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Replication study concerning the effects of homeopathic dilutions of histamine on human basophil degranulation in vitro☆

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Summary
Background: Various investigators have observed significant effects of highly diluted histamine on human basophil degranulation in vitro, compared to corresponding water controls. However, active and inactive dilution levels differed in most studies.
Objective: We aimed to reproduce former studies with flow-cytometry using rigorously controlled experimental conditions to minimise confounding factors.
Methods: In seven independent experiments, basophils of the same human donor were incubated with diluted histamine (up to 10^{-34} M) or water controls and activated with anti-IgE antibodies. Basophil activation was determined by using bicolour flow-cytometry. Experiments were blinded and performed with a randomised arrangement of the solutions on microtiter-plates.
Results: Histamine at the dilutions 10^{-2} M and 10^{-22} M was associated with a significant inhibition of basophil degranulation ($p = 0.018$, Wilcoxon signed rank test) of 23.1% and 5.7%, respectively, if compared to ''diluted'' water treated in an identical manner. However, if all controls were pooled, only histamine 10^{-2} M had a significant effect. Significant effects were seen for row numbers of the microtiter plates.
Conclusion: We were not able to confirm the previously reported large effects of homeopathic histamine dilutions on basophil function of the examined donor. Seemingly, minor variables of the experimental set up can lead to significant differences of the results if not properly controlled.

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Introduction

Human basophils are a rare granulocyte type accounting for 0.1—1% of peripheral blood leukocytes. This cell type has been extensively used to study the effects of highly diluted substances in vitro as applied in homeopathy and anthroposophical medicine. All studies are based on the fact that human basophils can be activated by anti-human-IgE antibodies.[1] This leads to a degranulation process consisting of a fusion of cytoplasmatic granules with the plasma membrane and the release of inflammatory mediators, such as histamine.

Different procedures can be used for measuring basophil activation: (1) By means of staining and microscopical counting of basophils, degranulated (i.e. activated) cells can directly be visualised and be put in relation to brightly stained (i.e. inactive) cells.[2] However, even if this method is used in a blinded manner, it may be prone to subjective influences. (2) Histamine release of activated basophils can be determined using fluorimetric assays of the histamine content in the surrounding media compared to histamine content of the cells.[3] (3) Newer flow-cytometric methods allow the measurement of basophil degranulation using larger cell numbers.[4,5] The fusion of cytoplasmatic granules of activated basophils with the plasma membrane leads to the expression of the marker CD63 on the cellular surface. The percentage of CD63 expressing basophils can be determined with flow-cytometry and correlates linearly with the histamine release of basophils.[4]

Table 1 gives an overview of published studies on this subject and the methodologies used.

In 1988, a research group around J. Benveniste published their observations of a significant basophil degranulation induced by anti-IgE at dilutions up to a level of 10^{-120} as assessed by microscopical counting.[6] This publication provoked an intensive correspondence. A team led by the publishing chief editor demonstrated that the variability of the positive results was smaller than the statistically expected error of microscopical cell count and found no effect of higher dilutions, when the experiments were blinded.[7] Whereas the initial finding were later replicated by Benveniste's group,[8] independent groups[9,10] concluded to be unable to reproduce these positive results.

Histamine, one of the inflammatory mediators released by basophils, can bind to H2 receptors on the surface of basophil granulocytes, when present in high concentrations ($>10^{-6}$ M). Thus, it regulates the basophil degranulation by exerting a feedback inhibition.[11,12] This finding led others to examine the effect of very high dilutions of histamine on basophils stimulated by anti-IgE.[13—22] All of the three methodologies described above were used to assess the extent of basophil activation. As can be seen in Table 1, blinding of the experiments was only systematically performed in studies using visual determination of basophil activation. In the initial flow-cytometric studies of the effects of high dilutions[15,16,18,19], anti-IgE antibodies were used to gate basophils. This method has the disadvantage that the labelling antibodies have a possibly confounding activating effect on basophils.[21] This problem has recently been overcome by a new flow-cytometric technique which identifies basophils by gating CD123 positive and CD2, CD14, CD16, CD19, HLA-DR negative cells.[20,21]

The aim of our study was to perform an independent replication of former studies, which used flow-cytometry for determining basophil activity.[15,19] We applied a rigorous protocol to minimize possible confounding factors. In particular, all experiments were performed with a blinded and randomised arrangement of dilutions and controls.

Methods

Material

Material and experimental procedures were adapted from Sainte-Laudy and Belon[15] and Brown and Ennis[19] in order to be able to compare the results. HEPES—EDTA buffer (1×), contained 127 mM NaCl (7.42 g/l), 5 mM KCl (0.37 g/l), 20 mM HEPES (Sigma, 4.76 g/l), 5 mM EDTA (Sigma, 1.46 g/l), 5000 IU/l Heparin (Liquemin®, Roche) adjusted to pH 7.4 with NaOH. HEPES—calcium Buffer, pH 7.4, contained 127 mM NaCl, 5 mM KCl, 20 mM HEPES, 20 mM $CaCl_2 \cdot 2H_2O$ (2.94 g/l), 5 mM $MgCl_2 \cdot 2H_2O$ (1.02 g/l). Histamine dihydrochloride and formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma and dextran was obtained from Pharmacia Biotech. Rabbit-anti-human IgE used to activate basophils was from DAKO. FITC-anti-IgE and PE-anti-CD63 used for flow-cytometry were from Caltag. FITC-anti-IgG and PE-IgG1 (Caltag) served as staining controls.

Preparation of histamine and sham dilutions

A 0.1-M solution was prepared by adding 184 mg histamine to 10 ml distilled water. The 10^{-2} M solution was obtained by 10-fold dilution of histamine 0.1 M with distilled water, followed by 10 s of vortexing at maximal speed. Concentrations 10^{-4} M to

Table 1 Overview of published studies investigating the effects of homeopathic solutions on human basophils and the methodologies used.

Study	Method	Pre-treatment	Homeopathic dilution	Measured parameter	Blinding reported
Davenas et al.[6]	Visual counting	—	Anti-IgE	% Degranulated basophils	Some of the experiments
Maddox et al.[7]	Visual counting	—	Anti-IgE	% Degranulated basophils	Yes
Poitevin et al.[13]	Visual counting	—	Lung histamine, *Apis mellifica*	% Degranulated basophils	Yes
Benveniste et al.[8]	Visual counting	—	Anti-IgE, *Apis mellifica*	% Degranulated basophils	Yes
Ovelgonne et al.[9]	Visual counting	—	Anti-IgE	% Degranulated basophils	Yes
Hirst et al.[10]	Visual counting	—	Anti-IgE	% Degranulated basophils	Yes
Sainte-Laudy and Belon[14]	Visual counting	—	Histamine	% Inhibition of basophil degranulation	Yes
Sainte-Laudy and Belon[15]	Flowcytometry (anti-IgE+/CD63)	—	Histamine	% Inhibition of basophil CD63 expression	No
Sainte-Laudy and Belon[16]	Flowcytometry (anti-IgE+/CD63)	Incubation with cimetidine	Histamine	Basophil CD63 expression	No
Belon et al.[17]	Visual counting	—	Histamine	% Inhibition of basophil degranulation	Yes
Sainte-Laudy[18]	Flowcytometry (anti-IgE+/CD63)	Incubation with cimetidine	Histamine, Histidine	% Activation of basophil degranulation	No
Brown and Ennis[19]	Flowcytometry (anti-IgE+/CD63)	—	Histamine	% Inhibition of basophil CD63 expression	No
Lorenz et al.[20]	Flowcytometry (CD123+, CD2−, CD14−, CD16−, CD19−, HLA-DR−/CD63)	Ficoll isolation, Comparison of two dilution media	Histamine	% Inhibition of basophil CD63 expression	Yes
Lorenz et al.[21]	Flowcytometry (CD123+, CD2−, CD14−, CD16−, CD19−, HLA-DR−/CD63)	Ficoll isolation	Histamine	Test stability, basophil CD63 expression	Yes
Belon et al.[22]	Visual counting	—	Histamine	% Degranulated basophils	Yes
	Flowcytometry (anti-IgE+/CD63)	Incubation with cimetidine and ranitidine	Histamine	Basophil CD63 expression, % inhibition of basophil CD63 expression	No
	Fluorimetrical measurement of histamine in supernatant and in cells	—	Histamine	Histamine release	No

10^{-34} M were prepared by serial 100-fold dilution with distilled water, followed by vortexing for 10 s at maximum speed. New pipette tips and tubes were used for each dilution step. Sham solutions (''diluted water'') were analogously prepared by serial dilution and vortexing of distilled water. Histamine and sham concentrations 10^{-2} M, 10^{-18} M, 10^{-20} M, 10^{-22} M, 10^{-26} M, 10^{-30} M, 10^{-32} M and 10^{-34} M were made isotonic by adding 1 part in 10 HEPES—EDTA buffer in a 10-fold concentration. Solutions were stored at 4 °C for 1—6 days.

Preparation of leucocytes

Thirty millilitres of fresh blood anticoagulated with EDTA from healthy non-allergic volunteers (after informed consent according to the declaration of Helsinki) was mixed (in the main experiments) with 7.5 ml dextran 6%. After sedimentation of erythrocytes for 90 min at room temperature, the leukocyte-rich plasma was collected and washed with HEPES—EDTA buffer and adjusted to a concentration of 70—100 million cells per millilitres.

Incubation protocol

Twenty millilitres of leukocyte suspension was aliquoted in the wells of a 96-microtiter plate, mixed with 20 μl of buffer control, histamine or sham solutions and incubated during 30 min at room temperature. Then, 20 μl anti-IgE (final concentration 0.2 μg/ml) in HEPES—calcium was added to the cells followed by incubation at 37 °C for 30 min. EDTA-buffer (''untreated water''), calcium buffer and fMLP (final concentration 8.3×10^{-6} M) served as negative and positive controls. The reaction was stopped by adding cooled HEPES—EDTA buffer. After centrifugation ($200 \times g$ and 4 °C, 10 min), the cells were labelled in a volume of 50 μl with 1 μg FITC-anti-IgE and 1 μg PE-anti-CD63 or control antibodies. After 20 min at 4 °C, the cells were washed with HEPES—EDTA buffer and analysed by flow-cytometry.

Flow-cytometry

Flow-cytometry was performed using Becton Dickinson FACSCalibur 4FL. Lymphocytes and basophils were gated according to their distribution in the FSC/SSC dot plot. From this, basophils were gated by their bright anti-IgE FITC fluorescence. Two hundred to five hundred basophils were counted, depending on the cell concentration available. The percentage of active basophils was calculated by setting an electronic gate between CD63+ and CD63− basophils corresponding to 1—2% positive cells stained with the PE-isotype matched control antibody.

Pilot experiments

During pilot experiments, the influence of the methodological set-up on basophil activation was assessed. No blinding was performed for pilot experiments. A possible difference of basophil responses when using either tubes, culture plates or microtiter-plates was investigated by comparing the results obtained with otherwise equally treated basophils of one donor. The influence of dextran sedimentation was studied in three subjects. The optimal amount of activating anti-IgE was tested by establishing a dose—effect curve for five subjects.

Principal experiments

For the principal experiments, precautions were taken in order to minimise effects due to factors other than homeopathic dilutions of histamine. Seven independent experiments were performed with the blood of one single donor to avoid inter-individual differences. This donor was chosen on account of the sufficiently high proportion of basophils in his leucocyte suspensions (mean $1.16 \pm 0.6\%$) and because his basophils showed the desired moderate response to activation with anti-IgE antibodies (mean activation $30.16 \pm 10.94\%$). The response to low histamine concentrations was not previously tested and was not a criteria for the selection of this donor. Histamine dilutions were compared with sham solutions (see above). Histamine and sham solutions were blinded with a letter code. All coded solutions were performed in triplicates instead of duplicates used in most studies. Experiments were done on 96-well plates with a computer-randomised arrangement for the position of each experimental condition, including the replicates, and this random arrangement was newly generated for each experiment. The outer wells of the plate were not used in order to avoid possible border effects. Minimisation of differences in delays of incubation was achieved by transferring the coded solutions and activators from mirror plates to the experimental plate using multi-pipettes, and by limiting the number of concentrations tested. The concentrations 10^{-2} M, 10^{-18} M, 10^{-20} M, 10^{-22} M, 10^{-26} M, 10^{-30} M, 10^{-32} M and 10^{-34} M were selected for our study, since they had shown the most pronounced effects in previous investigations (see Tables 1 and 3).

Table 2 Mean activation as determined by % CD63 positive basophils is shown for all seven independent experiments and every experimental condition (+: histamine and H_2O were made isotonic by adding EDTA buffer).

Pre-incubation	Activation	Experiment number							Mean	S.D.
		1	2	3	4	5	6	7		
Controls										
Buffer	Buffer	3.91	0.96	0.55	1.45	1.62	2.96	4.57	2.29	1.54
Buffer	aIgE	45.65	30.95	20.49	19.66	24.55	24.95	44.84	30.16	10.94
Buffer	fMLP	50.8	43.89	36.94	40.44	40	57.82	68.29	48.31	11.37
10^{-2} M										
Histamine+	aIgE	42.05	21.62	13.21	14.84	22.84	28.08	31.84	24.93	10.04
H_2O+	aIgE	48.7	27.09	28.97	17.76	24.77	37.35	41.96	32.37	10.76
Difference		*−13.66*	*−20.19*	*−54.40*	*−16.44*	*−7.79*	*−24.82*	*−24.12*	*−23.06*	*15.06*
10^{-18} M										
Histamine+	aIgE	47.04	28.82	28.92	17.32	23.69	27.32	38.73	30.26	9.80
H_2O+	aIgE	50.99	30.9	28.27	15.48	26.53	27.3	36.95	30.92	10.93
Difference		*−7.75*	*−6.73*	*2.30*	*11.89*	*−10.70*	*0.07*	*4.82*	*−0.87*	*8.00*
10^{-20} M										
Histamine+	aIgE	49.4	30.67	24.33	20.87	22.55	32.87	43.76	32.06	10.92
H_2O+	aIgE	45.85	27.52	24.27	22	24.84	34.74	39.83	31.29	9.02
Difference		*7.74*	*11.45*	*0.25*	*−5.14*	*−9.22*	*−5.38*	*9.87*	*1.37*	*8.32*
10^{-22} M										
Histamine+	aIgE	46.9	31.2	21.03	18.32	24.59	32.08	37.84	30.28	9.99
H_2O+	aIgE	50.63	31.27	22.5	19.3	28.16	33.46	39.25	32.08	10.56
Difference		*−7.37*	*−0.22*	*−6.53*	*−5.08*	*−12.68*	*−4.12*	*−3.59*	*−5.66*	*3.86*
10^{-26} M										
Histamine+	aIgE	53.54	28.92	29.18	20.24	23.93	39.55	42.66	34.00	11.74
H_2O+	aIgE	43.4	35.29	27.16	19.69	25.8	29.33	40.7	31.62	8.52
Difference		*23.36*	*−18.05*	*7.44*	*2.79*	*−7.25*	*34.84*	*4.82*	*6.85*	*17.78*
10^{-30} M										
Histamine+	aIgE	49.26	29.77	23.72	22.15	22.08	34.29	37.58	31.26	10.00
H_2O+	aIgE	53.36	33.77	22.79	16.61	24.26	34.33	38.12	31.89	12.14
Difference		*−7.68*	*−11.84*	*4.08*	*33.35*	*−8.99*	*−0.12*	*−1.42*	*1.06*	*15.29*
10^{-32} M										
Histamine+	aIgE	46.94	31.82	27.86	17.97	24.91	38.46	41.05	32.72	10.06
H_2O+	aIgE	47.53	31.69	24.28	18.37	26.67	31.63	42.17	31.76	10.15
Difference		*−1.24*	*0.41*	*14.74*	*−2.18*	*−6.60*	*21.59*	*−2.66*	*3.44*	*10.47*
10^{-34} M										
Histamine+	aIgE	51.75	30.94	26.38	17.62	21.15	32.38	43.94	32.02	12.18
H_2O+	aIgE	48.83	29.18	27.5	16.41	17.53	36.34	40.36	30.88	11.86
Difference		*5.98*	*6.03*	*−4.07*	*7.37*	*20.65*	*−10.90*	*8.87*	*4.85*	*10.02*

The difference between histamine and water dilution was calculated by the formula $(W_x - H_x)/W_x$, where W was the percent activitation of basophils pre-incubated with distilled water; H, percent activation of basophils pre-incubated with histamine and x the corresponding dilution step.

Table 3 Comparison of the results of the five previous studies using flow-cytometry to assess the effect of homeopathic histamine on human basophils and our investigation.

Histamine concentration (M)	Sainte-Laudy and Belon[15]	Sainte-Laudy and Belon[16]	Sainte-Laudy[18]	Brown and Ennis[19]	Belon et al.[22]	Actual study
10^{-2}	35**			34**	32*	23*
10^{-4}	26*			27**	28*	
10^{-6}	2			17*	21	
10^{-8}	4			7	13	
10^{-10}	4			13	13	
10^{-12}	3			15	19	
10^{-14}	−3			22*	19	
10^{-16}	6			15	16	
10^{-18}	4			18*	17	1
10^{-20}	6	n.s.		43***	30*	−1
10^{-22}	28*	n.s.		22	18	6*
10^{-24}	13	n.s.		14	18	
10^{-26}	13	n.s.	−11*	28**	17	−7
10^{-28}	2	n.s.	1	−8	−3	
10^{-30}	2	17*		26	14	−1
10^{-32}	2	21**		8	8	−3
10^{-34}	21*	10*		17	10	−5
10^{-36}	2	n.s.		−12	3	
10^{-38}	14	n.s.		16	9	
10^{-40}	12	n.s.		8	13	

Two further studies are not tabulated since they contain preliminary results only.[20,21] Mean inhibition of basophil CD63 expression by centesimal dilutions of histamine is indicated in percent compared to the corresponding water control (n.s.: not significant, no mean values reported). Data were estimated from graphs if not explicitly quoted.

* $p < 0.05$

** $p < 0.01$.

*** $p < 0.001$.

Analysis of the data

The main hypothesis (''there is no difference between the CD63-expression of basophils incubated with histamine and sham solutions, i.e. diluted water'') was statistically assessed for each pair of dilutions using the non-parametric Wilcoxon signed rank test (independent planned comparisons, therefore, without Bonferroni correction[23]).

In an additional analysis a posteriori, degranulation of basophils incubated with histamine was compared to the data of the EDTA-buffer control (''untreated water'') of the corresponding experiment and to the mean value of all sham solutions of the corresponding experiment. In this case, a Bonferroni correction was applied to the non-parametric Wilcoxon signed rank test since all homeopathic dilutions were compared simultaneously to one control.

Analysis of variance (*F*-test) was used for the statistical test of plate-effects: data were analysed for border-, row-, and column-effects. Columns were labelled with the letters B to G on 96-well plates and handled simultaneously with multi-pipettes, whereas rows were labelled with the numbers 2−11 and handled subsequently. Environmental effects (influence of temperature and age of the dilutions on basophil degranulation) were assessed by an exploratory data analysis (*F*- and *t*-tests).

Results

Pilot experiments

Influence of methodological parameters on basophil degranulation

The usage of polystyrene tubes, cell culture plates or conventional microtiter plates did not significantly influence basophil responses (results not shown).

No significant difference was found between degranulation of anti-IgE activated basophils obtained with or without dextran sedimentation. In an exploratory investigation, no major influence of dextran on the effect of histamine was observed. However, significantly more basophils could be obtained when dextran was used (mean increase of 289%). Therefore, in the principal experiments, erythrocyte-sedimentation was accelerated with

dextran. Dose—response curves of anti-IgE revealed that a concentration 0.2 μg/ml, which was also used in previous work of other groups,[15,19] induced a near maximal effect. Therefore, this concentration was used for our experiments.

Principal experiments

Effect of histamine dilutions on CD63 expression in basophils activated with anti-IgE

As shown in Table 2, CD63-expression was increased by anti-IgE (mean 30.16 ± 10.94%) and fMLP (mean 48.31 ± 11.37%) as compared to the buffer control (mean 2.29 ± 1.54%).

The effect of pre-incubating the basophils with different dilutions of histamine or water treated in an identical manner is shown in Fig. 1 and was analyzed in three different manners. The black bars show the effect of pre-incubation with histamine at the indicated concentration when compared to the corresponding concentration of ''diluted water''. A pharmacological concentration of histamine (10^{-2} M) inhibited basophil degranulation by 23% ($p = 0.018$, Wilcoxon signed rank test), in agreement with former studies. A rather weak, but statistically significant, inhibition of 5.7% was found for the homeopathic concentration of 10^{-22} M ($p = 0.018$, Wilcoxon signed rank test). None of the other homeopathically diluted histamine concentrations showed a significant effect.

A posteriori, the same data set was also analysed by comparing the effect of histamine to the corresponding ''untreated water'' control. In this case, none of the homeopathic histamine dilutions showed a statistically significant effect (Fig. 1, shaded bars). In a third analysis, all ''diluted water'' controls of one experiment were averaged and compared to the effect of the different histamine dilutions. Again, in contrast to the pharmacological histamine concentration, highly diluted solutions had no significant effect (Fig. 1, open bars).

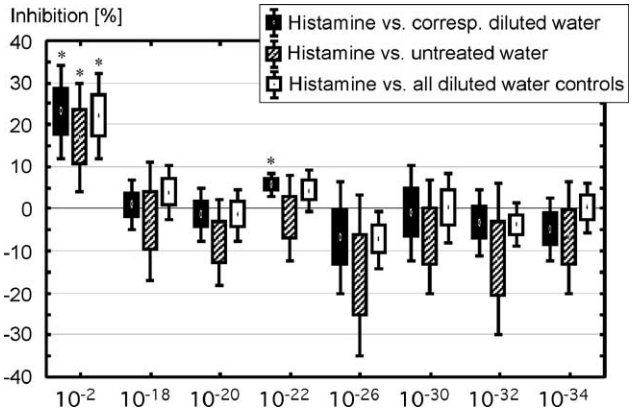

Figure 1 Inhibition of basophil degranulation (expressed as percentage of the corresponding control) observed after incubation of basophils with dilutions of histamine (mean ± standard error ± double standard error, $^{*}p < 0.05$). Black bars: histamine vs. corresponding diluted water control; shaded bars: histamine vs. untreated water control; open bars: histamine vs. the pool of all treated water controls.

Microtiter plate position effects

Microtiter-plate effects are a quite common phenomenon. We, therefore, analysed the raw data of the main experiments for column, row and border effects. Data of controls and pharmacological concentrations of histamine were excluded from this analysis.

Overall analysis of variance reveals no significant column ($p > 0.05$) or border ($p > 0.4$) effect, but a significant row effect ($p < 0.0001$). Overall, CD63-expression decreased for higher row numbers with an increment of −0.6% per row number. The row number effect additionally shows a significant interaction with the number of the experiment ($p < 0.0001$). Linear regression between basophil activation and row number yields a statistically significant correlation for four single experiments: a decrease for three experiments ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively) and an increase for one experiment ($p < 0.002$).

As expected, randomisation effectively eliminated these microtiter-plate effects. A comparison of histamine and water dilutions did not yield any significant difference ($p > 0.05$) for the row number, neither for all dilution levels pooled nor separately.

Inter-experimental variations

A closer inspection of Fig. 1 reveals an increased standard deviation for dilution levels $<10^{-24}$ M. We, therefore, performed an exploratory data analysis in order to determine possible modulating factors.

The experiments were performed in summer time with considerable variation of the room temperature, which was registered for each experiment. Whereas the temperature during the *preparation* of the dilutions does not seem to be an important factor, a high room temperature during *incubation* of basophils with histamine at a dilution level of 10^{-26} M compared to controls was associated with a significant ($p < 0.05$) increase of CD63 expression (negative inhibition). However, for all other dilution levels, no significant differences can be found between any pair of temperature ($p > 0.05$) and a two-way analysis of variance of the pure differences between histamine and water

dilutions does not yield a significant interaction between dilution level and incubation temperature ($p > 0.3$).

Histamine- and sham-dilutions were stored at 4 °C for 1–6 days before usage. A two-way analysis of variance does not yield a significant interaction between dilution level and storage time on the pure differences between histamine and water dilutions ($p > 0.1$). In particular, no significant differences can be found between a storage time of 1 day compared to storage times of 2–6 days ($p > 0.1$) for the pharmacological concentration of 10^{-2} M, indicating that molecular histamine was stable when stored at 4 °C. However, a qualitative comparison of a storage of 1 day to storage times of 2–6 days could suggest, that homeopathic histamine dilutions below 10^{-24} M enhance anti-IgE induced CD63-expression when prepared only 1 day before use, in contrast to the previously reported inhibitory effect of homeopathic histamine. A *t*-test for the dilution level 10^{-26} M yields a significant difference for a storage time longer than 1 day ($p < 0.002$).

Discussion

Several studies have reported significant effects of highly diluted solutions on basophil degranulation in vitro, using different rationales and different methodologies for the assessment of basophil activation (Table 1). The initially reported significant activation of basophil degranulation by homeopathic dilutions of activating anti-IgE-antibodies could not be reproduced when experiments were blinded and properly controlled.[6–10] In contrast, very high dilutions of histamine have shown significant effects on basophil activation in all published studies so far.

When assessing basophil degranulation by visual counting of stained basophils, a significant inhibition of basophil degranulation by histamine was found in some of the tested very low concentrations.[13,14] Furthermore, an overall inhibitory effect of highly diluted histamine was confirmed by a European multicentre study.[17,22] However, the results differed to a large amount between the four laboratories involved, which was ascribed to inter-individual differences of the blood donors.

As can be seen in Table 3, several studies using flow-cytometry to measure basophil activation observed a significant inhibitory or activating effect of histamine at some of the tested homeopathic concentrations. However, active and inactive dilution levels differed in most investigations. For example, a concentration of 10^{-26} M caused a significant inhibition in one study,[19] whereas a significant activation with the same concentration was found elsewhere[18] (see Table 3).

In a more recent publication of Belon et al.,[22] significant effects of highly diluted histamine were also found when basophil activation was measured with the histamine release method. However, these experiments as well as most of the flow-cytometric studies do not state to have performed blinding of diluted histamine and controls. The only exception are the recent studies of Lorenz et al.,[20,21] which contain only preliminary results.

In this study, we tried to reproduce the repeatedly reported inhibitory effect of highly diluted histamine solutions on anti-IgE induced basophil degranulation using the flow-cytometric method developed by Sainte-Laudy et al.[5] The blinding of the solutions and the (computer generated) randomisation of the experimental set-up including the position of replicates on the microtiter-plate by far exceeded the usual precautions to avoid artefacts in ''conventional'' in vitro research. In contrast to former studies (Table 3), no large effect of highly diluted histamine solutions on anti-IgE induced basophil degranulation as assessed by CD63 up-regulation can be found in our data under these conditions. But notably, even with this rigid protocol we found one minor, but statistically significant, inhibitory effect at a histamine dilution of 10^{-22} M, when compared to the effect of water ''diluted'' to 10^{-22} M. However, when the same data were compared a posteriori to other reasonable controls, statistical significance was lost. One may argue, that statistical significance was reached only because the standard deviation of the inhibition within this dilution level (10^{-22} M) happened to be small (Table 2 and Fig. 1). Since the *p*-value is rather modest ($p = 0.018$), the evidence for rejecting the null hypothesis (''no difference between histamine and water dilutions'') is not overwhelming, i.e. it is possible that this result is due to chance only.

One interest of our study was to raise hypotheses, which might explain the differing results in previous work and in our investigation (Table 3). Whereas at pharmacological concentrations the results were quite similar in the different studies, larger differences were found at higher dilutions, especially at 10^{-26} M, where inhibitory as well as activating effects were described. Four different explanations can be assumed: (1) A differing methodology might provoke divergent results. (2) The study results might depend on inter-individual differences of blood donors. (3) Unidentified confounding parameters might provoke a systematic error and even false positive results, which mimic

an effect of histamine at high dilutions. (4) External environmental factors could influence the experimental system.

(1) Methodological differences among previous studies might be responsible for the divergent results. Indeed, it has been suggested that the dilution medium may influence the effects of high dilutions on basophils.[20] However, identical solutions and antibodies were mostly used and the methodology developed by Sainte-Laudy and Belon[15] was followed closely in previous reports and in our study. In a pilot experiment, we ruled out a possible influence of using microtiter-plates (as used in[15]) or single polystyrene tubes (used in[19]). Unlike other groups,[15,16,18,19] we found an acceleration of leukocyte sedimentation with dextran to be necessary. In pilot experiments, we ascertained that this difference had no significant influence on basophil degranulation and on effect of substantial histamine. A negative influence of dextran on effects of highly diluted histamine formally cannot be excluded, though.

It is noteworthy, that dilutions in our and most previous studies using vortex instead of long lasting shaking were not produced according to homeopathic recommendations. However, major effects were reported with this technique (Table 3).

(2) One may argue that the optimally active homeopathic dilution may differ for cells isolated from different individuals, obscuring an effect when the different experiments are combined. In order to avoid this objection, the blood of one single donor was used for all our seven main experiments. On the other hand, it is also possible that the basophils of this single donor investigated are not or only weakly susceptible to homeopathic dilutions of histamine, explaining the smaller effects observed in our study.

(3) We observed highly significant effects related to the microtiter-plate position, although maximal efforts were taken to avoid such influences. We do not have a convincing explanation for this effect. Since row effects were observed, differences of the incubation time would seem most probable. However, by using multi-pipettes and mirror-plates, and by controlling the incubation times of each row with a clock, the differences in incubation time were maximally 60 s, which seems to be too small to cause larger effects. These difficulties illustrate the importance of randomisation and blinding of a sufficient number of replicates within each experiment, which effectively eliminated confounding plate effects in our study. Lacking randomisation might yield false positive results and might be responsible for the varying results among different investigations.

In this study, experimental procedures were adapted from earlier studies,[15,19] in which large effects of high histamine dilutions were reported. However, by using improved buffer solutions,[18,21] isolation of leucocytes[21] or basophils as well as by assessing basophil activation with an improved flow-cytometric system[20,21] or measurement of histamine release,[22] a more stable experimental system might be obtained in future studies.

(4) A closer inspection of Fig. 1 reveals an increased standard error for dilution levels $<10^{-24}$ M. An exploratory data analysis gives rise to the hypothesis, that the age of the histamine dilutions and the temperature during the incubation of the basophils might modulate their response to homeopathic dilutions of histamine ($<10^{-24}$ M).

Additional studies using strictly controlled experimental conditions, several blood donors and an improved methodology are needed to elucidate the open questions.

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References

1. Nakagawa T, Stadler BM, de Weck AL. Flow-cytometric analysis of human basophil degranulation. I. Quantification of human basophils and their degranulation by flow-cytometry. *Allergy* 1981;**36**:39—47.
2. Gilbert HS, Ornstein L. Basophil counting with a new staining method using alcian blue. *Blood* 1975;**46**:279—86.
3. Shore PA, Burkhalter A, Cohn Jr VH. A method for the fluorimetric assay of histamine in tissues. *J Pharmacol Exp Ther* 1959;**127**:182—6.
4. Knol EF, Mul FPJ, Jansen H, Calafat J, Roos D. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J Allergy Clin Immunol* 1991;**88**:328—38.
5. Sainte-Laudy J, Sabbah A, Vallon C, Guérin JC. Analysis of anti-IgE and allergen induced human basophil activation by flow cytometry. Comparison with histamine release. *Inflamm Res* 1998;**47**:401—8.
6. 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.

7. Maddox J, Randi J, Stewart WW. ''High-dilution'' experiments a delusion. *Nature* 1988;**334**:287—91.
8. Benveniste J, Davenas E, Ducot B, Cornillet B, Poitevin B, Spira A. L'agitation de solutions hautement diluées n'induit pas d'activité biologique spécifique. *CR Acad Sci Paris* 1991;**312**:461—6.
9. Ovelgonne JH, Bol AW, Hop WC, van Wijk R. Mechanical agitation of very dilute antiserum against IgE has no effect on basophil staining properties. *Experientia* 1992;**48**:504—50.
10. 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.
11. Lichtenstein LM, Gillespie E. Inhibition of histamine release by histamine controlled by H2 receptor. *Nature* 1973;**244**:287—8.
12. Tung R, Kagey-Sobotka A, Plaut M, Lichtenstein LM. H2 antihistamines augment antigen-induced histamine release from human basophils in vitro. *J Immunol* 1982;**129**:2113—5.
13. Poitevin B, Davenas E, Benveniste J. In vitro immunological degranulation of human basophils is modulated by lung histamine and *Apis mellifica*. *Br J Clin Pharmacol* 1988;**25**:439—44.
14. Sainte-Laudy J, Belon P. Inhibition of human basophil activation by high dilutions of histamine. *Agents Actions* 1993;**38**:C245—7.
15. 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.
16. 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.
17. 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.
18. Sainte-Laudy J. Stimulatory effect of high dilutions of histamine on activation of human basophils induced by anti-IgE. *Inflamm Res* 2001;**50**(Suppl. 2):S63—4.
19. 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.
20. Lorenz I, Schneider EM, Stolz P, Brack A, Strube J. Influence of the diluent on the effect of highly diluted histamine on basophil activation. *Homeopathy* 2003;**92**:11—8.
21. Lorenz I, Schneider EM, Stolz P, Brack A, Strube J. Sensitive flow cytometric method to test basophil activation influenced by homeopathic histamine dilutions. *Forsch Komplementärmed Klass Naturheilkd* 2003;**10**:316—24.
22. Belon P, Cumps J, Ennis M, Mannaioni PF, Roberfroid M, Sainte-Laudy J, et al. Histamine dilutions modulate basophil activation. *Inflamm Res* 2004;**53**:181—8.
23. Hsu JC. *Multiple comparisions: theory and methods*. New York: Chapman & Hall/CRC; 1996.