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ORIGINAL PAPER

Improvement of flow cytometric analysis of basophil activation inhibition by high histamine dilutions. A novel basophil specific marker: CD 203c

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Background: **Histamine is known to elicit a negative feedback effect on anti-IgE and allergen-induced basophil activation. A series of experiments performed between 1981 and 1995 using a manual method showed biological activity of highly diluted histamine. Most of the experiments used histermine in the range 10^{-30} (15C)–10^{-36} M (18C). These results were confirmed by automated flow cytometry, but this method is based on the selection of basophils by anti-IgE and analysis of basophil activation by anti-CD 63, showing significant but relatively low inhibition (approximately 14%), insufficient to convince the scientific community of the reality of the phenomenon.**
Objective: **We investigated if the use of CD 203c, a basophil specific, earlier marker than CD 63 of the activation cascade, increased the sensitivity of the method, testing two target histamine dilutions, 10^{-4} (2C) and 10^{-32} M (16C).**
Methods: **Basophils, obtained from buffy coats, were pre-incubated with the histamine dilutions and activated by two agonists: anti-IgE and fMLP (formyl-methionyl-leucyl-phenylalanine peptide). Basophil activation was stopped with EDTA. The cells were labelled with anti-IgE, anti-CD 13 and anti-CD 14 for basophil selection, and anti-CD 63 and anti-CD 203c for basophil activation. Results were expressed in up-regulation percentage for CD 63 or mean intensity of fluorescence (MFI) for CD 203c.**
Results: **Histamine 10^{-4} M (2C) and histamine 10^{-32} M (16C) were capable of inhibiting both IgE-dependent (anti-IgE) and IgE-independent (fMLP) basophil activation. The percentage inhibition depended on the activation marker used. The highest inhibition for histamine dilution 16C was observed with CD 203c (38%, $P<0.001$), approximately half the inhibition observed with histamine 2C (73%).**
Conclusion: **These new flow cytometric protocols confirmed that high dilutions of histamine may inhibit basophil activation and that the inhibitory effect is not restricted to IgE-dependent activation. The use of CD 203c instead of CD 63 increased the magnitude of the response.** *Homeopathy* (2005) **95,** 3–8.

Keywords: high dilutions; histamine; basophil activation; flow cytometry; CD 63; CD 203c

Introduction

Between 1981 and 1995, a large series of experiments examined the biological activity of highly diluted compounds, in which, theoretically, no molecule of the diluted compound should be present. Different biological models were investigated, but, in immuno-allergology,

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most experiments were based on direct, allergen-dependent basophil activation[1,2] or anti-IgE-induced activation modulated by successive dilutions of histamine.[3] Histamine is the major pre-formed mediator of anaphylactic reactions; it exerts a negative feedback of the activation response via the H2 histamine receptor.[4,5]

Most experiments were performed 'blind', including a multicentre trial.[6] But these experiments involve a time-consuming manual technique and are based on relatively low stained basophil counts (40–50). However, only small volumes of the leukocyte suspensions were required, and since stained basophils were stable for several days, this technique allowed the simultaneous testing of a large series of dilutions. These experiments led to the observation, mainly for centesimal dilutions (dilutions prepared with a dilution ratio of $\frac{1}{100}$), of two dilution ranges (10^{-4}–10^{-8} M and 10^{-30}–10^{-36} M) at which inhibition was found consistently. The specificity of the effect was confirmed by the reversibility of histamine-induced inhibition by an anti-H2 agent (cimetidine 10^{-5} M)[3] and the absence of activity of histidine, an inactive precursor of histamine, diluted in parallel and in the same conditions.[3]

These results were at the heart of a major controversy[7] after the publication claims concerning the biological activity of high dilutions of anti-IgE. Other groups were not able to reproduce these experiments, perhaps due to the necessity of long training for the method.[8,9]

During the last 10 years, flow cytometric analysis of human basophil activation has been carried out extensively. The first methods based on the flow cytometric analysis of the expression of a membrane activation marker were published in the early 1990s.[10] Basophils were selected on the basis of their high membrane density of bound IgE (by an anti-IgE antibody) and basophil activation was measured by CD 63 upregulation (CD stands for 'cluster differentiation').[11] CD 63 had the advantage that it is expressed at a very low density on resting basophils (less than 1%) but at a high density on activated basophils.

Validation of the IgE/CD 63 method demonstrated its extreme specificity and high sensitivity for allergy diagnosis[12–14] (usually more than 90% and reaching 100% for several allergens). We applied this first flow cytometric protocol to the study of the biological activity of high dilutions of histamine, leading to the confirmation of our previous results, although the degree of inhibition was lower (about 14% instead of 60–70%).[15]

Other markers have been described, notably, recently, CD 203c. It is basophil-specific and an earlier marker of the activation cascade than CD 63. We devised two other flow cytometric protocols using CD 203c IgE/CD 203c and CD 13/CD 14/CD 203c, the basophil being selected either by anti-IgE or anti-CD 13, and basophil activation being measured either by an anti-CD 63 or anti-CD 203c.

We present here the application of these protocols to the analysis of the biological activity of histamine in high dilutions, with basophil activation induced either by an IgE-dependent agonist (anti-IgE) or an IgE-independent agonist fMLP (formyl-methionyl-leucyl-phenylalanine peptide).

Material and methods

Preparation of histamine dilutions

Histamine dilutions were prepared in 20 ml polystyrene tubes with a dilution ratio of $\frac{1}{100}$ from a 0.1 M stock histamine hydrochloride solution to dilutions of 10^{-2} (1C)–10^{-36} M (18C). The first centesimal dilution (1C) was obtained by diluting the 0.1 M stock dilution 1:10. After each dilution step, tubes were vortexed (10 s, full speed). Histamine dilutions were kept at 4°C before use. All dilutions were made in deionized water. Controls were prepared according to the same procedure (ie 0.1 ml of deionized water was diluted in 0.9 ml of deionized water (1C) the other dilutions being prepared with a dilution ratio of $\frac{1}{100}$).

Preparation of leukocyte suspensions

Blood was taken on EDTA from at least five different donors in order to limit the individual variation of basophil releasibility. Leukocyte suspensions were obtained from buffy coats prepared by centrifugation of the whole blood (700*g*, 15 min). Cell pellets were resuspended in TRIZMA buffer (127 mM NaCl, 5 mM KCl, 20 mM TRIZMA, 5 IU/ml heparin; pH 7.4) and pooled. Leukocyte suspensions were also prepared from 'reconstituted' whole blood obtained by centrifugation (500*g*, 10 min). The plasma layer was discarded and replaced by the same volume of TRIZMA buffer and leukocyte suspensions were pooled. Macromolecules such as Ficoll were strictly avoided as they may lead to membrane fluidity modification and interfere with membrane receptors.

Pre-incubation with histamine dilutions

The tested histamine dilutions were isotonified by dilution $\frac{1}{2}$ in a 2X TRIZMA buffer just before the experiments. One volume of the leukocyte suspension (20 µl for sedimentation and buffy coat and 60 µl for reconstituted whole blood) was mixed with one volume of the tested isotonified histamine dilution and incubated for 30 min at room temperature or 10 min at 37°C together with agonist/histamine free controls and histamine free controls, all in triplicate.

Anti-IgE and fMLP-induced basophil activation

Anti-IgE (0.8 µg/ml ATAB, USA) (protocol 1) and fMLP (10^{-7} M) (protocols 2 and 3) (see below), diluted in TRIZMA-calcium buffer (127 mM NaCl, 5 mM KCl, 20 mM TRIZMA, $CaCl_2$ 5 mM, $MgCl_2$ 2 mM, 5 IU/ml heparin; pH 7.4) were added v/v, except for the

agonist free controls (TRIZMA buffer alone), and incubated v/v for 30 min at 37 °C. After addition of 50 µl of TRIZMA-EDTA buffer (127 mM NaCl, 5 mM KCl, 20 mM TRIZMA, EDTA Na_3 2.8 mM, 5 IU/ml heparin; pH 7.4), cells were labelled with 10 µl of the following antibody combinations diluted $\frac{1}{10}$ final in the sheath buffer (Isoflow, Beckman Coulter):

- Protocol 1: Anti-IgE FITC (Caltag, USA) and anti-CD 63 PE (Coulter-Immunotech, France).
- Protocol 2: Anti-IgE FITC (Caltag, USA)/anti-CD 203cPE (Coulter Immunotech, France).
- Protocol 3: Anti-CD 13 ECD, anti-CD 14 PC5, antiCD 203c PE (Coulter Immunotech, France).

FITC, PE, ECD and PC5 are fluorochromes (respectively, fluorescein isothiocyanate, phycoerythrin, phycoerythrin-texas red and pycoerythrin-cyanin 5.1) emitting at different wavelengths.

After incubation for 10 min at room temperature in the dark, erythrocytes were lysed by addition of 4 ml ammonium chloride buffer (NH_4Cl 154 mM, CO_3HK_2 10 mM, EDTA Na_3 0.08 mM, pH 7.2). After further incubation for 10 min at room temperature, 500 µl of sheath buffer (Isoflow, Beckman Coulter, USA) was added and leukocyte suspensions were centrifuged (400*g*, 5 min). Leukocytes were not fixed in order to avoid any morphological modification of the basophil population. Results were given by the cytometer in percentage of cells expressing a given marker in case of easy threshold setting between negative and positive cellular populations (CD 63), or in mean intensity of fluorescence (MFI) when the analysed marker was already present on resting cells, but upregulated by activation (as for CD 203c). The MFI is usually given by the cytometer in arbitrary fluorescence units according to a logarithmic scale and is expressed in decimal units ($10^{\log MFI}$). Results were expressed in ratio *vs* control.

We only included experiments fulfilling the following criteria: significant activation (>15% CD 63 expression or MFI ratio >5), inhibition induced by histamine 2C higher than 50% and number of counted basophils higher than 500.

Statistical analysis

Interassay statistical analysis was performed on the raw data by the distribution free Willoxon signed rank test using the XLStat software. For one experiment, the intraassay significance was calculated using the same test.

Results

Flow cytometric analysis with protocol 1

Thirty-six experiments were performed on different cell suspensions yielding 108 data sets related to histamine dilutions 15C and 16C, histamine free anti-IgE positive control and histamine and anti-IgE free negative controls. Histamine dilutions 15C and 16C (Figure 1) induce a significant inhibition of anti-IgE-induced CD 63 membrane expression (13.2%, $P<0.001$ for histamine 15C and 19.4%, $P<0.0001$ for 16C).

Comparison of the activity of histamine 16C for fMLP- and anti-IgE induced activation

The activity of histamine 16C was studied for anti-IgE- and fMLP-elicited basophil activation. A series of fMLP concentrations (10^{-7}–10^{-8} M) were compared to anti-IgE 0.8 µg/ml.

Histamine 16C inhibited fMLP-induced basophil activation, the inhibition being optimum for 10^{-7} M (12.6%, $P=0.02$). Under the same conditions, histamine 16C inhibited anti-IgE (0.8 µg/ml)-induced activation (15.2%, $P=0.02$).

Flow cytometric analysis of basophil activation with protocol 2

Gate setting

Basophils were selected (Figure 2) by gating cells situated within the lymphocyte population (gate A), positive cells (gate C) and CD 203c positive cells.[3]

Effect of histamine dilutions 2C and 16C on fMLP-induced basophil activation

In a series of eight successive experiments, histamine 2C and histamine 16C (Figure 3) inhibited fMLP-induced basophil activation, respectively, 63% ($P<0.001$) and 38% ($P=0.02$). Results were expressed in the ratio of linearized MFI *vs* the negative controls.

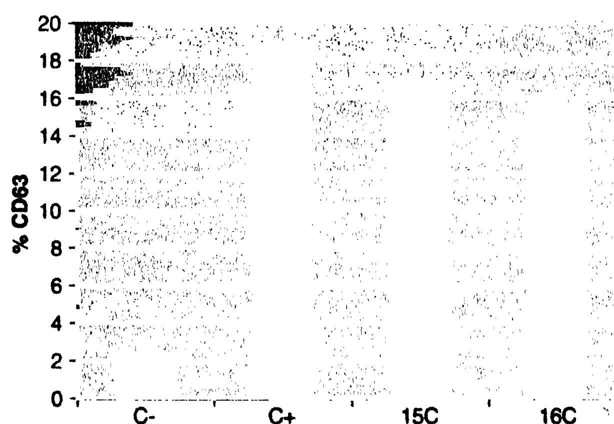

Figure 1. Anti-IgE induced CD 63 expression of anti-IgE+basophils in the presence of histamine dilutions 15C and 16C *vs* the histamine free positive control (C+) and the anti-IgE/histamine free control (C−). Basophil activation was analysed by protocol 1 and results were expressed in percentage activation (mean of 36 experiments). *$P<0.001$, ** $P<0.0001$.

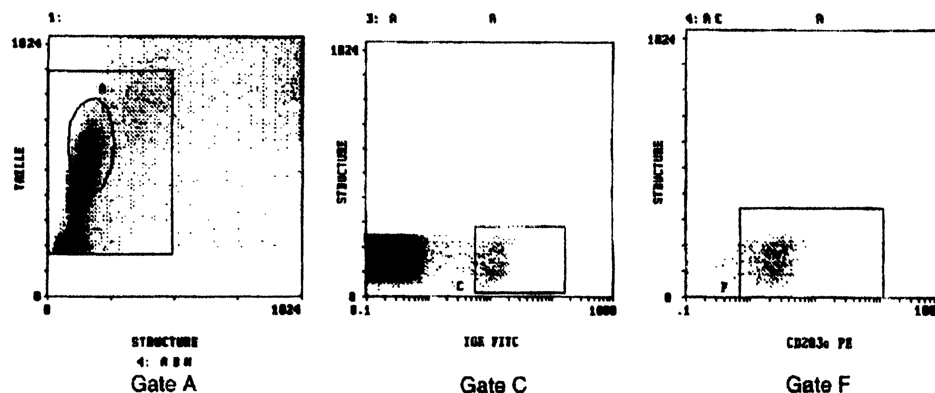

Figure 2. Gates setting of protocol 2: basophils were gated within the lymphocyte population (gate A) and selected by an anti-IgE FITC (gate C). Anti-IgE+/CD203+ were analysed (gate F).

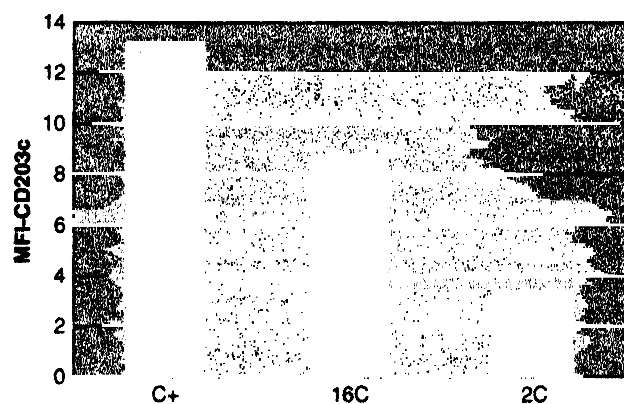

Figure 3. Inhibition of basophil activation by histamine dilutions 2C and 16C analysed by protocol 2 *vs* the fMLP histamine free control (C+) (mean of 8 experiments). $^*P = 0.02$, $^{**}P < 0.001$.

Flow cytometric analysis of basophil activation with protocol 3

Gate setting

Five dot plots recorded (Figure 4) gating cells situated within the lymphocyte region (gate A), selecting CD 13 positive cells (gate B), eliminating CD 14 positive cells (monocytes) (gate H), selecting the CD 203c positive cells (gate E) and calculating the MFI-CD 203c in an electronically purified CD 203c positive population (gate G).

Effect of histamine dilutions 2C and 16C on fMLP-induced basophil activation

Using protocol 3, the mean results obtained from five successive experiments showed that histamine 2C and histamine 16C inhibited fMLP-induced basophil activation, respectively (Figure 5) 76%, $P < 0.001$ and 39%, $P = 0.005$. Results were calculated by linearizing the log MFI-CD 203c values given by the cytometer and expressed in ratio *vs* the negative controls.

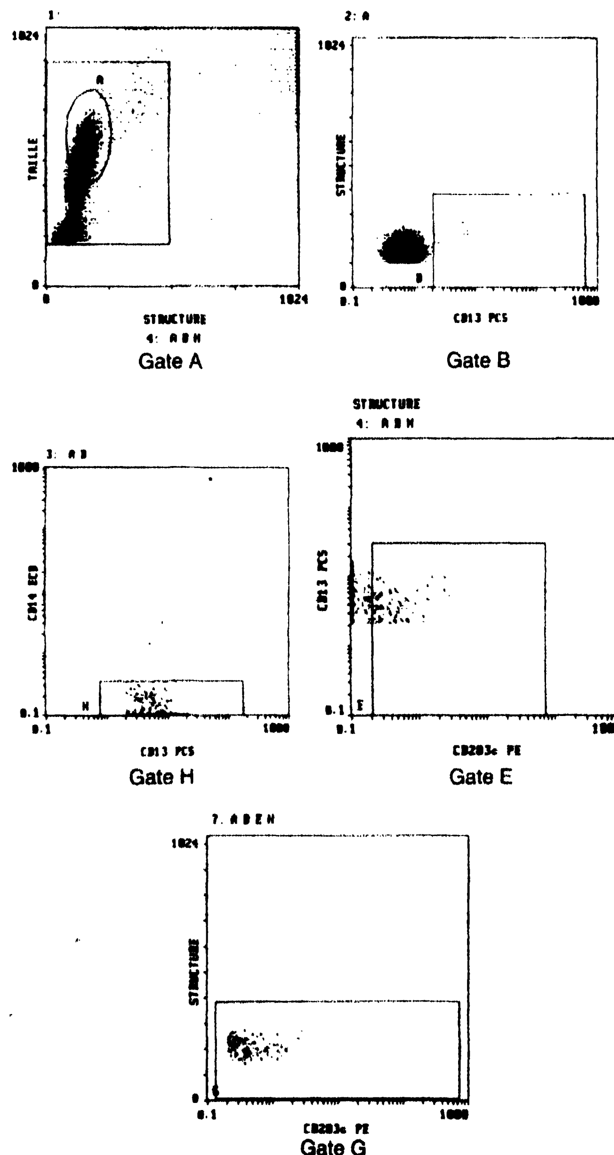

Figure 4. Flow cytometer's settings leading to lymphocytes region gating (gate A), CD 13+ cells selection (gate B), elimination of monocytes (gate H), selection of basophils (gate E) and analysis of the CD 203c+population (gate G). $^*P = 0.005$, $^{**}P < 0.001$.

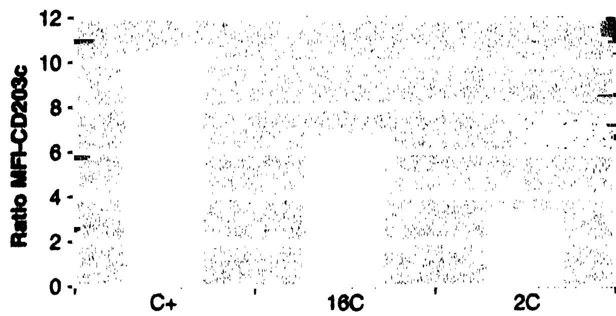

Figure 5. Inhibition of basophil activation by histamine dilutions 2C and 16C analysed by protocol 3 *vs* the fMLP histamine free control (C+) (mean of five experiments). $^{*}P=0.005$, $^{**}P<0.001$.

Confirmation of the inter-assay results obtained by the three colours protocol

Each test (controls, histamine 2C and histamine 16C) was repeated 10 times using the same leukocyte suspension. Compared to positive control, histamine 2C and histamine 16C induced a significant inhibition of fMLP-induced activation, respectively, 75%, $P<0.001$ and 34%, $P=0.01$.

Discussion and conclusion

Flow cytometric analysis of the effect of histamine 2C- and 16C-induced immunomodulation of basophil activation demonstrated biological activity in dilutions in which theoretically no molecule of histamine should be present, the inhibition magnitude depending on the marker used.

CD 63 (gp53 or lysosyme-associated membrane protein [LAMP)-3] is a member of the transmembrane-4 superfamily expressed by various cell types, including basophils, tissue mast cells, macrophages and platelets. Anti-CD 63 antibodies have recently been demonstrated to inhibit adhesion of mast cells to the extracellular matrix proteins fibronectin and vitronectin, and to inhibit IgE-dependent activation of adherent mast cells. CD 63 is a relatively late marker of the activation cascade preceding histamine release. CD 63 is an interesting activation marker for flow cytometry, as basophil activation induces a clear bimodal repartition of resting and activated basophils, leading to an easy separation of both populations and allowing the results to be expressed in percentages.

CD 203c (neural cell surface differentiation antigen E-NPP3) belongs to the ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPs) multigene family. It is a type II transmembrane metalloenzyme with broad substrate specificity. It catalyses the cleavage of phosphosulphate and phosphodiester bonds of numerous molecules including deoxynucleotides and nucleotide sugars. In peripheral blood, CD 203c is exclusively expressed on the surface of basophils. CD 203c is upregulated very quickly in response to specific allergen or anti-IgE stimulation. The kinetic of CD 203c expression is faster than CD 63. Upregulation and response to specific activators and inhibitors of CD 63 and CD 203c follow different kinetics and seem to be directed through alternative signal transduction pathways.[16-18] The CD 13 is a transmembrane glycoprotein expressed on polymorphonuclears and monocytes in normal blood. CD 14 is a glycoprotein linked to the membrane by a glycosyl-phosphatidylinositol. It is strongly expressed on monocytes/macrophages, but weakly on neutrophils.

The use of an anti-IgE for selecting basophils does not lead to pure basophil populations even if only the anti-IgE FITC bright cells are selected. The use of anti-CD 203c for protocol 2 showed that the anti-IgE FITC bright population contained an average of 90% of basophils: other contaminating cells were monocytes and dendritic cells. Moreover, and in contrast to a frequent criticism, there is no interference between the activating anti-IgE and the labelling anti-IgE, because the addition of EDTA before basophil labelling leads to an inhibition of the activation cascade. Therefore, protocol 1 led to the observation of a significant but relatively weak inhibition (19.4%).

One of the problems we encountered in standardizing the cytometric protocol was that anti-IgE induced basophil activation is only observed in 85–90% of the donors (anti-IgE responders) and, even using leukocyte suspension pools, a significant fraction of the experiments (5–10%) had to be discarded due to the absence of significant activation. In contrast, fMLP is a quasi-systematic basophil activation inducer, and we found that histamine 2C and histamine 16C similarly inhibited an IgE-dependant activation (anti-IgE) and IgE-independent activation (fMLP).

Therefore, we used fMLP instead of anti-IgE for protocols 2 and 3. Protocol 2 may be considered electronic purification by anti-CD 203c of the IgE positive population leading to the calculation of the MFI-CD 203c of a purified basophil population. This protocol showed significant inhibition of fMLP-induced activation with histamine 2C and histamine 16C. The magnitude of the inhibition induced by 2C was similar to that observed with the two other protocols (about 70%), but the magnitude of the inhibition induced by histamine 16C was higher with CD 203C (38%) than with CD 63 (14%).

For protocol 3, CD 13 was used as a selection antibody as it is present on all neutrophils and on monocytes/macrophages. Within the lymphocyte population, theoretically, the only contaminating cells are the monocytes/macrophages, and these were eliminated by anti-CD 14, since CD 14 is present on the membrane of these cells but not on neutrophils or basophils. The use of anti-CD 14 represented electronic purification of basophils. This protocol showed inhibition of a similar magnitude as observed with the anti-IgE/CD 63 protocol for histamine 2C, but was higher for histamine 16C (39%). These results were

confirmed by an intraassay statistical analysis. The magnitude of histamine 16C-induced inhibitions observed by two different protocols (2 and 3) using the same activation marker (CD 203c) but different selecting markers (IgE and CD 13) was similar (38% and 34%). The close relation observed between the observed effect and the nature of the activation marker argues in favour of its specificity.

The inhibition of basophil activation by histamine is IgE-receptor independent (fMLP-induced activation) and the involvement of histamine H2 receptors, in spite of the partial blocking of the phenomenon by cimetidine,[19] remains to be demonstrated.

The increasing magnitude of the inhibition observed for histamine 16C dilution using protocols 1, 2 and then 3 favour the use of an early marker of the activation cascade. The activity of high dilutions of histamine on basophil activation was recently confirmed by two independent groups,[20,21] and the biological activity of high dilutions has been repeatedly observed, in the same dilution ranges, with another *in vitro* model based on the inhibition of platelet aggregation by high dilutions of acetyl salicylic acid.[22]

These results, obtained using different protocols and different markers, may be considered as additional arguments in favour of the reality and the specificity of the biological effect of highly diluted compounds.

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