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Inhibition of basophil activation by histamine: a sensitive and reproducible model for the study of the biological activity of high dilutions

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Background: **At the beginning of this series of experiments we were looking for a model based on the use of purified commercially available compounds based on a fully described and accepted pharmacological model to study of the biological effect of high dilutions. Negative feedback induced by histamine, a major pro-inflammatory mediator, on basophils and mast cells activation *via* an H2 receptor me these criteria. The simplest way of measuring basophil activation in the early 1980's was the human basophil activation test (HBDT).**

Objectives: **Our major goal was first to study the biological effect of centesimal histamine dilutions beyond the Avogadro limit, on the staining properties of human basophils activated by an allergen extract initially house dust mite, then an anti-IgE and N-formyl-Met-Leu-Phe (fMLP). Technical development over the 25 years of our work led us to replace the manual basophil counting by flow cytometry. The main advantages were automation and observer independence. Using this latter protocol our aim was to confirm the existence of this phenomenon and to check its specificity by testing, under the same conditions, inactive analogues of histamine and histamine antagonists. More recently, we developed an animal model (mouse basophils) to study the effect of histamine on histamine release.**

Methods and results: **For the HBDT model basophils were obtained by sedimentation of human blood taken on EDTA and stained with Alcian blue. Results were expressed in percentage activation. Histamine dilutions tested were freshly prepared in the lab by successive centesimal dilutions and vortexing. Water controls were prepared in the same way. For the flow cytometric protocol basophils were first labeled by an anti-IgE FITC (basophil marker) and an anti-CD63 (basophil activation marker). Results were expressed in percentage of CD63 positive basophils. Another flow cytometric protocol has been developed more recently, based on basophil labeling by anti-IgE FITC (fluorescein isothiocyanate) and anti-CD203 PE (another human basophil activation marker). Results were expressed in mean fluorescence intensity of the CD203c positive population (MFI-CD203c) and an activation index calculated by an algorithm. For the mouse basophil model, histamine was measured spectrofluorimetrically.**

The main results obtained over 28 years of work was the demonstration of a reproducible inhibition of human basophil activation by high dilutions of histamine, the effect peaks in the range of 15–17CH. The effect was not significant when histamine was replaced by histidine (a histamine precursor) or cimetidine (histamine H2 receptor antagonist) was added to the incubation medium. These results were confirmed by flow cytometry. Using the latter technique, we also showed that 4-Methyl histamine (H2 agonist) induced a similar effect, in contrast to 1-Methyl histamine, an inactive histamine metabolite.

Using the mouse model, we showed that histamine high dilutions, in the same range of dilutions, inhibited histamine release.

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Conclusions: **Successively, using different models to study of human and murine basophil activation, we demonstrated that high dilutions of histamine, in the range of 15–17CH induce a reproducible biological effect. This phenomenon has been confirmed by a multicenter study using the HBDT model and by at least three independent laboratories by flow cytometry. The specificity of the observed effect was confirmed, *versus* the water controls at the same dilution level by the absence of biological activity of inactive compounds such as histidine and 1-Methyl histamine and by the reversibility of this effect in the presence of a histamine receptor H2 antagonist.** *Homeopathy* (2009) **98**, 186–197.

Keywords: Human basophil; Mouse basophil; High dilutions; Homoeopathy; Histamine; Flow cytometry; Histamine release; IL4 release

Introduction

Although some homeopathic remedies contain molecules of the diluted compounds, a major and provocative claim of homoeopaths is that high dilutions, on the basis of Avogadro's number, are statistically very unlikely to contain molecules of diluted compound, do exhibit a biological and therapeutic effects. Various *in vivo* and *in vitro* models have been proposed to investigate this question.

Our initial idea was to set up an *in vitro* model based on the similia principle which was sensitive, theoretically able to measure effects and simple enough to be easily repeated to test various different experimental conditions. When we began our series of experiments we already had extensive experience of the study of human basophil activation and it seemed that this model could fit our concept, knowing that different mediators produced by human basophils, and particularly histamine, were already used as homeopathic remedies (*Lung Histamine* and *Histaminum*).

We began in 1981 with basophil activation with various allergens such as honey bee venom and house dust mites using leukocyte suspensions taken from allergic patients. In our first series of experiments run in 1982, we tested, following homeopathic practice, histamine in the 7C dilution (*Histaminum* or Histamine hydrochloride 10^{-14} M). These experiments led to the first positive results obtained by our group, presented at the European Congress of Allergy and Clinical Immunology (London November 1982).[1] The magnitude of the observed effect was not dependent on the nature of the allergen. These results were well accepted, but there were no theoretical difficulties, 10^{-14} M being a concentration to which biologists were used, particularly in the field of cellular biology.

The same reasons lead us to test other biologically active molecule such as Platelet Activating Factor (PAF) and lyso-PAF (an inactive structural analogous of the PAF) released by various cells such as mast cells and eosinophils. In contrast to *Histaminum*, high dilutions of PAF inhibit basophil activation (unpublished results) in the range of 8CH–11CH the observed inhibition peaks at 10C with no effect between 5 and 8CH and no effect either between 11 and 15CH. On the basis of our previous results obtained with histamine, we extended the range of the tested dilutions up to 20CH and observed, in addition to the inhibition peak around 7CH, a second inhibition peak in the range of 15CH–17CH. All these experiments were performed on blood samples taken from allergic patients.

In parallel, the group of Benveniste, who was asked to reproduce the experiments performed in our lab, began to work on anti-IgE stimulated basophils, to have the possibility of using samples from healthy donors, blood taken from allergic patients being rather difficult to obtain in sufficient quantity. After a first technical step of responder selection (patients responding normally to anti-IgE), basophil activation was induced by targeted anti-IgE, concentrations, depending on the anti-IgE used, were between 1 μg/ml and 0.01 μg/ml.

In 1984, we began to observe a significant activity of high histamine dilutions (in which statistically it is very unlikely that there remains any molecule of the diluted compound, meaning beyond 11C) on anti-IgE induced basophil activation. As these scientifically provocative results met a quite sceptical reception, we decided to accumulate the experiments in order to confirm these results and to convince other laboratories to repeat these experiments in the same and in other technical conditions.[2–5] In parallel Poitevin *et al.* published positive results concerning the effect of *Lung Histamine* and bee extract (*Apis mellifera*) on anti-IgE stimulated human basophils.[6,7]

After the publication of the Davenas *et al.* Nature paper in 1998[8] and the scientific storm, which followed,[9–12] it was extremely difficult to present and publish results dealing with high dilutions biological activity. We had to wait until the early 1990's to publish our results[13–16] by that time we had substantially modified the model, particularly by replacing manual counting of stained cells with an automated technique, flow cytometry, which we began use in our laboratory for diagnosis of allergy after the description of a novel basophil activation marker, CD63. With some modifications, this technique has been used ever since. We present here, in detail, the different steps of this research.

Human basophil pharmacology

Basophils, present in low concentration (less than 1% in human blood) had to wait until the 1990's to be recognised as a key cell in the immune system after the demonstration that they secrete Th2 like cytokines, in particular interleukin4 (IL4) and IL13 when stimulated[17]

in addition to the two major mediators, histamine and leukotriene C4 (LTC4). In contrast to what was generally believed until recently, the presence of blood basophils in mice has been clearly demonstrated. Today several animal models exist including basophils from mice bone marrow, basophil deficient mice and mice knockout for various markers. Human basophils lack the PGD2 pathway (in contrast to mice basophils) and PAF release is still questioned. Moreover, they do not release tryptase and chymase, in contrast to mast cells. They are classified as granulocytes and characterized by the presence of intracytoplasmic granules with metachromatic staining properties with several dyes including toluidin blue, astra blue and Alcian blue which bind to the negative charges exhibited by the heparinoid fragments present on the granule's surface. They are generally considered blood cells but, in several pathological conditions, and particularly at the site of inflammatory reactions, they have also to be considered tissue cells. They are found at the site of nasal and cutaneous late reactions.[18,19]

Basophils bear specific high affinity receptors for IgE at high density, other blood cells such as eosinophils and neutrophils also bear this receptor, but at a much lower density. Basophil sensitivity is defined as the number of cell surface IgE related to half the maximum histamine release has been studied by McGlashan.[20] It is in the range of 300–40,000 molecules per basophil with a median value of 1900; 40 molecules per basophils are sufficient to trigger induce release. Membrane IgEs or IgE receptors have to be cross-linked by the agonist, which thus needs to be bivalent at least. The binding constant of IgE for FCεRI is particularly high ranging from 1.4 to 2.7×10^9/M.[21] In order to analyse basophil activation, flow cytometry seemed the ideal technique as it is automated, allowing electronic selection of the analysed populations and visualization and so is not a 'black box', in contrast to basophil staining and counting or mediator release measurement (histamine and leukotrien release). When we began our research on high dilutions, in the early 1980's, the two available methods were human basophil activation test (HBDT) based on basophil staining properties and histamine release.

In 1991, Knol[22] described the basophil activation marker CD63 and showed that following anti-IgE, anti-FCεRI or fMLP (N-formyl-Met-Leu-Phe)-induced activation, CD63 anchored in the granule stroma of resting basophils, was expressed on the plasma membrane, without permeabilization, due to the fusion of these granules with the membrane after transcytoplasmic migration. A considerable advantage of this marker is that it is poorly represented on resting basophil membranes and but expressed at high density on activation. For potent allergens (proteins, neuro-muscular blockers)[23–31] there is a good correlation between histamine release and CD63 expression, suggesting parallelism of the two phenomenon. In other circumstances, (non physiological conditions, weak allergens), discrepancies observed between the two phenomenon are in favour of a graded phenomenon, not an 'all or nothing' relationship.[32] The activation cascade does not necessarily stop at the endpoint (mediator release) but may stop at an intermediate level between this endpoint and the early events of basophil activation (calcium influx, membrane lipid rafting). Since the discovery of the CD63 marker, other activation markers have been described such as CD203c described in 1999 by Buhring,[33] which is an ectoenzyme i (ectonucleotide pyrophosphatase/phoshodiesterase 3) of unknown biological function. Other activation markers such as CD107 and CD164 were also described more recently.[34]

The mechanism analysed by these different methods are quite different: histamine release analyses the endpoint of the activation cascade, CD63 the fusion of intracellular granules with the cell membrane, CD203c membrane remodelling due to the formation of lipid rafts and clusters and stained basophil counting the negative charges neutralization due early cation (calcium) influx (Alcian blue is a cationic dye used at low pH) and degranulation leading to granule extrusion.

On the basis of these data concerning basophil pharmacology, we thought, at the beginning of these studies, that basophils were the appropriate cells for studying the highly diluted compound-induced immunomodulation, as basophil activation is extremely sensitive to low concentration of the main effectors (40 IgE molecules per cell which correspond to around 100 FCεRI cross-links) and so represents a signal amplification system mediated by an antibody (IgE) which has a particularly high affinity for its specific receptor. Moreover, basophils are extremely sensitive to IgE-mediated stimulation, and it has been shown that histamine release can be initiated by less than 100 molecules of antigen.[20,21]

Preparation of high dilutions

We compared several times the effect of preparation protocol on the biological activity of high dilutions, including dynamisation by manual or automatic succussion either in glass tubes or in polystyrene vials, or stirring by a usual laboratory vortex in glass tubes or in polystyrene vials. We never found any significant difference between these different preparation methods but as all these experiments were performed on short series of basophil counts performed by basophil staining they should use flow cytometry control.

In our laboratory, histamine dilutions were systematically prepared in polystyrene vials by diluting 1:100 a mother dilution of the tested compound. For histamine, for example, a first 0.01 M dilution was prepared from the commercially available histamine hydrochloride (Sigma, USA). This represented the 1C dilution. At each dilution step there was extensive vortexing (10 s, full speed). The dilutions were kept at 4°C until use and stored for a maximum of 2 months. We have recently shown by flow cytometry[35] that histamine dilutions shows a marked loss of activity after 1 month storage. Ever since, the expiration date of our dilutions was set at one month and experiments performed on mouse basophils were all

performed with freshly prepared histamine dilutions (less than 1 week).

Analysis of human basophil activation by their metachromatic properties

Between the two methods available in the early 1980's, we chose basophil staining properties, as the method was sufficiently fast to be repeated within the same working day. After 1 year of research, we obtained the first reproducible results with HBDT but failed to show the same effect with histamine release, at least in our hands. We started by testing different types of highly diluted compounds such as specific allergens on basophils taken from allergic patients. After the first positive results, we changed our strategy with the idea that such scientifically provocative results had to be obtained by a well accepted pharmacological reaction already used for conventional pharmacology, for this reason we chose the inhibition of basophil activation by histamine. The negative feedback mechanism of histamine of its own secretion by basophils and mast cells was widely described and accepted. It has been established that histamine is capable of inhibiting anti-IgE or allergen-induced activation *via* H2 receptor for histamine.[36-38] Another histamine receptor (H4) has been described recently on human and mice basophils. In addition, histamine is readily available as a chemically pure compound.

We began to test the effect of successive centesimal histamine dilutions on blood leukocytes prepared by simple sedimentation at 1 g. Cell suspensions were washed twice with Hepes buffer (127 mM NaCl, 5 mM KCl, 20 mM Hepes, 5 IU/mL heparin; pH 7.4) and histamine dilutions were incubated at room temperature with aliquots of the leukocyte suspensions for 30 min. Basophil activation was elicited either by a target allergen concentration (for basophils from allergic patients) or by targeted anti-IgE. These agonists were diluted in hepes-calcium buffer (127 mM NaCl, 5 mM KCl, 20 mM Hepes, $CaCl_2$ 5 mM, $MgCl_2$ 2 mM, 5 IU/mL heparin; pH 7.4). The experiments were performed in microtiter plates and basophils were first stained by toluidin blue and then, due to the difficulty, for untrained technicians, of counting the stained basophils, we changed to Alcian blue, basophils being much more easily differentiated from the other blood cells by this method (Figure 1).

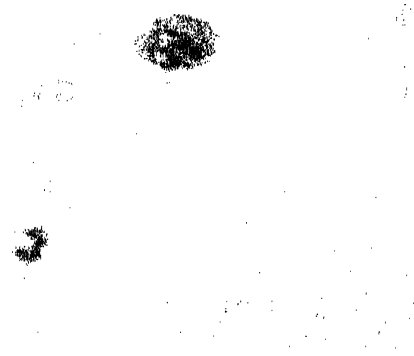

Figure 1 Human basophil stained by Alcian blue among unstained Polymorphonuclear cells.

After obtaining the first positive effects of histamine dilution with the 7CH[1] we tested, in the same conditions, serial histamine dilutions beyond the statistically likely presence of one molecule per litre of the diluted compound (Avogadro's number). We obtained curves showing recurrent inhibition zones separated by inactive dilution ranges, as shown in Figure 2, for house dust mite induced basophil activation.[13,14] The same type of curve was also observed for anti-IgE elicited basophil activation.[15] We decided to continue with an anti-IgE as the use of an anti-IgE had several advantages over the allergen-induced activation, including: better definition of the agonist and the possibility of using healthy donors blood instead of being dependent on the availability of blood taken from allergic patients. We observed, by repeating the same experiment ten times, that the preincubation of the leukocyte suspensions with cimetidine, a classical histamine H2 receptor antagonist 10^{-5} M, resulted in the reversion of the inhibition induced by histamine dilutions in the range of 6C–9C but also in the range of 15CH–17CH ($p < 0.001$, Wilcoxon rank test).[39] So, there were clear similarities between histamine induced inhibition of basophil activation at conventional concentrations and at high dilutions.

In spite of these results in favour of the specificity of the observed phenomenon, a simple non-specific 'dilution effect' independent of the diluted compound was one of the main criticisms. In order to answer this criticism, we tested in parallel and in the same conditions the same histamine dilutions and dilutions of histidine, the carboxylated histamine precursor, knowing that this amino acid had no described effect on basophil activation. Again in 10 successive experiments, the inhibition induced by histamine was significant[36] at 16CH and 17CH ($p < 0.05$) whereas no significant effect was observed for histidine. Intra-assay significance of the inhibition observed with histamine 16CH was also calculated, with similar results.[16]

In 1988, Benveniste's group, which had agreed, a few years earlier, to repeat our experiments published the now infamous 'Nature paper'.[8] This used a completely different model based on a direct activation of human basophils by anti-IgE dilutions In spite of the 'scientific storm' elicited by this publication, we persevered with our research in this field and organised a multi-center European trial. The four laboratories participating used the same protocol and the same dilutions prepared by an independent group. In spite of laboratory to laboratory variation of the magnitude of the observed inhibition, the results were significant (pooled data) and in 3 of the 4 participating laboratories.[37,38]

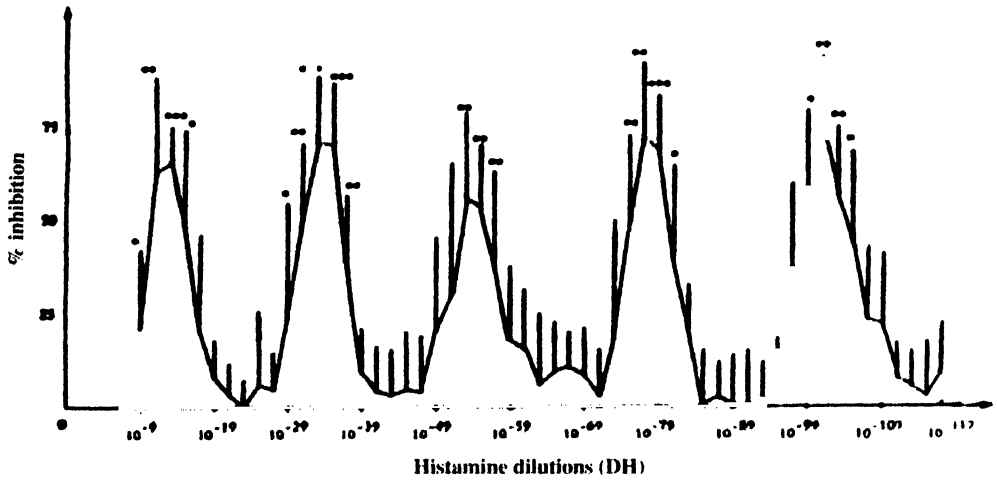

Figure 2 Effect of histamine dilutions from 10 $^{-10}$ to 10 $^{-120}$ M, showing recurrent inhibition of activation. Basophil activation triggered by house dust mite extract. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

In parallel, to begin to explore the relationships between the solvent (deionized water) and diluted compound, we tested the effect of heat on the histamine dilutions and showed that heating (30 min in a water bath) at different temperatures, histamine 7CH and 17CH continued to exhibit significant inhibition up to 60°C but not significant for the two dilutions heated to above 70°C (unpublished results).

All these results were obtained by a manual model based on the visualization by the use of specific dye of a very early event in the basophil activation cascade, the influx of positively charged ions. As activated basophils do not bind these dyes due to local neutralization of negative charges present on the surface of the granule stroma, the proportion of activated cells is calculated from the difference between the total stained cells and the cells stained after activation. But using this method spontaneous basophil activation (without agonist) cannot be calculated. The reference basophil population comprises only non activated basophils, in contrast with the methods we later adopted, which take account of the total basophil population. Nevertheless, and in spite of the fact that most of these experiments were performed blind, the model itself was strongly criticized. As our group was working in parallel on new flow cytometric methods for allergy diagnosis[23] after the brilliant description of CD63 expression on activated basophils membrane by E Knol[22], we modified our model by replacing the staining of non activated basophils and the microscopic counting of the stained cells by the analysis of membrane markers of immunomodulation by flow cytometry.

Analysis of human basophil activation by flow cytometry

Flow cytometry is a method for analysing intracellular and membrane events based on the irradiation by a laser beam (generally a 488 nm laser) of cells passing through a micro capillary. The scattered light is recorded at narrow and wide angles giving size and structure, the emission of fluorescent light at 4 or 5 different wavelengths ranges, depending on the conjugate used, gives the targeted marker's density. These machines are capable of analysing about 100,000 cells within 2–3 min. Flow cytometers need precise setting depending of the reagents and on the protocols used. In addition, thresholds have to be set between the negative and the positive populations (fluorescent cells) in order to express the results in percentage of positive cells. Flow cytometric results reliability is hightly dependent of the quality of the cytometer settings (thresholds, photomultiplier voltage, compensations etc).

Leukocyte suspensions were obtained by centrifugation of whole blood taken on EDTA. The buffy coat (leukocyte layer situated between the plasma and erythrocytes layers) was pipetted out and washed twice with Hepes buffer (127 mM NaCl, 5 mM KCl, 20 mM Hepes, 5 IU/mL heparin; pH 7.4) and then incubated v/v with histamine dilutions for 30 min at room temperature.

Aliquots (30 µL) of the cell suspensions were then mixed v/v with target concentrations of the agonists, either an anti-IgE (0.3 µg/ml, Caltag, USA) or fmlp (10–7 M, Sigma) diluted in hepes-calcium buffer (127 mM NaCl, 5 mM KCl, 20 mM Hepes, CaCl2 5 mM, MgClt 2 mM, 5 IU/mL heparin; pH 7.4) and incubated for 30 min at 37°C. After addition of an EDTA buffer in order to stop the calcium dependent basophil activation, basophils were labeled with a mixture of antibodies (15 min at room temperature in the dark). Erythrocytes were then lysed by an ammonium chloride buffer and, after centrifugation, cell pellets were resuspended in 500 µl of sheath buffer (Isoflow, Beckman Coulter 500 µL).

We first used, for basophil labeling a mixture of an anti-IgE FITC (Caltag USA) and an anti-CD63 PE (Coulter USA) in order to select basophils by their high membrane density of bound IgE and quantify the magnitude of basophil activation by the expression of CD63 on activated basophil membrane. Results were expressed in % CD63 positive basophils.[23] There is little or no interference in this protocol between the anti-IgE used for stimulating basophils and the anti-IgE FITC used for basophil labeling

as there is a marked difference of concentration between these two antibodies (0.4 μg/ml and 50 μg/ml) and EDTA is added to the cell suspensions before the addition of anti-IgE FITC, preventing any additional activation by the second anti-IgE. Moreover, basophils were labeled at room temperature and basophil activation is highly temperature dependent (35–37°C).

For the flow cytometric method as for the previous one all the experiments performed showing a significant basophil activation (>30% for Alcian blue and >15% for CD63 and MFI-CD203c) were included in the statistics. A typical flow cytometric protocol is represented in Figure 3. With this protocol, our main first objective was to reproduce the results obtained by basophil staining, within the first 2 years of research, we obtained encouraging results (Figure 4, unpublished results).

In order to confirm these preliminary results, we repeated the same experiment 47 times in triplicates (141 data points) and showed that histamine 15CH and 16C inhibited significantly (respectively 13.2%, $p = 0.00026$ and 19.4% $p < 10^{-5}$) anti-IgE induced human basophil activation (Figure 5, unpublished results) These results were followed by the confirmation of most of the results observed with the HBDT,[39-44] comprising the antagonist effect of cimetidine[45] favouring an H2 histamine receptor mediated effect as cimetidine is a potent H2 antagonist. The specificity of the biological effect of histamine was confirmed by testing in parallel high dilutions of histamine and histidine (inactive carboxylated precursor of histamine). Histamine significantly inhibited human basophil activation (17.6%, $p = 0.028$) whereas the effect of hisdidine was not significant (unpublished data, Figure 6). The inhibition induced by histamine 16 CH was of the same order of magnitude as the inhibition observed for the previous series of experiments (Figure 6, unpublished results).

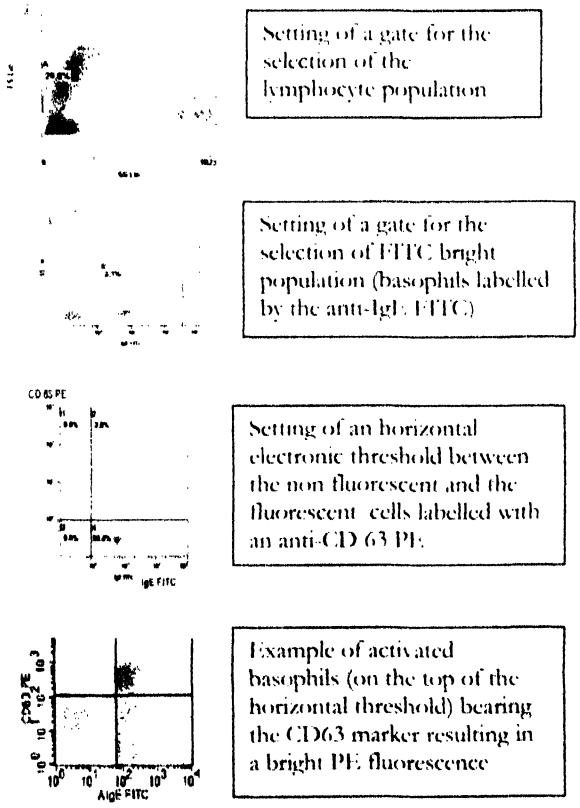

Figure 3 Set up of flow cytometric protocol based on the double anti-IgE and anti-CD63 staining.

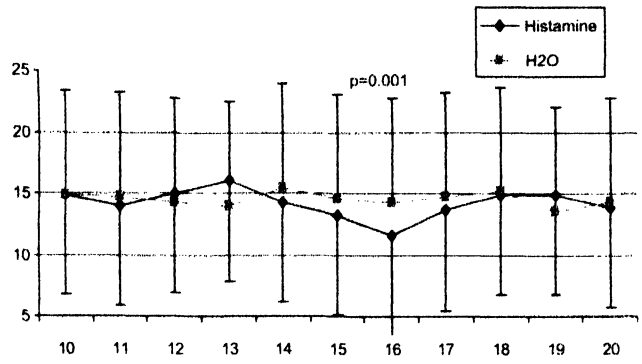

Figure 4 Effect of histamine dilutions from 10CH (10) to 20CH (20) on anti-IgE induced human basophil activation *versus* the water controls diluted in the same conditions. Compared to water control 16C, the effect of histamine 16C was significant ($p < 0.001$). Results are mean of 10 experiments ± SD.

In order to get information on the relationship between the solvent (water) and the diluted compounds (here histamine) we checked, in parallel with the experiments performed by L Rey,[46] the effect of the addition of lithium ion to the incubation medium, the lithium ion being a hydrogen bond scavenger. The inhibition by histamine 15 and 16CH alone was significant ($p = 0.013$, $p = 0.017$) whereas it was not significant in the presence of lithium 10 μg/ml ($p = 0.62$ and 0.56) (Figure 7, unpublished data). The effect of lithium 10 μg/ml on the positive control was not significant.

Following the description of new human basophil activation markers, we investigated the interest of the CD203c[33] which was tested according to the protocol represented in Figures 8 and 9. In contrast to the IgE/CD63 flow cytometric protocol, the two main changes were, apart the use of another activation marker: preparation of the tested leukocytes by a buffy coat (centrifugation of the total blood and separation of the layer of white cells situated between the plasma

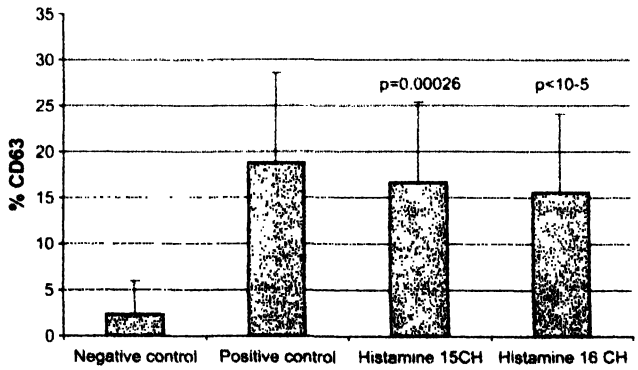

Figure 5 Inhibition of anti-IgE induced human basophil activation by histamine 15CH and 16CH. Results expressed in %CD63 ± SD *versus* the positive and negative controls prepared with water 16C.

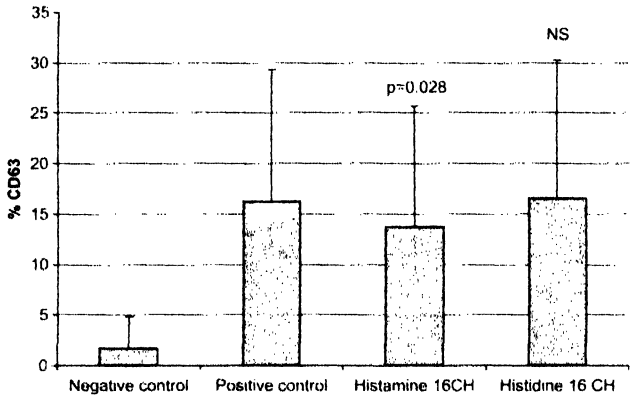

Figure 6 Comparison of the effect of high dilutions of histamine and histidine on anti-IgE induced human basophil activation (mean of 13 experiments in triplicates). Results expressed in% CD63 ± SD *versus* positive and negative controls prepared in water 16C. NS = not significant.

and the erythrocytes pellet) and the use of the peptide fMLP instead of an anti-IgE for basophil activation.

This protocol led to results[47–49] confirming results obtained with CD63, the magnitude of histamine 16CH being higher with CD203c (32%, $p = 0.004$, (Figure 10)) than with CD63. Moreover, if these results were expressed in activation index calculated after linearization of the MFI-CD203c (data are given by the cytometer in log units) the inhibition induced by histamine 16CH and histamine 2CH reached respectively, 78% and 96% These results also showed that the effect of histamine in high dilution was not significant on the IgE marker measured by the MFI-IgE, which was not susprising as IgE downregulation is closely linked to an IgE dependent activation, in contrast of fMLP-induced activation.

More recently, we demonstrated that among the different methylated histamine derivatives (1-Methyl, 3-Methyl and 4-Methyl histamine) tested at the 16CH dilution, only 16CH 4-Methyl histamine[49] (Figure 11) had a significant effect on fMLP-induced basophil activation. These results showed that inhibition of biological effects on human basophils by high dilutions of histamine was similar to the activity of H2 or H4 agonists and that the effect is specific, as the

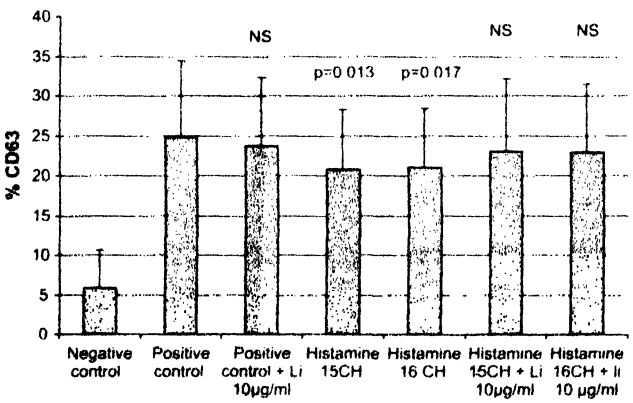

Figure 7 Antagonist effect of lithium 10 µg/ml on inhibition of anti-IgE induced human basophil activation by histamine 15CH and 16CH. Results expressed in %CD63 ± SD, NS = not significant.

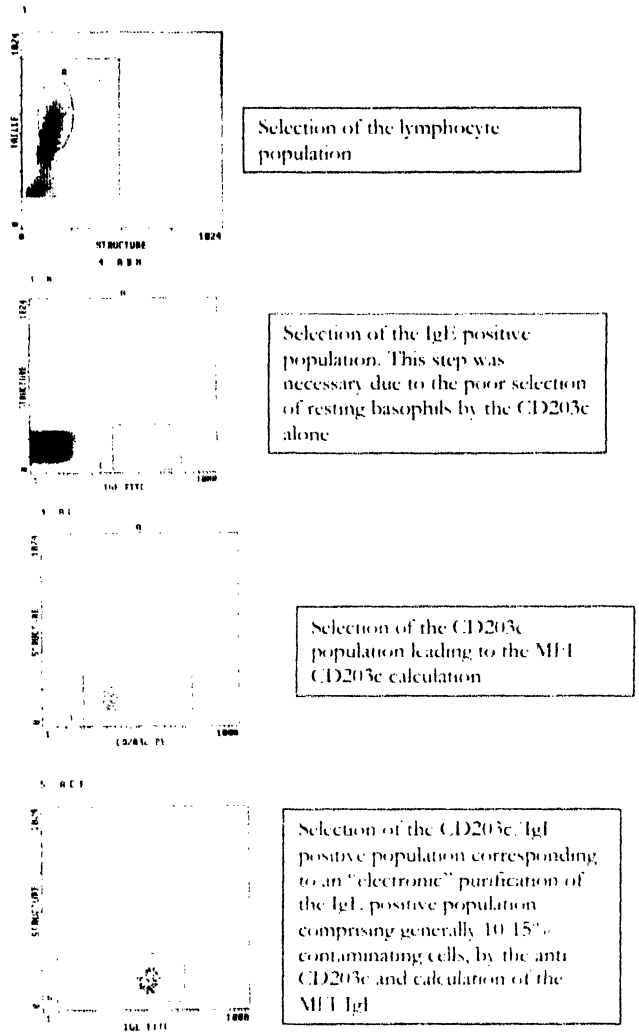

Figure 8 Set up of flow cytometric protocol for the analysis of CD203c up-regulation on activated human basophil membrane.

1-Methyl and 3-Methyl derivatives had no activity in the same conditions.

Since 2004, we have worked on a mouse model (UMR-CNRS 8147 hospital Necker, Paris),[50] particularly on the release of histamine by basophils prepared from the bone

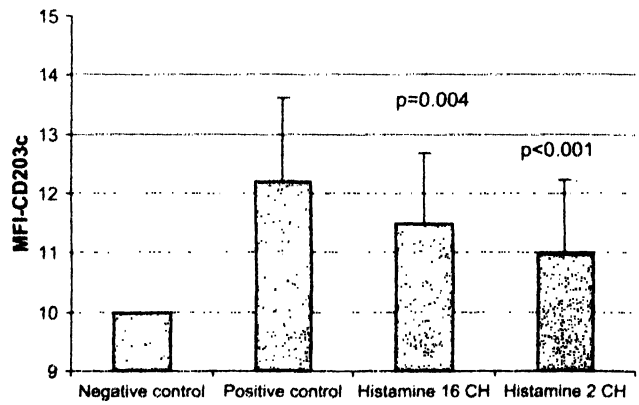

Figure 9 Inhibition of fMLP-induced basophil activation by histamine 16CH and histamine 2CH. Results expressed *versus* negative and positive controls prepared in water 16 C and expressed in MFI-CD203c ± SD. Negative controls set at 10. Statistical significance calculated on the raw data by Wilcoxon rank test.

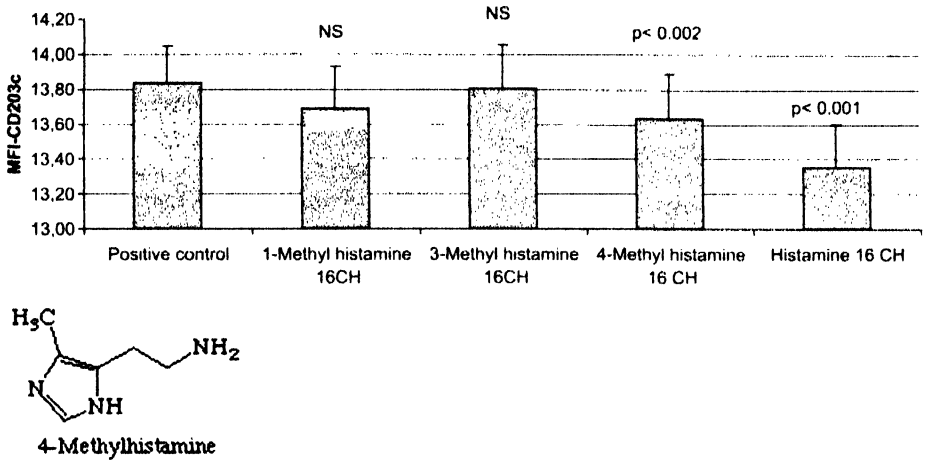

Figure 10 Effect of 1, 3, 4-methyl histamine 16CH and histamine 16CH on fMLP-induced basophil activation. Basophil activation expressed in MFI-CD203c ± SEM, mean of 10 experiments in triplicates. Negative controls (not shown) were set at 10 to compare the different experiments performed on different blood donors. NS = not significant.

marrow (total bone marrow leukocytes and bone marrow derived basophils) and stimulated by IgE or IL3. Histamine was determined by spectrofluorimetry.[51,52] We recently demonstrated, with mice bone marrow basophils (unpublished results, Figure 8), that histamine 15CH inhibits ($p < 0.001$) histamine production induced by IL3 ($p < 0.001$) and by IgE ($p = 0.042$) with an inhibition of 8.4%, compared to water 15CH. The inhibition is similar to that observed for CD63 up-regulation inhibition (14%).

The results published by Benveniste group in 1988 have never been reproduced elsewhere[53,54] and experiments described in 1991 by the same group[55] have been widely criticized, mainly on statistical grounds. In contrast, the effect of histamine measured by flow cytometry has been reproduced and published by two independent groups, one[56] using the IgE/CD63 protocol and the other[57] using a more sophisticated protocol based on the negative selection of basophils by a cocktail of antibodies (CD 2, 14, 16, 19, HLADR), a positive selection by an anti-CD123 and CD63 as an activation marker. In both cases, basophil activation was elicited by an anti-IgE. Moreover, the group of M Ennis in Belfast[55] has shown that the biological effect of high dilutions is inhibited by heating the dilutions. A recent report from an Italian group[58] confirmed, using also a sophisticated and standardized cytometric protocol with CD203c as an activation marker, the biological activity of histamine high dilutions including histamine 16CH prepared by a commercial dynamizer. Another, Swiss, group[59] observed a significant effect of histamine high dilutions when compared to the related water controls. When the mean of all the water controls compared to the whole series of histamine dilutions tested, the effect was not significant. A plate effect was hypothesised but according to the authors, was eliminated by randomization. This plate effect has been known for a long time by serologists[60] and is due to the manufacturing process which leads to electrostatical inhomogeneities between the 96 plastic wells. In addition, leukocytes were prepared by dextran sedimentation and, according to our experience, polymers as dextran and ficol may lead to non-specific activation of leukocytes and modifications of t membrane properties. It is for this reason that we first used simple spontaneous sedimentation and then, for the CD203c protocol a buffy coat, cell suspensions always being tested in disposable tubes. One argument supporting these hypotheses is that the magnitude of inhibition observed for both histamine 2CH (23.1%) and histamine 16CH (5.7%) were much lower than percentage inhibition observed in other laboratories. Results obtained by these different laboratories are summarized below in Table 1.

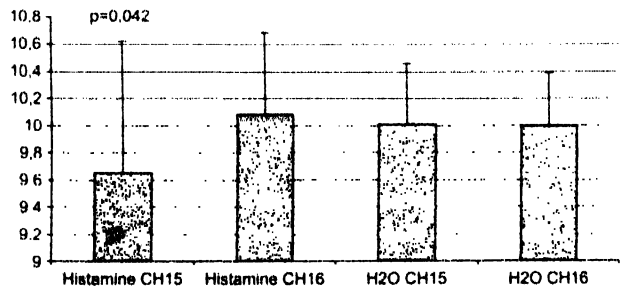

Figure 11 Effect of histamine 15CH and 16CH on histamine production by mouse total bone marrow cells stimulated by IgE *versus* water controls tested at the same dilution level.

Relationships between results and hypotheses related to the mode of action of high dilutions

Hypothesis derived from biological experiments

Results obtained with histamine high dilutions model on inhibition of human and mouse basophil activation lead to the conclusions that:

- High dilutions of histamine have a reproducible biological activity on human and mice basophils, these results based on an enormous amount of experiments conducted over a long period (28 years), on a large number of different leukocyte suspensions and with at least, three main protocols. The quantitation method used since the early 1990's is automated and independent of the observer.

Table 1 Published results related to the inhibition of basophil activation by histamine dilutions

Author	*Protocol*	*Measured parameter*	*Histamine control*	*Percentage inhibition*	*Histamine dilution*	*Percentage inhibition*
Sainte-Laudy 1993	Staining	Number stained cells	ND		18CH	53.4% $p < 0.001$
Sainte-Laudy 1997	FCM IgE/CD63	%CD63	ND		16CH	21.5% $p = 0.0067$
		%CD63			16CH + cimetidine	<5 NS
Brown 2001	FCM IgE/CD63	%CD63	2CH	34% $p < 0.01$	10CH	43.8 $p < 0.001$
Guggisberg 2005	IgE/CD63	%CD63	2CH	23,1 $p = 0.018$	11CH	5,4 $p = 0.018$[1,2]
Sainte-Laudy 2006	IgE/CD203c	MFI-CD203c	2CH	56% $p < 0.001$	16CH	32% $p = 0.0004$
Chirumbolo 2009	CD123/HLADR/ CD63/CD203c/CD45	MFI-CD203c	2CH	72% $p < 0.001$	16CH	19% $p = 0.009$
		%CD63	2CH	66% $p = 0.001$	16CH	14% NS

ND = not done, NS = not significant.
[1] significance calculated *versus* the related water control.
[2] Not significant *versus* the whole series of water controls.

- The effect is specific and the hypothesis of an artefact due to a non-specific effect between the solid phase (glass, polystyrene) is in contradiction with the inactivity of histidine, 1-Methy histamine and 3-Methyl histamine and the reversibility of histamine high dilutions activity by a known histamine antagonist (cimetidine or ranitidine).
- The systematic addition of a histamine 10^{-4} M control has shown a close parallelism between the pharmacological effects of histamine tested at conventional concentrations and diluted to 15CH or 16CH.

These results are consistent with the existence of common target for molecular and sub molecular histamine dilutions (in human H2 or H4 histamine receptors or, in mice, a protein carrier).[50] The curve consistently observed with the HBDT may indicate that this effect could also be observed for dilutions higher than the 15C–17C range.

There are hundreds of biological experiments dealing with the effect of high dilutions but only a few so far, show reproducible positive effects. The main models are the effect of various arsenic trioxide dilutions (*Arsenicum album*) in humans, animals and plants intoxicated by arsenic trioxide at sub lethal doses and the effect of acetylsalicylic acid dilutions in human or animals (rats and mice). These results, obtained from completely different models may be considered as supplementary arguments in favour of the existence of the biological activity of high dilutions. As with allopathic agents, the cellular targets and the mechanism involved should depend on the nature of the diluted compound, an argument in favour of this hypothesis being, for high dilutions, the variable delay necessary for observing the effect, 10–30 min for basophil activation *in vitro*, to some hours and lasting a few days for arsenic *in vivo* and few minutes to 2 h for acetylsalicylate *in vivo*.

An enzyme, the cyclooxygenase 2 (COX2) has been hypothesised as a target for acetylsalicylate[61–65] and a mechanism involving the genome have been suggested for high dilutions of *A. album*.[66–68] This latter mechanism could involve various class of nuclear or cytoplamic small RNA such as iRNA[69,70] which may interfere *via* the H bounds, with the messenger RNA [mRNA]. This system is faster, in eukaryotic cells, than complete genetic expression, which initially requires synthesis of mRNA from the related DNA and its diffusion from the nucleus to the cytoplasm.

Hypotheses derived from physical experiments

The study of relationships between dissolved compound and water and the study of the structure of water have been the main field of interest of the physicists to try to explain the biological activity of high dilutions. Thermoluminescence has shown that the spectra related to dynamized water 15CH is different from a dilution 15CH of NaCl or LiCl.[71,72] Other results obtained by calorimetry or conductivimetry lead to similar conclusions.[73,74] A modification of the structure of water has been demonstrated by NMR for high dilutions of histamine.[75] A fascinating theory is the possible link between the formation of microbubbles during dynamisation and the formation of stable clusters,[76] although this hypothesis has been questioned by several authors. The stability of histamine dilutions (for at lest 1 month at +4 C), the preliminary results related to the effect of heat and of an H bond scavenger (LiCl) and the specificity of the observed effect make necessary the hypothesis of a specific template generating a reproducible and recurrent message. It seems that, in the current state of our knowledge concerning the structure of water, any other hypothesis may rapidly become redundant.

Conclusions

The experiments described here have repeatedly lead to similar results in terms of immunomodulation (inhibition) and in terms of active dilution ranges (15C–18C) in spite of the different protocols used during this long period and the fact that the experiments have been performed on hundreds of different blood samples. The only common element between all these experiments is, except for the experiments performed at the very beginning, the use of the same pharmacological model based on a well known feedback mechanism (histamine induced inhibition of human basophil activation) by a well chemically characterized compound (histamine).

We have systematically pursued possible artifacts, but such an explanation is not consistent with results obtained for histamine dilutions compared with the water controls diluted in the same conditions, the absence of effect of histamine analogues and derivatives such as histidine, 1-Methyl histamine and 3-Methyl histamine, and the reversibility of the biological activity in the presence of an histamine H2 antagonist (cimetidine or ranitidine).

These results and results arising from other biological models including the effects of high dilutions of acetyl salycilic acid on platelet function and arsenic detoxication models, argue strongly for the existence of biological activity of dilutions in which, statistically, according to Avogadro's number, no molecule of the diluted compound can remain. The results are consistent with structural modifications of the solvent (water in our experiments), which are stable for at least 2 months at 4°C. In these conditions, perturbations of the hydrogen bound may be key further physical experiments are necessary to investigate this hypothesis. Even if this hypothesis were true, there is a second stgae, which concerns the relationships between this of physico-chemical message and living cells, *in vitro* on *in vivo*. Experiments on basophils and neutrophils show that this effect is fast (less than 30 min) with pharmacokinetics not very different from those of the molecular form of the tested compound.

These results should, at least, be considered an encouragement for developing other protocols, organizing multi-center trials and gathering multi-disciplinary specialists leading to a new and synthetic approach to this phenomenon.

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