-8} and 10^{-30} to 10^{-34} theoretical molar concentrations). The inhibition by histamine was reversed by an H_2 receptor antagonist (cimetidine) and a structural analogue such as histidine showed no effect [21].

Given the controversial nature of these results, a multi-centre blind investigation of the inhibition of basophil degranulation by high dilutions of histamine was performed. In order to further investigate the effect, using a flow cytometric assay of basophil activation, further studies were performed in three of the participating laboratories. Part of this work has been published in the form of refereed extended abstracts [21–23].

Materials and methods

Subjects

All participants were healthy control subjects of either sex, without any major diseases.

Study 1: Multi-centre trial based on the measurement of basophil activation by alcian blue staining

This trial was planned and managed by the coordinator (MR), who did not participate in the experiments. Four independent European laboratories participated in the trial (Laboratory 1: France; Laboratory 2: The Netherlands; Laboratory 3: UK; Laboratory 4: Italy). In order to ensure that all participants were counting the cells in an identical manner, they underwent a training period in the French laboratory. Furthermore, samples were sent to the laboratories and the counts were verified by the French laboratory before the start of the trial.

Histamine dilutions and controls were prepared independently in 3 separate laboratories and then coded randomly by the coordinator. Histamine hydrochloride (50 mg, Sigma) was dissolved in distilled water (5 ml) and vortexed for 15 s (full speed). To obtain the dilutions for the trial, this solution was serially diluted (1/100 v/v) up to 19 times. All steps were performed in disposable 20 ml polystyrene tubes and using disposable polystyrene tips. The dilutions 15, 16, 17, 18 and 19 were coded by the coordinator; these are equivalent to a final theoretical molar concentration of 10^{-30} – 10^{-38} M. In parallel, dilutions of distilled water alone were prepared in an identical manner and coded (controls). On receipt of the coded dilutions and controls, each participating laboratory stored them at 4°C. Prior to use, the dilutions and controls were made isotonic by dilution (1/10 v/v) in Hepes buffer (NaCl 127 mM, KCl 5 mM, Hepes 20 mM, pH 7.4).

All reagents, including disposable plastics, were from identical sources. Following completion of the experiments the data were returned to the coordinator and analysed by an independent statistician (JC), who was not involved in any other part of the study.

The basophil degranulation test has previously been published in detail [24]. Cell suspensions (250 μ L) were incubated for 30 min at room temperature in the presence or absence of histamine dilutions. After brief mixing (vortex medium speed, 3 s), aliquots (20 μ L) were added to a microtitre plate and mixed with 20 μ L anti-human IgE (anti-IgE, polyclonal anti-IgE affinity purified ATAB, USA, 1, 0.2, 0.04 μ g/mL). The plates were covered with sealer tape and incubated for 30 min at 37°C. Thereafter, alcian blue solution (100 μ L) was added to each well [24]. Stained basophils (not activated) were counted using a haemocytometer (Fuchs Rosenthal). Approximately 80 cells were

counted in each well. Positive (anti-IgE alone) and negative (buffer alone) controls were always included.

Study 2: Multi-centre trial based on the measurement of basophil activation by flow cytometry

Given the results of the above study, a further multi-centre study was devised. This was based on a new flow cytometric method designed for allergy diagnosis, which has high specificity is not subjective [see references cited in 25]. The study investigated the inhibition of basophil activation induced by anti-IgE and measured by an anti-IgE/CD63 flow cytometric method.

Three laboratories took part in this study (Laboratories 1, 3 and 4). Histamine dilutions were prepared in the individual laboratories as described above with a range of concentrations tested (theoretical final concentrations 10^{-2} – 10^{-40} M). The methods used have also been described in detail [26]. Leukocyte suspensions were obtained by sedimentation (1–1.5 h) of EDTA blood. The cell suspensions were washed twice with EDTA buffer (127 mM NaCl, 5 mM KCl, 20 mM Hepes, 5 mM EDTA, 5 IU/mL heparin; pH 7.4) and then incubated with equal volumes of histamine solutions for 30 min at room temperature. In the experiments performed to evaluate the effect of H_2 receptor antagonists, the drugs were co-incubated with histamine before adding anti-IgE, at the stated concentrations. Aliquots (20 μ L) were then mixed with anti-IgE (0.2 μ g/mL, Dako, UK; ATAB, USA or Sigma, Milan, Italy) for 30 min at 37°C. Cells were washed and labelled with 10 μ L anti-IgE FITC (0.5 μ g / 10^6 cells, Caltag, USA or Sigma, Milan, Italy) and 10 μ L anti-CD63 PE (1 μ g / 10^6 cells, Caltag, USA or Coulter, Milan, Italy) for 20 min at 4°C. Buffer (Isoflow, Beckman Coulter; FACSflow BD Biosciences; 500 μ L) was added. Basophils were selected by their brightly fluorescent anti-IgE FITC (high mean channel fluorescence MCF). The percentage of activated cells was calculated by positioning an electronic gate between CD63⁻ and CD63⁺ cells. Each experiment was performed at least 4 times in duplicate. Negative controls consisted of isotype-matched, directly conjugated non-specific antibodies.

Study 3: Measurement of basophil activation based on the evaluation of histamine release and modulation of histamine effect by H_2 -receptor antagonists

A/Effect of high dilutions of histamine on anti-IgE induced histamine release

These experiments were performed in Laboratory 4. Samples of 30 mL of venous blood were taken from healthy donors whose basophils responded to anti-IgE and who had not used any medication in the previous 4 weeks. The blood was collected by venepuncture from a superficial antecubital vein and anticoagulated with adding EDTA (0.5 M to 10 mL of blood). About 15 mL of plasma were obtained by natural sedimentation (2 h at room temperature) and collected in conical plastic tubes. A pellet rich in leukocytes was obtained by centrifugation at 900 rpm using a 4227 R refrigerated centrifuge (Beckman Inst, Inc, Palo Alto, Ca, USA) at 20°C for 10 min in a buffer of the following composition: HEPES 20 mM, NaCl 127 mM, KCl 50 mM, sodium heparin 5 IU mL⁻¹, bovine serum albumin 1.5 mg mL⁻¹, pH 7.4 (Buffer A). Erythrocytes were lysed by a solution of the following composition: NH₄Cl 152 mM, KHCO₃ 10 mM, EDTA 100 mM (Buffer B) Leukocyte-rich suspensions were washed with buffer A and then incubated with equal volumes of the drugs under study (histamine final concentration 10^{-10} – 10^{-40} M) for 30 min at 37°C in a shaking water bath [3]. Aliquots (20 μ L) were then incubated with anti IgE (Sigma, Milan Italy) for 30 min, and then processed for the evaluation of the release of histamine. Basophil activation was assessed by measurement of histamine release [1].

Histamine was measured fluorimetrically using the method of Shore et al. [27] as modified by Kremzer and Wilson [28]. In the supernatants, *o*-phthalaldehyde was added directly to the samples after alkalisation. The same procedure was used for the cells after extraction with