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ORIGINAL PAPER

Basophil models of homeopathy: a sceptical view

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This paper examines the activation and inhibition of activation of human basophils. After a brief description of human basophils, different methods to determine basophil activation are discussed with a special emphasis on the use of flow cytometric methods, as these circumvent the potential problems of assays based on the loss of colour by activated basophils. The activation of human basophils by ultra-high dilutions of anti-IgE is discussed. The majority of the paper describes the inhibition of basophil activation by ultra-high dilutions of histamine. The results from published papers are described and discussed.

After over 20 years research trying to find out if high dilutions of histamine have a negative feedback effect on the activation of basophils by anti-IgE, what do we know? The methods are poorly standardized between laboratories – although the same is true for conventional studies. Certainly there appears to be some evidence for an effect – albeit small in some cases – with the high dilutions in several different laboratories using the flow cytometric methodologies. After standardization of a number of parameters, it is recommended that a multi-centre trial be performed to hopefully put an end to this "never-ending story". *Homeopathy* (2010) **99**, 51–56.

Keywords: Homeopathy; *In vitro*; Basophils

Introduction

This could be an exceedingly short paper, since in my opinion, from a conventional scientific background, when there are no molecules of the active agent left in a solution; there can not be any biological effects. However, a search in PubMed combining homeopathy with basophil revealed 15 items. Interestingly this did not include the now infamous article in Nature or the papers that attempted to repeat the work.[1–3] Changing the search to homeopath* and basophil increased the total to 21. Including phrases such as 'high dilutions' or 'extremely low doses' only resulted in 33 publications. Witt and co-workers used several different databases in their review and found a total of 75 publications and further evaluated 67 of them.[4] One of their sources was the HomBRex database which specialises in basic research in homeopathy and as of February 2009 contained 1301 experiments in 997 original articles including 1172 biological studies.[5] Using the CAM (Complementary and Alternative Medicine) Database (http://cambase.dmz.uni-wh.de/opencam/start_en.html) and putting in basophil resulted in 95 hits. The question of publication bias is also worth considering – is it easier to publish a paper with negative results or with positive results? Normally, trials or studies with negative results are difficult to publish. However, it is possible that the opposite is true for studies using ultra-high dilutions.

The principle of homeopathy is that the patient is treated by substances which when given to healthy subjects produce effects that are similar to the symptoms seen in the sick patient. The remedies are deemed more effective when diluted with vigorous shaking (succussion). Histamine, *via* the H2 receptor, inhibits basophil activation when used at pharmacological concentrations.[6] This negative feedback mechanism on basophil activation is also observed when high dilutions of histamine are used.[7] In the *in vitro* studies with human basophils, although ultra-high dilutions are used, most of the effects are qualitatively similar to those obtained with pharmacological doses. To my mind, this opposes the fundamentals of homeopathy, where my

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understanding is that there are opposing effects caused by pharmacological doses and those caused by ultra-high dilutions. This may well be thought of as a naïve view by those more familiar with homeopathic principles. Readers are referred to the review by Bellavite and colleagues for a complete discussion on mechanisms.[8]

This paper will concentrate on some of the many studies using human basophils.

Basophils

Basophils, first described by Paul Ehrlich in 1879, are circulating white blood cells. In inflammatory processes, basophils are recruited from the blood and enter the tissues. They are important cells in allergy. On their surface they express the high affinity receptor for IgE FcεRI. Cross-linking of IgE bound to its receptors results in the release of histamine, newly synthesized lipid mediators, cytokines and other biologically active molecules. In laboratory studies, cross-linking is usually achieved by the means of anti-IgE although in studies investigating allergic responses the allergen is employed.

Basophil activation has been studied in a number of different ways: changes in basophil staining with alcian blue,[9] measurement of histamine release[10] and flow cytometric methods to determine changes in basophil surface markers after activation.[11,12] The alcian blue staining method as described by Gilbert and Ornstein in 1975 provided a rapid method for the counting of basophils.[9] Basophil activation is accompanied by a loss of the staining affinity for basic dyes.

In 1967, Lichtenstein *et al*. described the use of histamine release method for the detection of ragweed allergy.[10] Protocols for determining basophil histamine release have varied extensively; whole blood, white cell preparations and purified basophils have been used. Histamine has been measured using many different techniques e.g., radioenzymatic assays, HPLC (High Performance Liquid Chromatography), radioimmunoassays, ELISAs (Enzyme Linked Immunosorbent Assay), fluorescent assays (with or without purification of the sample, manual or automated, glass fibre based assays).[13]

In 1991, Bochner and Sterbinsky compared changes in surface expression of CD11b (Cluster of Differentiation 11b) and histamine release following basophil stimulation.[14] This initiated research into the use of basophil activation as determined by changes in surface markers. Since this type of change can be detected using flow cytometry, far fewer cells are needed than for histamine release studies. This is a major advantage when dealing with basophils which are present in low concentrations in the blood. Changes in the expression of CD63 have been widely used.[11,12] Upon basophil activation there is a rapid translocation of CD63 from the cytosol to the surface. There is compelling evidence that this occurs in parallel with histamine release.

However, as CD63 is not specific for basophils, they have to be identified usually by using labelled anti-IgE. This is a potential drawback of the method, as anti-IgE could itself activate the basophils. The technique therefore employs low concentrations of anti-IgE which do not cause basophil activation. Other studies have employed CD123 as a basophil marker together with HLA-DR (Human leukocyte antigen DR). In a comparative study using 2 commercially available kits for allergy diagnosis, there were fewer percentage positive basophils from grass pollen allergic patients using the CD123/HLA-DR/CD63 verses anti-IgE/CD63.[15]

In 1999, Bühring and colleagues reported that the surface marker CD203c was upregulated after basophil activation.[16] This marker is reported to be found only on mast cells, basophils and their progenitor cells. There are major differences in terms of the changes in surface expression of CD63 and CD203c; the former reaches a maximum response after about 20–30 min stimulation and the latter after 5–15 min.[17] In contrast to CD63, CD203c is constitutively expressed on the basophil cell surface and is also upregulated by IL-3 (interleukin 3) preincubation.[18]

Thus in conventional pharmacological experiments there are a number of ways to ascertain basophil activation using flow cytometry. However, to date the methodologies have not been completely defined. For example, as discussed by Brown and Ennis,[12] about 70% of studies using CD63 have preincubated the basophils with IL-3. There is a variation in the preparation of the samples with some studies using whole blood, others using leukocyte preparations and a few using purified basophils.

Although using whole blood results in a quick assay there can be problems due to the low number of basophils per sample, interference by serum components and thrombocytes also express CD63. It has been reported that the sensitivity of whole blood or leukocyte preparations in allergy tests depends on the allergen studied.[19] Even the incubation times are not standardized with CD63 periods between 10–45 min have been used and with CD203c 5–30 min.[12] In addition, basophil reactivity can vary markedly between donors and some donors (10–20%) do not respond to stimulation with anti-IgE.[20] There is debate as to whether the bell-shaped dose response curves seen in histamine release studies are also found when using flow cytometric methods with the CD63 or CD203c.[19,20]

Given these potential problems even when using conventional pharmacological concentrations, should we be surprised that there are variations in data obtained using high dilutions? Surprisingly, the published data are relatively consistent.

Initial studies – the Benveniste story

In 1988, Poitevin and colleagues published a paper in the British Journal of Clinical Pharmacology in 1988[21] which was a follow-up to an earlier paper which had reported that incubation of basophils with high dilutions of the homeopathic drug *Apis mellifica* was able to inhibit allergen-induced basophil degranulation.[22] In this paper, they reported that very low concentrations of anti-IgE (ca. 10–100 molecules per well) activated basophils and that this was inhibited by very high dilutions of the preparations

lung histamine and *Apis mellifica* (i.e., no active molecules likely to be present). Despite being published in a very well respected journal, this paper did not attract any attention.

In contrast, also in 1988, a large group lead by Benveniste published a paper in Nature on the activation of human basophils by ultra-high dilutions of anti-IgE.[1] This paper was welcomed by the homeopathic community as a landmark publication. Alas the joy was short-lived when a team of investigators, including a magician, visited the French laboratory and eventually published a report describing the work as ''pseudo-science''.[23]

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.[3] Their results did, however, contain a source of variation that they could not explain. Both the original paper and the subsequent paper resulted in a flood of letters and comments too numerous to be listed here.

A further group investigated the actions of very dilute anti-IgE dilutions in a work published in 1992.[2] They performed 24 blinded experiments compared a series of anti-IgE dilutions and found no effect on basophil degranulation regardless of whether the solutions had been strongly agitated or not.

In contrast, Benveniste and colleagues reported further studies on the activation of basophils by highly dilute anti-IgE and found that this was positive in 7 of 18 experiments (39%) which was significantly different from the highly dilute anti-IgG experiments (6%). Apis mellifica at ultra-high dilutions was able to inhibt anti-IgE induced activation in 10 of the 19 (53%) of the experiments.[24]

Inhibition of basophil activation by histamine – basophil staining studies

The majority of the studies performed with human basophils have concentrated on the inhibition of basophil activation by histamine. These studies are based on the early findings of Lichtenstein and Gillespie who demonstrated that micromolar histamine concentrations caused an almost 75% inhibition of basophil activation and the inhibition was reduced in the presence of 10^{-5} M buramide (an H_2 receptor antagonist) but not in the presence of 10^{-5} M diphenhydramine (an H_1 receptor antagonist).[6] It should be noted that it is possible to get basophil activation without histamine release with weak or low dose stimuli.[25]

This work was pioneered by Sainte-Laudy and colleagues beginning in the 80s and continuing to the present day. However, many of the initial publications are not easy to find, although they are reported in the review by Witt.[4] Overall, using the histamine degranulation assays, as standardized by Sainte-Laudy,[26] it was found that histamine at both conventional pharmacological concentrations and at high dilutions inhibited allergen and anti-IgE induced basophil activation.[7,27] Examining a range of dilutions from 5c to 59c, the response was periodic in form, with maxima at ca. 7c, 17c, 28c, 40c and 52c. The effect was not mimicked by using histidine and, similar to the work by Lichtenstein and Gillespie,[6] the H_2 receptor antagonist Cimeditine (10^{-5} M) reversed the inhibition caused by the high dilutions of histamine. The 17c dilution contains no histamine and is equivalent to a theoretical concentration of 2.5 10^{-35} mM.

I first heard about this work at the 1984 meeting of the European Histamine Research Society where Sainte-Laudy bravely presented his data to a crowd of extremely sceptical and rather hostile scientists and clinicians. Certainly, I together with others was carefully performing my calculations and realized that there were no molecules of histamine in the solutions for which he was claiming activity. Sainte-Laudy continued to present his data, which became even more difficult after the publication of the Benveniste Nature paper.[1]

Apart from the natural scientific objections to solutions containing essentially water having a biological effect, a number of other issues were raised: (1) the biological validity of the test; (2) the reproducibility of the phenomenon, (3) the subjectivity of cell counts and (4) that the data nearly all came from the same laboratory. In answer to these points, at that time, this form of examining basophil activation was a recognized procedure. Sainte-Laudy had performed repeated experiments, indeed in a series of 6 experiments he repeated each measurement 16 times and got the same answer.

As far as subjectivity is concerned, this is a valid objection. To understand this, I will describe the assay. Cell suspensions are preincubated with test dilution at room temperature for 30 min and are then incubated with anti-IgE for 30 min at 37°C. Alcian blue solution is added to each well. Stained basophils (not activated) are counted using a haemocytometer. Approximately 80 cells are counted for each well. Positive and negative controls are always included. Thus there is potential for subjectivity as it can be difficult to distinguish between stained and unstained basophils.

In order to answer points (3) and (4), it was decided to perform a multi-centre European Trial and it is at that point that I 'dipped my toes into the waters' of homeopathic research. As an ardent sceptic, I was invited to take part in the trial, which involved one coordinating laboratory and 4 laboratories performing the research. This study has been published.[28]

In brief, all the laboratories were trained in the basophil counting method, with the counts verified by Sainte-Laudy's laboratory. The dilutions were made in 3 different laboratories and coded by the coordinator (histamine and water solutions made up identically from 15c–19c). All study materials were from the same source and shipped to the performing laboratories. The data were returned to the coordinator and then analysed by an independent biostatistician. When the results for the histamine solutions were compared to those for the water solutions, there was a small but statistically significant inhibition of basophil degranulation caused by the lowest concentration of anti-IgE used in 3 of the 4 laboratories. When all the data were combined together, there was a statistically significant inhibition for the histamine containing solutions. Thus this multi-centre study indicated that high dilutions of histamine did indeed have biological effects.

Inhibition of basophil activation by histamine – flow cytometry studies

The advent of the flow cytometric methods to examine basophil activation has sparked even more interest in the area. Sainte-Laudy and colleagues published a number of papers in this area in the mid 90s and have continued to publish their work.[29–36] In 2001, Sainte-Laudy published a short communication which demonstrated a potentiation of anti-IgE induced basophil activation that was significant at a dilution of 13c and although also present at 14c did not achieve statistical significance. This potentiation was not caused by histidine dilutions and was inhibited by pharmacological concentrations of the H2 receptor antagonist cimetidine.[37] This curious and unexpected effect compared to those previously published by Sainte-Laudy is known in homeopathic literature as hormesis and is discussed in the review by Bellavite and colleagues.[8]

In the multi-centre trial described above, 3 of the laboratories independently examined the effects of high dilutions of histamine and to a varying degree all demonstrated inhibition of basophil activation with these dilutions.[28] Flow cytometry is employed in most immunological laboratories and there have now been a series of independent laboratories investigating the phenomenon. These will be discussed in detail.

In a short paper published in 2001, Brown and Ennis used the two colour method (anti-IgE FITC (Fluorescein Isothiocyanate) and anti-CD63 R-PE) to examine the effect of high dilutions of histamine on basophil activation.[37] Statistically significant inhibition was seen at pharmacological concentrations (10^{-2}, 10^{-4}, 10^{-6} M) and at high dilutions (10^{-14}, 10^{-18}, 10^{-20} and 10^{-26} M). Heating the solutions to 70°C for 30 min significantly reduced the inhibition caused by the 3 solutions tested (10^{-2}, 10^{-30}, and 10^{-36} M). In contrast, there was little or no effect on the inhibition following freezing the solutions at −70°C then thawing to room temperature (performed twice). This study did not use blinded samples which is a disadvantage.

Lorenz and co-workers developed a method to examine a large number of basophils using a 3 colour flow cytometric system: they selected the CD2, CD14, CD16, CD19 and HLA-DR negative cells and examined CD63 expression in CD123 (IL-3 receptor) positive cells.[38] They assessed a very large number of basophils (25,000–30,000) per batch and recommended that at least 10,000 cells be examined to reduce measurement errors. The cells were obtained from buffy coat donors.

Inhibition was clearly observed between 10^{-2} and 10^{-11} M histamine but also at 10^{-22}–10^{-25} M histamine. Interestingly, they found that the coefficient of variation was smaller when polypropylene tubes were used instead of polystyrene ones. In their second paper, Lorenz and co-workers examined the effect of the diluent for the histamine containing solutions, comparing the more traditional brandy type diluent with an ethanol based diluent.[39]

Certainly, I would prefer not to employ ethanol as a solvent if it was not necessary and their first paper had used water as the diluent. The rationale for this work was that ethanol water dilutions are used commonly in homeopathic preparations and that Hahnemann had recommended the use of brandy.

The study was performed with blinded samples but only using buffy coats from 4 individuals. A mixture of responses was obtained indicating no effect, inhibition and even potentiation of the responses to anti-IgE by histamine. However, they were unable to use the same cells for all tested dilutions, thus in one experiment cells from one subject were used for D3–D16 and another for D17–D30 (nb this was performed in duplicate therefore 2 donors each were studied). In bother papers, the authors use decimal (D) potencies, instead of centesimal (C) potencies as in all other publications. Overall, using brandy as the diluent produced more inhibition than ethanol water. The data presented in this study are equivocal and may be due to the odd choice of diluent.

The approach taken by Guggisberg and co-workers was different; they used the cells from the same individual for main 7 replicate experiments reported in their paper[40] and the two colour method as described by Sainte-Laudy and used by Brown and Ennis[29,37] All samples were coded and tested in triplicate. The solutions were assigned to random positions in the plates using a computer programme and the outer wells were not used. There was considerable variability in the basophil activation with anti-IgE in the 7 repeated experiments: it ranged from 19.7% to 45.7%. Similarly, the variation in the inhibition even at pharmacological concentrations was very large: 10^{-2} M histamine causing between 7.8% and 54.4% inhibition.

Given this large variation, it is hardly surprising that the high dilutions also caused a variety of effects. Their data were analyzed by 3 different methods and in one of them a small but statistically significant inhibition of 5.7% was observed at 10^{-22} M histamine. Although, the method avoided problems due to inter-individual variations; certainly their repeated experiments differed in terms of anti-IgE induced activation and inhibition by pharmacological amounts of histamine. It is possible that the individual was not particularly responsive to histamine.

The paper included a good summary of previously published data using flow cytometric methods to determine the inhibitory effects of histamine (see Table 3 in their publication). It clearly demonstrates that the histamine dilutions causing significant inhibition vary between the reported studies. This may be due to the susceptibility of basophils from different donors to histamine. Factors such as the type of water used for the dilutions may also play a role. However, given that 3 of the studies came from the same laboratory this is unlikely. This is an area that must be further investigated.

A recent report has compared detection of basophil activation using both CD203c and CD63.[41] For each experiment the blood of 4 non-atopic subjects was mixed, with a different series of donors used for each experiment and each condition studied in triplicate. Using the CD203c activation, histamine at 2c (10^{-4} mol/l), 12c–16c (10^{-24} mol/l–10^{-32} mol/l) significantly inhibited the anti-IgE induced activation. In

contrast, only the 2c concentration inhibited the response as measured by CD63, although it should be noted that the positive control (anti-IgE alone) was very low ($8.22 \pm 9.53\%$).

Concluding remarks

After over 20 years research trying to find out if high dilutions of histamine have a negative feedback effect on the activation of basophils by anti-IgE, what do we know? The methods are poorly standardized between laboratories – although the same is true for conventional studies as described above. Certainly there appears to be some evidence for an effect – albeit small in some cases – with the high dilutions in several different laboratories using the flow cytometric methodologies.[29,37,38,41] How much of the effect is due to artifacts remains to be investigated. Some authors have employed anti-IgE dilutions which caused little basophil activation, others used greater concentrations.

Measuring basophil activation using CD63 or CD203c examines different parts of the activation pathways,[12] the recent paper by Chirumbolo and colleagues would suggest that employing the CD203c markers could prove useful.[41] Sainte-Laudy and Belon examined 4 different protocols for basophil activation.[33] Depending on the protocol used, the inhibition observed with 16c histamine (10^{-32} M) varied between non-significant (12.4%) to 63% (19.4%, 39%, 63%). The greatest inhibition was observed using the CD203c protocol with basophils labelled with anti-IgE.

Where next? To try and solve the question of whether highly diluted solutions of histamine exert a negative effect on basophil activation, a multi-centre trial is required. Prior to the start of the trial a number of issues need to be clarified including:

(1) Use of one cell donor or many.
(2) Use of multiple donors for experiments or repeat using just one donor.
(3) Methods to prepare histamine solutions.
(4) Use a number of anti-IgE dilutions or just one and whether anti-IgE is the best agonist to use.
(5) The type of water used as the diluent.
(6) Method to detect activation.
(7) Systematic negative control experiments should be performed to assess the stability of the experimental system.

Obviously, all sites should use the same batches of reagents such as anti-IgE; all solutions should be prepared externally by an independent laboratory and encoded so that studies are performed in a blinded and randomized fashion; all results should be sent to an independent statistician to analyze. This set of approaches may help to solve what seems to be a never-ending story.

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