Mechanical agitation of very dilute antiserum against IgE has no effect on basophil staining properties

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Abstract. A previously reported[2] effect of mechanically agitated dilutions of antiserum raised against IgE was investigated using the loss of metachromatic staining properties of human basophil leukocytes as a model.
A series of 24 blind experiments was performed in which we determined the number of toluidine blue–stainable basophils after incubating with vortexed or non-vortexed dilutions of anti-IgE. Tenfold serial dilutions were used, in the range 10^{21} to 10^{30} (6.6×10^{-26} to 6.6×10^{-35} M anti-IgE).
We found no evidence for a different effect of strongly agitated dilutions, compared to dilutions made with minimal physical agitation. In fact, in our hands no effect of extreme dilutions was shown at all. We conclude that the effect of extreme dilutions of anti-IgE, reported by Davenas et al.[2], needs further clarification and that in this process the reproducibility of results between experimenters should be carefully determined.
Key words. Basophils; degranulation; extreme dilutions; in vitro tests; homeopathy.

Extremely dilute solutions of agonists have been reported to produce significant biological effects, both in vitro[2,7–11] and in vivo[3–6]. The investigated solutions were usually prepared by a stepwise dilution procedure, with vigorous shaking of the solution between each dilution step. This procedure has been assumed to be necessary to obtain an active solution, and theoretical models[12] have been designed to explain the effect of shaking.
The most widely-known research on the effect of extreme dilutions is that reported by Davenas et al.[2]. These authors investigated the loss of metachromatic staining properties of human basophil leukocytes. Antibodies against IgE are able to initiate the process of degranulation in basophils. After degranulation the granules in these cells are no longer stainable. The decrease in stainability allows the process of degranulation to be assessed, and was described by Beauvais et al.[13] to be a more sensitive parameter than the amount of histamine released. Davenas et al.[2] found a loss of staining properties after exposure to antiserum diluted 10^{120} fold (molarity 2×10^{-126} M). They mentioned that this effect was observed only when serially (tenfold or one hundred fold) diluted solutions were vortexed for at least 10 s between 2 dilution steps. These data have been heavily criticized after an unsuccessful attempt to reproduce them[14]. In response to criticism by Jaques[15], who suggested that biological activity of the very dilute anti-IgE solution was due to a dissolved contaminant, Benveniste et al.[16] published a large series of blind replication trials. In this series a significant difference in response was found between identical cells treated with extreme dilutions of anti-IgE or anti-IgG. These dilutions had been made and agitated with an automat, ruling out differences that might have arisen as a result of contaminating substances in some solutions.
In this paper we report an attempt to reproduce a response in the basophil stainability test, as it was previously described[2], with minute concentrations of serum raised against IgE. Our investigation focusses on the possible effect of shaking a solution between two dilution steps. We hope this work will contribute to the discussion about the activity of extremely dilute agonists.

Materials and methods

Preparation of leukocytes. Venous blood was obtained from a group of 12 healthy donors. Individual donors gave blood between 1 and 5 times. Briefly, the procedure was as follows: 20 ml of blood was collected using 2 Greiner vacuette tubes (containing sodium heparin (14 IU/ml); EDTA-Na_2 and EDTA-Na_4 (Merck) had each been added to give a final concentration of 2.5 mM. The blood was allowed to sediment after adding 4 ml of a 4.5% solution of dextran T-500 (Pharmacia) in HBSS (Hank's Balanced Salts Solution (Seromed) without calcium and magnesium, to which EDTA-Na_2 (Merck) and HEPES (Seromed) had been added to give a concentration of 2.3 and 10.0 mM, respectively, pH adjusted to 7.40). The leukocyte-rich plasma was recovered and diluted by adding an equal volume of HBSS and washed twice with HBSS by centrifugation (10 min at 400 g). The pellet was finally resuspended in HBSS.
Preparation of dilutions of antiserum. Goat antiserum against human IgE (Fc) was purchased from Nordic Immunology, Tilburg, the Netherlands (batch number 3398). It was specified to contain approximately 10 mg of total IgG per ml, of which 3 mg was precipitable anti-IgE. Based on the 10 mg and the molecular weight of IgG, the concentration of active antibody cannot have been higher than 6.6×10^{-5} M. This last figure is used for further calculations of molarities. The serum was diluted serially, 1:10, with HBSS containing 11 mM $CaCl_2$, in disposable plastic tubes (Greiner 623201). Two series of dilutions were made. In the first each dilution was shaken by vortexing (Heidolph Reax 2000, 2400 rpm) for 25 s between each dilution step. In the second series mechanical forces were minimized by pipetting very gently and

tilting the test tubes slowly 10 times to mix the contents after diluting. These two series of solutions provided paired data for statistical tests. Dilutions of antiserum are designated by 'V' or 'N' (vortexed or non-vortexed) followed by number of 1:10 dilution steps. For example: in N21 the antiserum had been diluted 10^{21} times, without vortexing between each step.

Blinding. The investigation was performed as a blind study with respect to the dilutions: after the two series of dilutions had been made, the tubes containing the dilutions were coded by an independent person who replaced tube labels by labels bearing a random number. The code was revealed after each test was completed.

Basophil stainability test. The test was performed using the method described in Davenas et al.[2]. Of each antiserum dilution to be tested, 20 µl was deposited on the bottom of a well of a microwell plate (Nunc). The microwell plate and the leukocyte suspension were then preincubated separately at 37 °C for 5 min. After this 20 µl of cell suspension was added to each well, and the microwell plate was incubated again at 37 °C for 30 min. Subsequently 90 µl of staining solution (for 100 ml, 100 mg of toluidine blue (Fluka) dissolved in 25% ethanol and adjusted to pH 3.2–3.4 with glacial acetic acid) was added to each well and the suspensions were thoroughly mixed. After the microwell plate had been sealed with Scotch tape to prevent evaporation of the alcohol, it was placed at 4 °C to stain basophils overnight.

After staining, each sample was pipetted into a Fuchs–Rosenthal hemocytometer. Red-stained basophils were counted by eye (1/4 of the area was counted, corresponding to 0.8 µl of suspension).

In each experiment the test was performed on dilutions N3 and N21 through N30 and on V3 and V21 through V30 (22 samples total). Dilutions V21, N21 through V30, N30 were chosen to be certain that if any effect was found, this could not be due to any remaining molecules of antibody in solution: concentrations ranged from 6.6×10^{-26} M through 6.6×10^{-35} M (maximal possible molarities). Dilutions N3 and V3 were used to evaluate the sensitivity of the basophils to anti-IgE by comparing the mean count obtained with V3 and N3 with the mean count obtained with V21–V30 and N21–N30, using the following formula:

$$\text{sensitivity} = \frac{\text{mean count (N21–N30, V21–V30)} - \text{mean count (N3, V3)}}{\text{mean count (N21–N30, V21–V30)}} \cdot 100\%$$

Duplicate counting. To assess the reliability of the basophil counting procedure all samples were counted simultaneously by a second investigator in 14 of the experiments. In these experiments 2 counting chambers were filled from each sample. The 2 researchers (X and Y) were blind to each other's results.

Statistical methods. Components of variance associated with differences in counts between and within blood donors were assessed using nested Analysis of Variance. To stabilize the variance, square root transformation of the counts was applied in these analyses[17]. Differences in counts between the various dilutions were evaluated using Friedman's test. The effect of vortexing was assessed with Wilcoxon's test. P-values given are two sided.

Results

A set of 24 experiments was performed, in which the effect of vortexed dilutions of antiserum raised against IgE was compared with the effect of non-vortexed dilutions on the stainability of human basophils. 2 sets of 10 serial dilutions were compared. One was prepared with extensive vortexing between each dilution step (V21 through V30); in the other, mechanical forces on the solutions were minimized (N21 through N30). The maximal possible molarity of antibodies in these dilutions was 6.6×10^{-26} M (total IgG, including non IgE- specific antibodies, in dilutions N21 and V21). In the dilution range used, a basophil can hardly pick up an antibody molecule because of the very low concentration of these molecules. Dilutions V3 and N3 were used to evaluate the sensitivity of the basophils in each experiment. Basophil counts are based on red-stained, i.e. non-degranulated basophils as viewed in a Fuchs–Rosenthal counting chamber. To assess the objectivity of the counting procedure, 14 experiments were simultaneously evaluated by a second investigator. The study was conducted blind with respect to dilutions and to the results of the other investigator.

The table shows the mean basophil count obtained with the vortexed dilutions (combining V21–V30) and with the non-vortexed dilutions (combining N21–N30) for each experiment and each investigator. When V21 through V30 are compared with N21 through N30 using Wilcoxon's test, there is only one experiment where a small p-value is obtained with data collected by investigator X (experiment 5, $p = 0.02$). When Wilcoxon's test is carried out on experimenter Y's data, experiment 16 leads to a small p value. As a p value can be expected to be less than 0.05 by chance alone once in every 20 experiments[18], these findings cannot be considered to signify an effect of vortexing.

When counts from the same and from different blood donors were compared it was evident that there are significant differences between donors. For the mean value of counts derived from vortexed dilutions the standard deviation (sd) associated with between-donor differences was 15.7 ($p = 0.01$), with a within-donor sd of 6.2. From the non-vortexed dilutions similar figures were derived, respectively 18.7 ($p = 0.02$) and 7.1. Significant donor

Experiment no.	Donor	Number of stained basophils: V21 through V30 mean	sem			N21 through N30 mean	sem			p(*)		Basophil sensitivity (%)	
Investigator		X		Y		X		Y		X	Y	X	Y
1	l	69.0	2.16			66.6	4.50			0.61		− 0.3	
2	c	75.2	5.00			80.3	4.34			0.39		69.8	
3	d	83.0	3.16			88.5	3.95			0.44		13.7	
4	b	78.2	4.02			88.0	5.14			0.21		68.7	
5	a	61.1	2.56			72.5	2.98			0.02		73.1	
6	e	87.4	3.14			92.0	5.77			0.48		15.3	
7	f	73.5	1.76	77.0	2.57	70.1	3.01	73.0	3.27	0.39	0.33	14.4	6.7
8	a	61.4	2.91			64.8	4.03			0.44		58.8	
9	h	25.4	2.09	22.8	1.82	20.7	2.17	23.7	1.87	0.29	0.65	52.3	54.8
10	i	54.1	2.35			47.6	2.89			0.17		86.2	
11	a	77.5	3.15	84.3	6.28	73.6	3.54	89.3	3.66	0.48	0.58	78.2	57.4
12	c	69.4	2.56	66.5	2.87	64.3	2.27	61.5	3.83	0.13	0.29	76.1	60.2
13	a	89.6	3.77	77.9	4.06	95.2	4.81	81.8	5.49	0.29	0.68	58.9	49.9
14	e	93.1	3.08			100.7	6.46			0.26		− 2.8	
15	b	98.5	5.83	89.1	5.52	89.9	5.38	102.5	3.14	0.20	0.08	33.1	38.9
16	a	57.7	2.46	67.7	3.62	53.9	2.35	58.5	2.52	0.26	0.04	74.0	49.3
17	f	68.2	5.47	57.6	2.79	57.4	2.59	65.0	3.65	0.19	0.12	2.1	2.9
18	a	67.9	2.27	78.6	4.43	77.8	4.89	84.9	4.54	0.14	0.24	60.2	52.3
19	j	107.7	3.15	111.2	5.21	110.6	3.59	111.7	4.35	0.77	0.26	−5.4	2.6
20	d	63.9	2.52	68.4	4.48	68.4	2.21	68.6	4.37	0.24	0.86	22.9	13.9
21	g	123.3	3.45	141.7	4.45	125.7	3.46	132.3	5.54	0.77	0.14	78.7	76.6
22	k	42.9	2.17	57.6	3.42	46.0	4.12	61.1	4.15	0.45	0.68	58.4	62.9
23	b	67.3	2.68			68.7	4.73			0.80		36.8	
24	g	81.8	5.27	91.7	4.68	90.5	3.62	88.1	3.85	0.29	0.51	68.7	65.5
total		74.1	6.2(**)	78.0	7.6(**)	75.6	7.1(**)	79.1	8.6(**)	0.26	0.85	43.8	44.8

Counts of red-stained basophils determined in 0.8 µl of staining solution after incubation with vortexed and non-vortexed sequential 10-fold dilutions of antiserum against IgE. Mean and standard error of the basophil counts obtained with these dilutions are shown for each experiment. Twenty-four experiments were evaluated by investigator X using basophils from 12 donors (donors 'a' to 'l'), of which 14 were simultaneously evaluated by researcher Y. * Wilcoxon's test per individual experiment and of all experiments together. ** SEM allowing for donor differences.

differences between counts derived from vortexed dilutions and those obtained from non-vortexed dilutions were examined. This is true both for all counts separately and for the mean counts from each experiment. Therefore it is permissible to analyze the experiments as one group, without considering the basophil donor.

To verify that a possible vortexing effect was not related to the sensitivity of a particular batch of basophils to the antiserum, the comparison shown in figure 1 was made. The difference between counts derived from vortexed and non-vortexed dilutions does not appear to be related to the basophil sensitivity ($r = 0.10$, $p = 0.66$). The same conclusion was reached when analyzing counts at each dilution separately (data not shown).

The lower part of figure 2 shows the mean counts obtained with each dilution. No significant differences were present in basophil counts derived from dilutions 21 through 30, either for the vortexed dilutions, or for the non-vortexed dilutions, or for their differences. Counts derived from vortexed dilutions have also been expressed as a percentage of the counts obtained without vortexing (upper part of fig. 2). The mean percentage is lower than 100, which would be expected if the vortexing/diluting procedure had an effect on the stainability of basophils. Standard deviations are too high, however, to maintain this conclusion.

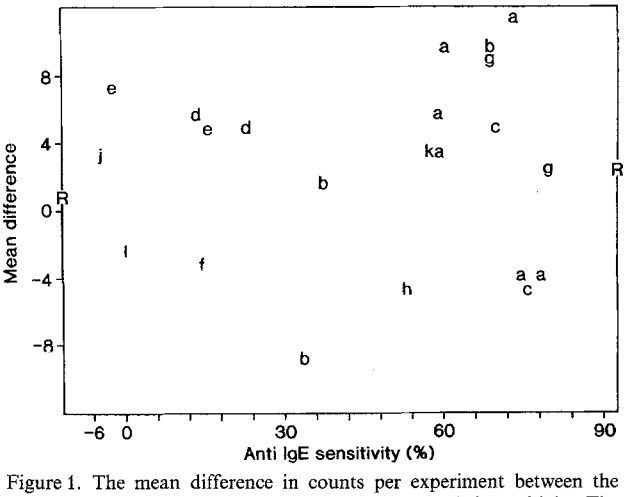

Figure 1. The mean difference in counts per experiment between the vortexed and the non-vortexed samples according to their sensitivity. The data were collected by investigator X and are also shown in the table; the letters represent the tested donors. The 2 letters 'R' on the ordinate indicate the intercepts of the regression line. Correlation statistics: $r = 0.10$ ($p = 0.66$).

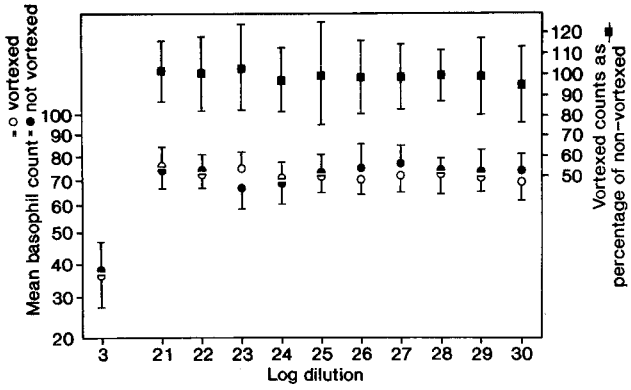

Figure 2. Dilution-wise analysis of the experiments performed by investigator X. Upper part (right ordinate): Basophil counts obtained with the vortexed dilutions expressed as a percentage of the counts obtained with non-vortexed dilutions. The latter were used as controls after proving them to be free of dilution-related effects with Friedman's test. For each experiment the mean basophil count of the non-vortexed samples was calculated. The count for each vortexed sample was expressed as a percentage of this mean. Per dilution the mean percentage and its standard deviation were calculated for all 24 experiments.
Lower part (left ordinate): For each dilution (V3, V21–V30 and N3, N21–N30) the mean count (± SEM) was calculated over all the experiments. The SEM takes account of differences between blood donors. The square-root transformed ordinate shows the mean count (± SEM) of 0: vortexed dilutions, + : non-vortexed dilutions. The number of dilutions is plotted on the abscissa.

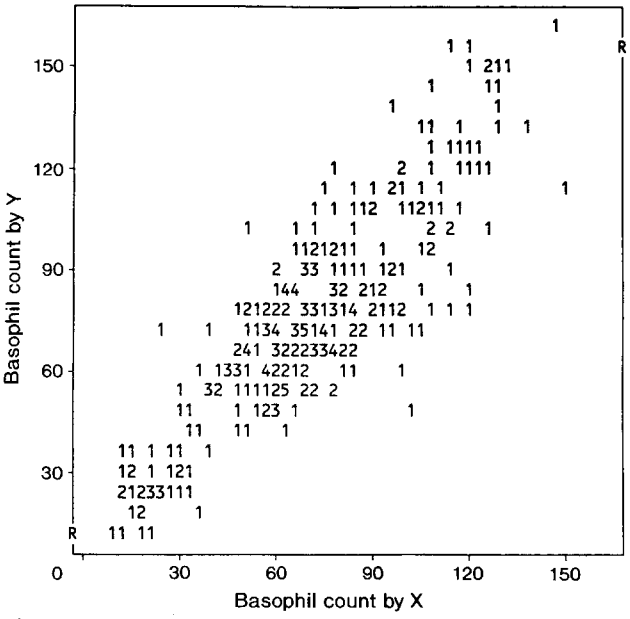

Figure 3. Correlation of counts obtained with the same samples but by both researchers X and Y. All samples from the 14 experiments (i.e. dilutions V3, V21–V30 and N3, N21–N30; 308 samples in total) that were evaluated in duplicate are plotted. The correlation is $r = 0.85$ ($p < 0.0000$; 'R' indicates intercepts of the regression line). The ciphers in the plot indicate the quantity of samples at the given positions.

In figure 3 a comparison is made between the sets of counts obtained by the two investigators. All counted samples have been plotted ($r = 0.85$). On the average, researcher Y counted 4 more basophils per sample than X ($p < 0.001$, Wilcoxon's test) which is about 5% of the mean. In individual experiments the counts generally showed a greater variation than might be expected if the number of cells in a counting chamber followed a Poisson distribution, indicating that small errors were made in counting and pipetting.

Discussion

In this investigation of extremely dilute solutions, we tested the hypothesis that agitation of a solution preserves the activity of the solute in some way. Our experiments present no evidence for a different effect on basophils of dilutions made by vortexing compared with dilutions made with minimal physical agitation. In fact, we conclude that in our experiments extreme dilutions had no activity at all.

No effect could be observed when data were analyzed per experiment, even when considering the sensitivity of batches of basophils to the antiserum, nor could an effect be observed when data were analyzed per dilution. Using the runs test we also verified that there were no trends in the counts obtained with each sequence of dilutions (data not shown). Similar conclusions have been reached by Scheepens et al.[19].

Benveniste et al.[16] selected the experiments that were used for the statistical analysis according to the following predefined criteria: there should be at least 35 basophils in control counting chambers, there should be less than 25% premature degranulation upon adding Ca^{2+} to the reaction medium, and at least 2 of the dilutions $10^2\times$, $10^3\times$ and $10^4\times$ should cause 40% or more degranulation. The last criterion implies the hypothesis that basophils insensitive to measurable amounts of antiserum are also insensitive to extreme dilutions, and 42% of the experiments were rejected on this basis. In our case, evaluating only the 1000 times dilution, this would be 58%, which is somewhat more but still comparable. There are some remarks to be made concerning the reliability of our negative results. In this study we are dealing with potentially small effects, i.e. variations in numbers of red-stained (i.e. non-degranulated) basophils. As the basophils can be difficult to discern, it is quite possible that detection of a vortexing effect with this model is sensitive to the skills of the researcher. We find a 5% difference in countings by 2 researchers and a correlation of 0.85. Although these data are quite reasonable they indicate that countings do differ between persons. Therefore, we cannot exclude the possibility that there was a vortex-related effect in our experiments which has gone unnoticed. The observation in the team of Benveniste that one person was better at performing positive experiments than the others (personal communication) could support this line of thought. A test of the possible experimenter-dependence of these experiments could be for someone who normally achieves positive results to count a number of experiments simultaneously with other workers.

A second possible factor influencing the results is the quality of the toluidine dye. Beauvais et al.[13] demon-

strate that toluidine-blue staining can be a sensitive method, detecting pre-degranulation events; but that some batches of toluidine have too high an affinity for the basophil granules and will therefore only detect true exocytosis[20]. Lastly, the pH and the cell density in the reaction medium might have some influence. The effects of these conditions have not yet been determined.

Summarizing, we conclude that we have no evidence for an effect of antiserum in high dilutions, and we have some doubts about the reproducibility of the basophil degranulation model. This is not the first instance of an experiment with extreme dilutions proving to be difficult to reproduce[5]. We are of the opinion that if conclusive information about this subject is to be obtained, it is necessary that an experimental model be investigated by a number of independent laboratories.

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Sister chromatid exchanges in lymphocytes of normal and alcoholic subjects

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Abstract. The effects of alcohol consumption, cigarette smoking and age on sister chromatid exchange (SCE) frequency in human lymphocytes were assessed by means of multiple linear regression. An increase in SCE rates was associated with alcohol consumption ($p = 0.0001$), smoking ($p = 0.0231$), and, to a small extent ($p = 0.057$), age. These three confounding factors explain 48% of the inter-personal variation in SCE rates among subjects studied.
Key words. Sister chromatid exchange (SCE); human lymphocytes; alcohol consumption; smoking; aging.

A sister chromatid exchange (SCE) is the cytological manifestation of DNA breakage and rejoining at homologous sites of the two chromatids of a single chromosome. One application of SCE analysis is the monitoring of human populations exposed to chemical mutagens and carcinogens[2]. Such exposure may be associated not only with occupational factors but with common lifestyle factors as well.

Cigarette smoking is now generally agreed to be a SCE-inducing factor[3], but it is still controversial whether alcohol consumption also contributes to elevated SCE rates. Increased rates of chromosome aberrations[4] and SCEs[5] were found in peripheral blood lymphocytes of alcoholics as compared to a control population. However, moderate alcohol consumption ('binge drinking') has been reported to be a non-significant factor contributing to the inter-personal variation in SCE rates[6–8]. Similarly, alcoholism in mothers did not affect the SCE rate of their newborn infants[9]. Obe and Ristow[10] related the induction of SCEs in vitro to the action of acetaldehyde, a metabolic derivative of alcohol[11], but not to the action of ethanol. Another in vitro study[12] showed that induction of SCEs by alcoholic beverages could be explained not only by their content of ethanol, but also by the presence of the other SCE-inducing compounds. It has been suggested[13] that the agents responsible for the increase of chromosomal damage in alcoholics may be ingredients other than ethanol found in alcoholic beverages. However, no significant differences related to the kind of beverage consumed (wine, beer, vodka, whiskey)