Inflamm. res. 53 (2004) 181–188
1023-3830/04/050181-08
DOI 10.1007/s00011-003-1242-0

Inflammation Research

Original Research Papers

Histamine dilutions modulate basophil activation

P. Belon [1], J. Cumps [2], M. Ennis [3], P.F. Mannaioni [4], M. Roberfroid [5], J. Sainte-Laudy [6], F.A.C. Wiegant [7]

[1] Boiron, 20 rue de la Libération, 69110 Sainte-Foy-Les-Lyon, France
[2] UCL 7369, 73 avenue Emmanuel Mounier, 1220 Brussels, Belgium
[3] Department of Clinical Biochemistry, Institute of Clinical Science, The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BJ, UK, Fax: ++ 44 28 90 236143, e-mail: m.ennis@qub.ac.uk
[4] Department of Pharmacology, Pieraccini 6, 50139 Florence, Italy
[5] Laboratoire de biotoxicologie, UCL 7369, 73 avenue Emmanuel Mounier, 1220 Brussels, Belgium
[6] Laboratoire d'Immunologie et d'Allergologie, 5 boulevard Montparnasse, 75006 Paris, France
[7] University of Utrecht, Department of Molecular Cell Biology, PO Box 80.056, 3508 TB Utrecht, The Netherlands

Received 11 December 2002; returned for revision 29 January 2003; accepted by A. Falus 12 November 2003

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 multicentre study by flow cytometry, was performed independently in 3 laboratories. Study 3 examined the histamine release (one laboratory) and the activity of H_2 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

Correspondence to: M. Ennis

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 H_2 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 planed 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 nM, 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 µL) 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 µL) were added to a microtitre plate and mixed with 20 µL anti-human IgE (anti-IgE, polyclonal anti-IgE affinity purified ATAB, USA, 1, 0.2, 0.04 µg/mL). The plates were covered with sealer tape and incubated for 30 min at 37 °C. Thereafter, alcian blue solution (100 µL) 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 µL) were then mixed with anti-IgE (0.2 µg/mL, Dako, UK; ATAB, USA or Sigma, Milan, Italy) for 30 min at 37 °C. Cells were washed and labelled with 10 µL anti-IgE FITC (0.5 µg / 10^6 cells, Caltag, USA or Sigma, Milan, Italy) and 10 µL anti-CD63 PE (1 µg /10^6 cells, Caltag, USA or Coulter, Milan, Italy) for 20 min at 4 °C. Buffer (Isoflow, Beckman Coulter; FACSflow BD Biosciences; 500 µL) 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 ml, KCl 50 ml, 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 µL) 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*-phtaldialdehyde was added directly to the samples after alkalisation. The same procedure was used for the cells after extraction with

0.1 M HCl, using the method of Bergendorff and Uvnas [29]. Histamine release was expressed as percentage of the total present in the cells plus supernatants. Spontaneous histamine release ranged between 5–10% and was subtracted from all the values.

B/Modulation of histamine effect by H_2 receptor antagonists

These experiments were performed in Laboratories 1 and 4. In addition to the protocol described for study 2, drugs (ranitidine 10^{-4} M and cimetidine 10^{-5} M) were co-incubated with histamine before adding anti-IgE, at the stated concentrations.

Statistical analysis

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

This study was designed to investigate the inhibitory effect of histamine dilutions on basophil degranulation triggered by anti-IgE. To examine such an effect, the anti-IgE must have caused a degranulation. Thus, all data were validated by calculating the coefficient of variation (cv) of the absolute basophil count in the absence of anti-IgE for each laboratory. The minimum percentage degranulation was set at 3.4 cv for the 0.1% level of risk. Only data exceeding these values were included in the analyses, those less than these values are described as censored. Statistical analysis was performed at 0.1% level of risk and two sets of tests were performed based on both parametric (GLM procedure, multivariate analysis) and distribution free non-parametric (Kruskal-Wallis) procedures.

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

Statistical analysis was performed using SPSS statistic software (Release 8.0.0 for Windows, standard version; SPSS Inc., 1989–1997). Comparison of two groups of data was performed using paired-value Wilcoxon test. Differences between three or more groups of data were analysed using Kruskal-Wallis test, subsequent analysis used the Dunn's Multiple Comparison Test. P values less than 0.05 were considered statistically significant. Data are reported as mean ± SEM for the number of experiments shown in brackets.

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

Data are reported as mean ± SEM for the number of experiments shown in brackets. Data were analysed either using the Kruskal-Wallis test, with subsequent analysis with Dunn's Multiple Comparison test; one way analysis of variance with subsequent analysis with Dunnett's Multiple Comparison Test or the Wilcoxon test.

Results

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

The relative standard deviation was similar for 3 of the laboratories (9.6%, 9.4% and 9.5%) and slightly higher for the fourth laboratory 11.4%. From the 3906 collected data points, 476 were eliminated as missing data (where either treatment or control counts were missing, the partner data were eliminated) and 724 as censored data (where no activation was observed after anti-IgE stimulation). No outliers were detected by Grubb's test applied to the control data. Thus 2706 data points were regarded as validated.

Combining all data in the presence or absence of histamine dilutions, the overall effect was highly significant ($p \leq 0.0001$). In laboratories 1, 3 and 4 the presence of histamine in the initial solutions significantly inhibited anti-IgE induced basophil degranulation at the lowest anti-IgE concentrations tested (Table 1). In laboratory 4, significant differences were seen at all tested anti-IgE concentrations. The results for the individual histamine dilutions and laboratories are shown in Table 2. At the highest concentration of anti-IgE tested, histamine dilutions caused a slight increase in degranulation in laboratory 1, had no effect in laboratory 2, caused an inhibition at 1 concentration (10^{-36} M) in laboratory 3 and were inhibitory at all concentrations in laboratory 4. The percentage degranulation was highest in laboratory 1 and lowest in laboratory 4. At the second concentration of anti-IgE tested (0.02 µg/mL), inhibition was only seen in laboratory 4. The control activation was lowest in laboratory 4. At the lowest anti-IgE concentration tested (0.04 µg/mL), some inhibition was seen in all laboratories but was most marked in laboratories 1 and 4.

Table 1. Comparison of percentage basophil degranulation in the presence and absence of histamine dilutions.

	Anti IgE (1 µg/mL)		Anti-IgE (0.2 µg/mL)		Anti IgE (0.04 µg/mL)	
	Control	+ histamine	Control	+ histamine	Control	+ histamine
Lab 1	77.4 ± 1.10 (n = 81)	81.6 ± 1.06** (n = 81)	67.6 ± 0.89 (n = 141)	66.1 ± 1.00 (n = 141)	46.2 ± 0.96 (n = 54)	34.1 ± 2.5** (n = 54)
Lab 2	53.4 ± 0.62 (n = 168)	54.9 ± 0.59 (n = 168)	54.5 ± 0.74 (n = 162)	53.9 ± 0.82 (n = 162)	50.3 ± 0.82 (n = 156)	47.8 ± 2.42 (n = 156)
Lab 3	54.3 ± 1.23 (n = 66)	53.2 ± 2.19 (n = 66)	57.3 ± 1.26 (n = 63)	57.0 ± 1.61 (n = 63)	51.3 ± 0.94 (n = 93)	47.3 ± 1.46* (n = 93)
Lab 4	50.9 ± 0.80 (n = 150)	40.0 ± 1.22** (n = 150)	53.2 ± 0.95 (n = 144)	41.8 ± 1.44** (n = 144)	46.4 ± 1.29 (n = 75)	37.4 ± 2.14** (n = 75)

Basophil degranulation was assessed by Alcian Blue counts. The data compare the combined results for all histamine and water dilutions. Data are expressed as mean ± SEM, for the number of data points n. Statistical analyses were performed using the T test. *$p < 0.005$, **$p < 0.0001$.

Table 2. Comparison of percentage degranulation in the presence and absence of histamine dilutions in the four different laboratories and examining the individual anti-IgE dilutions used.

Laboratory	Anti IgE	Histamine (M)	Control	N	+ histamine	N
1	1 µg/mL	10^{-30}	76.2 ± 2.0	15	80.8 ± 3.1	15
		10^{-32}	78.6 ± 2.2	18	85.7 ± 0.9	18
		10^{-34}	75.2 ± 3.7	15	83.2 ± 1.6	15
		10^{-36}	77.2 ± 2.5	15	81.7 ± 1.5	15
		10^{-38}	79.1 ± 2.0	18	76.7 ± 3.3	18
	0.2 µg/mL	10^{-30}	72.5 ± 1.3	33	71.2 ± 1.2	33
		10^{-32}	63.8 ± 1.7	18	63.1 ± 0.9	18
		10^{-34}	66.0 ± 2.1	36	67.5 ± 1.7	36
		10^{-36}	60.7 ± 3.6	15	59.4 ± 2.1	15
		10^{-38}	69.3 ± 1.4	39	64.5 ± 2.8	39
	0.04 µg/mL	10^{-30}	43.8 ± 2.4	9	49.4 ± 4.0	9
		10^{-32}	47.0 ± 2.3	12	44.4 ± 2.4	12
		10^{-34}	48.4 ± 4.5	6	10.7 ± 4.6	6
		10^{-36}	46.5 ± 1.5	12	29.8 ± 5.7	12
		10^{-38}	46.0 ± 1.5	15	29.5 ± 4.6	15
2	1 µg/mL	10^{-30}	54.3 ± 1.2	33	55.2 ± 8.2	33
		10^{-32}	51.8 ± 1.4	33	54.4 ± 1.2	33
		10^{-34}	55.9 ± 1.3	33	56.2 ± 1.0	33
		10^{-36}	52.7 ± 1.3	36	52.2 ± 1.3	36
		10^{-38}	52.1 ± 1.6	33	56.6 ± 1.6	33
	0.2 µg/mL	10^{-30}	54.1 ± 1.7	30	53.7 ± 1.7	30
		10^{-32}	52.9 ± 1.9	33	52.8 ± 2.0	33
		10^{-34}	55.2 ± 1.5	33	52.2 ± 2.2	33
		10^{-36}	54.4 ± 1.5	36	55.4 ± 1.5	36
		10^{-38}	56.0 ± 1.7	30	55.5 ± 1.6	30
	0.04 µg/mL	10^{-30}	49.0 ± 2.0	36	47.8 ± 3.0	36
		10^{-32}	51.4 ± 1.7	24	46.2 ± 2.8	24
		10^{-34}	48.9 ± 2.1	30	46.8 ± 2.8	30
		10^{-36}	52.7 ± 1.6	30	50.8 ± 2.0	30
		10^{-38}	50.0 ± 1.6	36	47.1 ± 3.0	36
3	1 µg/mL	10^{-30}	59.8 ± 3.4	11	55.4 ± 3.3	11
		10^{-32}	51.7 ± 2.5	12	57.6 ± 2.9	12
		10^{-34}	56.8 ± 2.5	12	63.9 ± 4.0	12
		10^{-36}	56.7 ± 2.8	14	37.8 ± 6.8	14
		10^{-38}	48.9 ± 2.0	17	53.9 ± 3.3	17
	0.2 µg/mL	10^{-30}	62.4 ± 2.7	12	57.7 ± 3.4	12
		10^{-32}	55.4 ± 2.5	15	58.4 ± 2.7	15
		10^{-34}	58.3 ± 3.0	12	57.5 ± 4.9	12
		10^{-36}	56.1 ± 2.1	12	57.7 ± 4.5	12
		10^{-38}	54.7 ± 3.5	12	55.4 ± 2.9	12
	0.04 µg/mL	10^{-30}	51.8 ± 1.7	27	48.4 ± 2.2	27
		10^{-32}	50.0 ± 3.0	12	46.6 ± 4.0	12
		10^{-34}	50.2 ± 1.4	27	44.3 ± 3.5	27
		10^{-36}	57.3 ± 3.6	6	52.2 ± 1.8	6
		10^{-38}	50.9 ± 2.3	21	48.7 ± 2.8	21
4	1 µg/mL	10^{-30}	47.2 ± 1.6	36	37.8 ± 2.7	36
		10^{-32}	52.6 ± 1.9	18	37.3 ± 5.0	18
		10^{-34}	52.1 ± 1.5	36	38.7 ± 2.1	36
		10^{-36}	52.9 ± 2.4	24	40.8 ± 3.1	24
		10^{-38}	51.3 ± 1.6	36	44.2 ± 1.9	36
	0.2 µg/mL	10^{-30}	51.3 ± 2.4	30	47.3 ± 3.5	30
		10^{-32}	53.1 ± 2.4	21	33.7 ± 3.7	21
		10^{-34}	56.4 ± 1.9	33	43.3 ± 2.0	33
		10^{-36}	51.8 ± 2.1	21	39.0 ± 3.5	21
		10^{-38}	52.9 ± 1.9	39	42.2 ± 3.1	39
	0.04 µg/mL	10^{-30}	48.4 ± 2.9	18	40.1 ± 4.9	18
		10^{-32}	51.8 ± 6.5	3	14.5 ± 3.5	3
		10^{-34}	47.3 ± 2.3	21	42.0 ± 3.1	21
		10^{-36}	43.6 ± 4.3	9	42.4 ± 5.7	9
		10^{-38}	44.6 ± 2.1	24	32.3 ± 4.0	24

Basophil degranulation was assessed by Alcian Blue counts. Data are expressed as mean ± SEM, for the number of data points n.

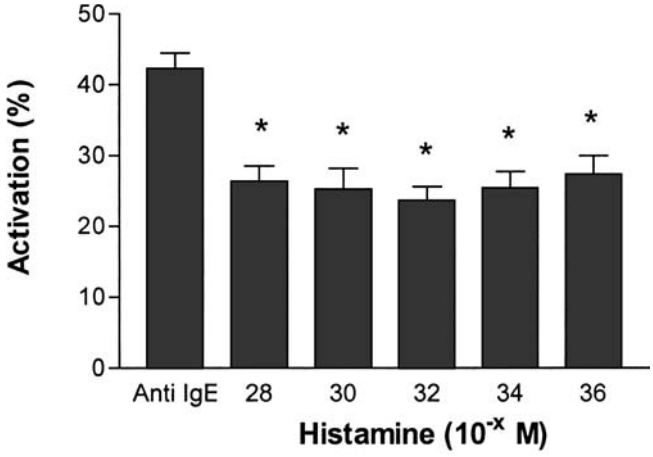

Fig. 1. Percentage basophil activation (assessed by flow cytometry) after incubation with anti-IgE in the absence (anti IgE) or the presence of histamine (theoretical molar concentrations are given). Data are shown from 29 experiments performed in duplicate. Anti-IgE refers to studies performed in the absence of histamine. Using the Kruskal-Wallis test, there were significant differences from the control release ($p < 0.0001$). Subsequent analysis with Dunn's Multiple Comparison Test revealed that all histamine containing samples were significantly different from the anti-IgE induced control (* = $p < 0.001$).

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

A/Laboratory 4

In this laboratory, the effect of 5 histamine concentrations (10^{-28}–10^{-36} M) was tested in 29 experiments, all performed in duplicate. Using the Kruskal-Wallis test, there were significant differences from the control activation in the absence of the histamine dilutions ($p < 0.0001$). Subsequent analysis with Dunn's Multiple Comparison Test revealed that all histamine containing samples were significantly different from the anti-IgE induced control ($p < 0.001$) (Fig. 1). The mean maximum percent inhibition observed was 42.8%.

B/Laboratory 3

A wide range of histamine concentrations was tested (10^{-2}–10^{-40} M). All experiments were performed in duplicate 15–42 times. Using a one way analysis of variance, there were significant differences from the control activation ($p < 0.05$). Subsequent analysis with Dunnett's Multiple Comparison Test revealed significant differences at only 3 concentrations (10^{-2}, 10^{-4} and 10^{-20} M) (Fig. 2). A dose response curve could not be established due to the protocol design (histamine dilutions were tested in groups). Furthermore, the degree of basophil releasibility varied between subjects.

C/ Laboratory 1

In a series of 36 experiments performed in triplicate, 4 of the 5 tested histamine dilutions were significantly different from anti-IgE plus water using the Wilcoxon test (Fig. 5).

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

A/Laboratory 4

Histamine release was significantly inhibited after preincubation with histamine dilutions (Fig. 3). Using the Kruskal-Wallis test, there were significant differences within the dataset ($p = 0.001$). Subsequent analysis with Dunn's Multiple Comparison Test revealed significant differences with all histamine dilutions tested (10^{-10}–10^{-40} M) ($p < 0.05$).

B/Laboratories 1 and 4

Further studies indicated that preincubation with the histamine antagonists ranitidine (10^{-4} M) or cimetidine (10^{-5} M) reduced the histamine induced inhibition of basophil activation (Fig. 5a). Using the Kruskal-Wallis test, there were significant differences within the dataset ($p < 0.0001$).

In another series of ten experiments, pre-incubation with cimetidine significantly (Wilkoxon paired test) reversed the effect of histamine for the dilution 10^{-32} M (Fig. 5b). The his-

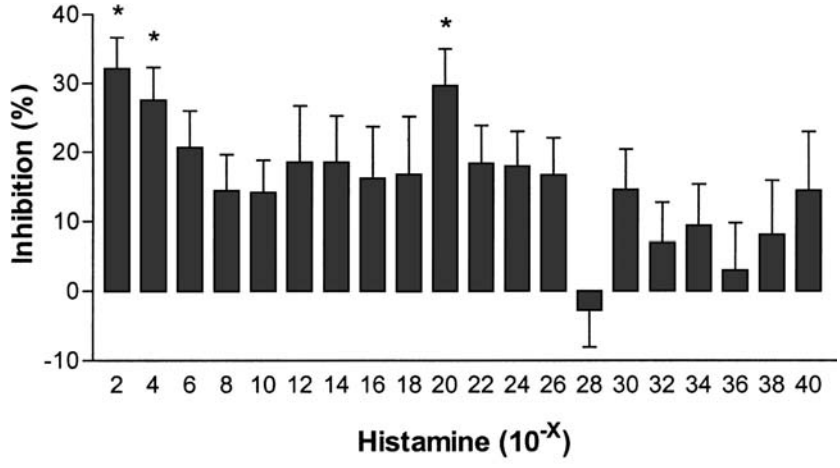

Fig. 2. Inhibition of basophil activation caused by histamine dilutions. Data are given as mean ± SEM for 15–42 experiments in duplicate. The unblocked (control) activation was 63.0 ± 2.8%. Activation of basophils was assessed using by examining the percentage of cells expressing CD 63 using flow cytometry. Using a one way analysis of variance, there were significant differences from the control activation ($p < 0.05$). Subsequent analysis with Dunnett's Multiple Comparison Test revealed significant differences (* $p < 0.05$).

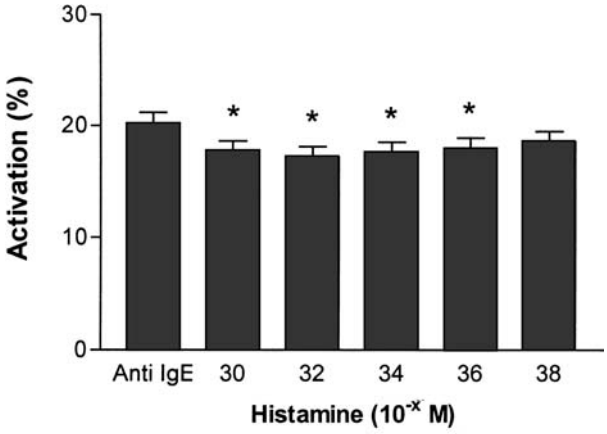

Fig. 3. Percentage basophil activation after incubation with anti-IgE in the absence (anti IgE) or the presence of histamine (theoretical molar concentrations are given). The data are shown as means for 36 experiments performed in triplicate. Activation of basophils was assessed using by examining the percentage of cells expressing CD 63 using flow cytometry. All tested dilutions except 10^{-38} M were significantly different from anti-IgE plus water using the Wilcoxon test ($p < 0.01$).

tamine induced inhibition was significant ($p = 0.0017$) in the absence of cimetidine and not significant in the presence of cimetidine compared to the anti-IgE control. In addition, the percentage activations observed in the presence and the absence of cimetidine for histamine dilution 10^{-32} M were also significantly different ($p = 0.0024$).

C/Laboratory 1

In further series of experiments the activity of histamine was compared to the activity of a structural analogue (histidine) tested in parallel on the same leukocyte suspensions. A significant inhibition was observed for histamine dilutions and no effect was observed with histidine dilutions. Comparing 9 experiments, the effects of histidine 16C versus histamine 16C (15.7 ± 13.7% verses 13.7 ± 12.0), we found a statistically significant difference ($p = 0.028$) and no difference

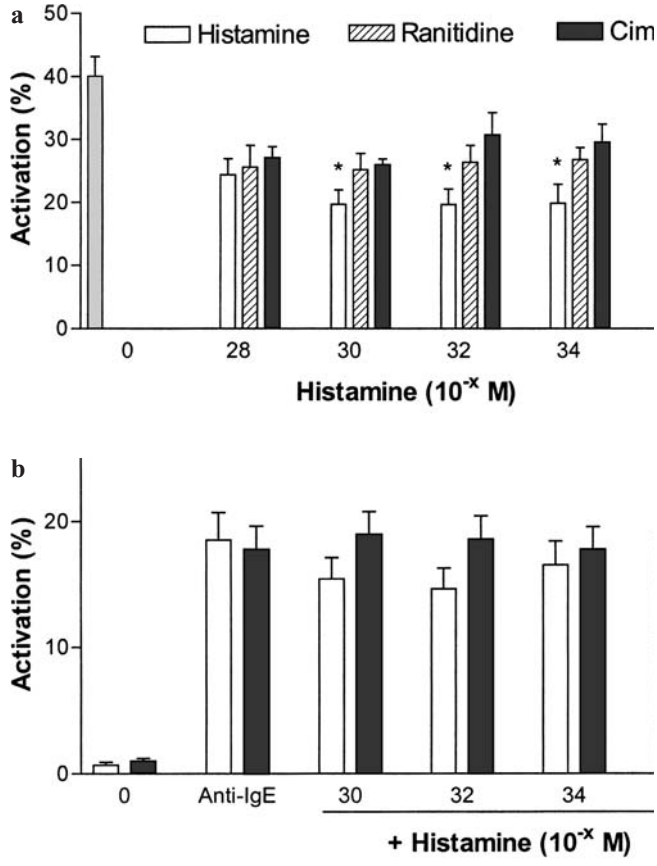

Fig. 5. Effect of preincubation with the histamine antagonists ranitidine (10^{-4} M) or cimetidine (10^{-5} M) on histamine induced inhibition of basophil activation induced by anti-IgE (0.2 µg/mL). **a** Data are shown as mean ± SEM for 6 experiments. Activation of basophils was assessed using by examining the percentage of cells expressing CD 63 using flow cytometry. * = $p < 0.01$ compared to anti-IgE alone using Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. **b** Data are shown as mean ± SEM of 10 experiments. Open columns are in the absence of cimetidine and filled columns in the presence of cimetidine.

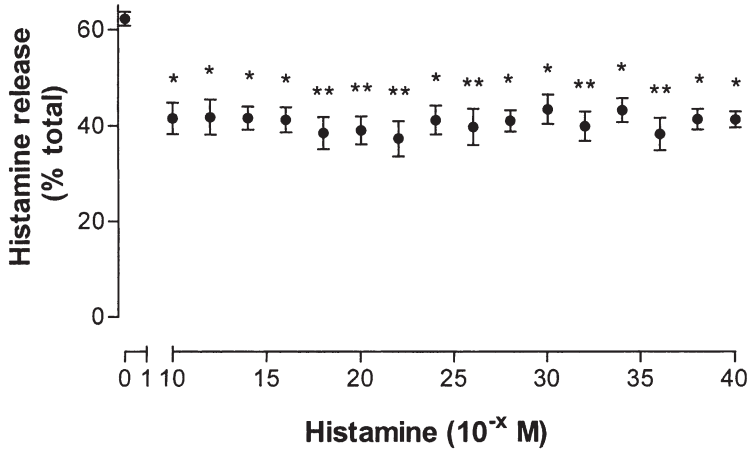

Fig. 4. Effect of preincubation with histamine dilutions on histamine release. Data are given as mean ± SEM for n = 14 experiments. The concentration of anti-IgE was 0.2 µg/mL. Using the Kruskal-Wallis test, there were significant differences from the control histamine release ($p = 0.001$). Subsequent analysis with Dunn's Multiple Comparison Test revealed significant differences with all histamine dilutions tested (10^{-10}–10^{-40} M) (*$p < 0.05$, **$p < 0.001$).

between histidine 16C and the water control (15.7 ± 13.7% verses 16.26 ± 13.1%).

Discussion

Previous studies from some of the authors of this paper have indicated that high dilutions of histamine are able to inhibit basophil activation, as measured either by examining alcian blue staining or by examining the expression of CD63 [3, 4, 23, 30]. In the first study, we attempted to avoid potential sources of problems/bias by arranging for all the solutions to be prepared and coded by an independent laboratory. The coded solutions were sent to the participating laboratories in a blinded fashion. Furthermore, all participants had received previous training in the basophil counting technique and the data were analyzed by an independent statistician. At the lowest concentration of anti-IgE tested, the histamine dilutions, when compared as a block to the water dilutions, produced a small but statistically significant inhibition of basophil degranulation in 3 of the 4 laboratories. This was not apparent at the higher anti-IgE concentrations tested. This observation is perfectly in keeping with the mechanism of release of endogenous substances. Whenever a release occurs, it is more difficult to down-regulate the release when it is at its maximum extent. When the different dilutions were considered separately, it was clear that the degree of inhibition depended on the amount of activation present. In one laboratory (laboratory 4), which had the lowest activation, the histamine dilutions almost always inhibited basophil degranulation. Nevertheless the intensity of the observed effect varies from one laboratory to the other. We believe that this is due to the varying sensitivity of basophils depending from the donors.

The protocol of this multi-centre study was designed to allow every laboratory to easily perform the experiments. In the first studies we performed, we used antigen activated basophils from allergic patients. This was not possible in this multi-centre control because most of the participating laboratories did not have access to a source of basophils from allergic patients, so we developed a technique using basophils from healthy donors stimulated with three different anti-IgE concentrations.

The controversy over the manual method used to assess basophil activation, together with its time consuming and repetitive nature, prompted the use of a machine based method to assess basophil activation. The flow cytometric analysis of basophil activation has gained acceptance in the field of allergy diagnosis [e. g. see papers cited in 25]. Three of the original participating laboratories separately studied the effects of high histamine dilutions i. e. all experiments were performed completely independently. All three laboratories observed inhibition of anti-IgE induced basophil activation in the presence of high dilutions of histamine. However, the results were not identical between the laboratories. Thus, in Laboratory 1, only 5 dilutions were tested (10^{-28}–10^{-32} M) and all produced a statistically significant inhibition of between 20–25% (median) with ca. 40% activation (range 20–77%). In Laboratory 3, a much wider range of dilutions was tested with significant inhibition observed at 10^{-2}, 10^{-4} and 10^{-20} M. However, in this series of experiments the average basophil activation response was over 60% (range 27–98%). In Laboratory 1, significant inhibition was observed at almost all concentrations tested. In a limited series of experiments in one laboratory, it was shown that histamine release was also inhibited by preincubation of basophils with a much larger range of histamine dilutions. However, the actual dilution tested did not seem to influence the amount of inhibition observed.

It is clear from Figure 1 that ultra-high dilutions of histamine inhibit basophil activation (CD63) without any dose-response relationship. It is equally clear from Figure 4 that high dilutions of histamine inhibit the immunological release of histamine without any dose-response relationship. From the experiments with cimetidine and ranitidine (shown in Fig. 5), H_2-receptor antagonists only partially revert the inhibitory action of ultra-high dilutions of histamine, without obeying to the PA_2/PD_2 rules. This was despite the antagonists being present in a vast excess (10^{-4}–10^{-5} M compared to 10^{-28}–10^{-36} M). The reversibility of histamine inhibition by cimetidine observed in Laboratory 4 was confirmed in Laboratory 1. This reinforced the specificity of the observed phenomenon confirmed by the absence of activity of histidine dilutions [23]. These findings confirm the previously published results exhibiting an inhibition by cimetidine of the basophils activation determined by staining method [21].

The experiments performed during the 80's were repeated using an automated method (flow cytometry). The results obtained by staining basophil by alcian blue were confirmed using the same method for the multi-centre trial in terms of inhibition of histamine on basophil activation. The effect of cimetidine and the absence of histidine activity were confirmed using a flow cytometric methods.

Although the mean activity of high histamine dilutions were relatively low (around 20% on average), the aim of the study was not to compare the activity of histamine to other inhibitors of basophil function but to demonstrate that high dilutions of histamine had a significant activity. We were unable to examine basophil cell lines in this model due to the lack of expression of CD63 and the low reactivity observed after passive sensitisation of transfected lines.

Other biological models have examined the effects of high dilutions:

- Doutremepuich and co-workers [6, 31, 32, 33] demonstrated that, in the rat, high dilutions of aspirin (15 C 10^{-30} g/l) have pro-aggregant and prothrombotic activity. They showed that the effect of an injection of 100 mg/kg aspirin is totally inhibited by the extemporaneous injection of aspirin 15 C.
- Jonas and co-workers [34] demonstrated that preincubation of neuronal cells with high dilutions of glutamate (10^{-22} and 10^{-30} g/L) protects against subsequent exposure to toxic levels of glutamate. High dilutions of cyclohexamde (10^{-27} M) also protected against the effects of glutamate exposure [35].
- Using a radioactive tracer method high dilutions of As_2O_3 (10^{-14} g/l) enhanced the elimination of orally administered arsenious anhydride [36].

We are not yet able to propose any theoretical explanation of these findings. Despite searching for artefacts, we have been unable to find any. Taking into account the ultralow concentration of molecules in such dilutions, we hypothesize that biological information must come from solvent which is pure

water in our experiments. Thus we have investigated not only biological models but also physical models.

Analyzing the intensity and emission spectrum of ice thermoluminescent glow, Rey demonstrated [37, 38] that this spectrum is directly linked to the network structure within the ice. Rey [39] also demonstrated that various frozen sodium chloride dilutions and various frozen high lithium chloride dilutions exhibit different thermoluminescent spectra: as a working hypothesis, the author suggests that the phenomenon results from a marked structural change in the hydrogen bound network. We are aware that these findings do not constitute a complete explanation of the observed phenomena but are merely steps to firstly validate the activity of high dilutions and secondly to second try to understand the underlying causes of the activity.

The findings presented in this paper suggest that high dilutions may indeed exert an effect on cellular activity. We are however unable to explain our findings and are reporting them to encourage others to investigate this phenomenon. On the basis of previous results, we plan to test with the flow cytometric technique the effect of other agents such as H_1 agonists and antagonists and autocoïds.

Acknowledgements. This work was supported by grants from the French Institute of Homeopathic Research.

References

[1] Lichtenstein LM, Gillespie E. The effects of the H1 and H2 antihistamines on 'allergic' histamine release and its inhibition by histamine. J Pharmacol Exp Ther 1975; 192: 441–50.
[2] Masini E, Blandina P, Brunelleschi S, Mannaioni PF. Evidence for H2-receptor-mediated inhibition of histamine release from isolated rat mast cells. Agents Actions 1982; 12: 85–8.
[3] Sainte-Laudy J, Belon P. Inhibition of human basophil activation by high dilutions of histamine. Agents Actions 1993; 38: C245–7.
[4] Sainte-Laudy J, Belon P. Analysis of immunosuppressive activity of serial dilutions of histamine on human basophil activation by flow cytometry. Inflamm Res 1996; 45 (Suppl 1): S33–4.
[5] Linde K, Clausius N, Ramirez G, Melchart D, Eitel F, Hedges VL et al. Are the clinical effects of homeopathy placebo effects? A meta-analysis of placebo-controlled trials. The Lancet 1997; 350: 834–43.
[6] Doutremepuich CH, Aguejouf O, Belon P. Effects of ultra-low dose aspirin on embolization in a model of laser-induced thrombus formation. Seminars in Thrombosis and Hemostasis 1996; 22 (Suppl 1): 67–70.
[7] Ovelgönne JH, Bol AWJM, Hop WCJ and Van Wijk R. 'Mechanical agitation of very dilute antiserum against IgE has no effect on basophil staining properties'. Experientia 1992; 48: 504–8.
[8] Hirst SJ, Hayes NA, Burridge J, Pearce FL, Foreman JC. Human basophil degranulation is not triggered by very dilute antiserum against human IgE. Nature 1993; 366: 525–7.
[9] Davenas E, Beauvais F, Amara J, Oberbaum M, Robinzon B, Miadonna A et al. Human basophil degranulation triggered by very dilute antiserum against IgE. Nature 1988; 333: 816–8.
[10] 'High-dilution' experiments a delusion. Nature 1988; 334: 287– 90.
[11] Only the smile is left. Nature 1988; 334(6181): 375–6.
[12] Coles P. Benveniste controversy rages on in the French press. Nature 1988; 334(6181): 372.
[13] When to publish pseudo-science. Nature 1988; 334(6181): 367.
[14] Explanation of Benveniste. Nature 1988; 334(6180): 85–6.
[15] Pool R. Unbelievable results spark a controversy. Science 1988; 241(4864): 407.
[16] Cherruault Y, Guillez A, Sainte-Laudy J, Belon P. Etude mathématique et statistique des effets de dilutions successives de chlorhydrate d'histamine sur la réactivité des basophiles humains. Bio-Sciences 1998; 7: 63–72.
[17] Sainte-Laudy J, Sambucy JL, Belon P. Biological activity of ultra low doses : I/Effect of ultra low doses of histamine on human basophil degranulation triggered by D. Pteronyssinus extract. Ultra Low Doses 1991; Taylor and Francis: 127–38.
[18] Sainte-Laudy J, Belon P. Biological activity of ultra low doses: II/Effect of ultra low doses of histamine on human basophil degranulation triggered anti-IgE. Ultra Low Doses 1991; Taylor and Francis: 139–43.
[19] Hadjaj B, Cherruault Y, Sainte-Laudy J. Basophil degranulation control Int J Biomed Comput 1992; 31: 89–97.
[20] Hadjaj B, Cherruault Y, Sainte-Laudy J. Control of basophil degranulation. Int. J Biomed Comput 1993; 32: 151–9.
[21] Sainte-Laudy J. Modulation of allergen and anti-IgE induced human basophil activation by serial histamine dilutions. Inflamm. Res 2000; 49 (Suppl 1): S5–6.
[22] Belon P, Cumps J, Ennis M, Mannaioni PF, Sainte-Laudy J, Roberfroid M et al. Inhibition of human basophil degranulation by successive histamine dilutions: results of a European multi-centre trial. Inflamm Res 1999; 48(Suppl 1): S17–8.
[23] Brown V, Ennis M. Flow-cytometric analysis of basophil activation: inhibition by histamine at conventional and homeopathic concentrations. Inflamm Res 2001; 50 (Suppl 2): S47–8.
[24] Sainte-Laudy J. Standardization of basophil degranulation for pharmacological studies. J Immunol Methods 1987; 98: 279–82.
[25] Crockard AD, Ennis M. Laboratory-based allergy diagnosis; should we go with the flow? Clin Exp Allergy 2001; 31: 957–77.
[26] Sainte-Laudy J, Sabbah A, Vallon C, Guerin JC. Analysis of anti-IgE and allergen induced human basophil activation by flow cytometry. Comparison with histamine release. Inflamm Res 1998; 47: 401–8.
[27] Shore PA, Burkhalter A, Cohn Jr, V H A method for the fluorometric assay of histamine in tissues. J Pharmacol Exp Ther 1959; 127: 182–6.
[28] Kremzner LT, Wilson JB. A procedure for the determination of histamine. Biochim Biophys Acta 1961; 50: 364–7.
[29] Bergendorff A, Uvnas B. Storage of 5-hydroxytryptamine in rat mast cells. Evidence for an ionic binding to carboxyl groups in a granule heparin-protein complex. Acta Physiol Scand 1972; 84: 320–33.
[30] Sainte-Laudy J, Belon P. Application of flow cytometry to the analysis of the immunosuppressive effect of histamine dilutions on human basophil activation: effect of cimetidine. Inflamm Res 1997; 46(Suppl 1): S27–8.
[31] Doutremepuich C, Aguejouf O, Pintigny D, Sertillanges MN, De Seze O. Thrombogenic properties of ultra low dose of acetylsalicylic acid in a vessel model of laser induced thrombus formation. Thrombosis Research 1994; 76; 2: 225–9.
[32] Belougne-Malfatti E, Aguejouf O, Doutremepuich F, Belon P. Doutremepuich C. Combination of two doses of acetyl salicylic acid : experimental study of arterial thrombosis. Thrombosis Research 1998; 90: 215–21.
[33] Aguejouf O, Malfatti E, Belon P, Doutremepuich C. Time related neutralization of two doses acetyl salicylic acid. Thrombosis Research 2000; 100: 317–23.
[34] Jonas WB, Lin Y, Tortella F. Neuroprotection from glutamate toxicity with ultra-low dose glutamate. Neuroreport 2001; 12: 335–9.
[35] Marotta D, Marini A, Banaudha K, Maharaj S, Ives J, Morrissette CR et al. Non-linear effects of cycloheximide in glutamate-treated cultured rat cerebellar neurons. Neurotoxicology 2002; 23: 307– 12.
[36] Cazin JC, Cazin M, Gaborit JL, Chaoui A, Boiron J, Belon P et al. A study of the effect of decimal and centesimal dilutions of arsenic on the retention and mobilization of arsenic in the rat. Human Toxicology 1987; 6: 315–20.
[37] Rey L. Thermoluminescence de la glace. C. R. Acad Sci Paris, 2000; tome 1, série IV: 107–10.
[38] Rey L. Low-temperature thermoluminescence. Nature January 1998; 391: 418.
[39] Rey L. Thermoluminescence of ultra high dilutions of lithium chloride and sodium chloride. in Physica A 2003; 323: 67–74.