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In vitro immunological degranulation of human basophils is modulated by Lung histamine and Apis mellifica

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1 The effect of high dilutions of two homeopathic drugs Lung histamine (Lung his) and Apis mellifica (Apis mel) used for the treatment of allergic diseases has been assessed on *in vitro* human basophil degranulation. Experiments were conducted blind.
2 Basophil degranulation induced by 1.66×10^{-9} M anti-IgE antibody was significantly inhibited in the presence of 5 Lung his (5th centesimal dilution of Lung his) and 15 Lung his (15th centesimal dilution of Lung his) by 28.8% and 28.6% respectively and by 65.8% in the presence of 9 Apis mel (9th centesimal dilution of Apis mel). Basophil degranulation induced by 1.66×10^{-16} to 1.66×10^{-18} M anti-IgE antibody was also inhibited by high dilutions of Lung his and Apis mel with an inhibition of nearly 100% with 18 Lung his (18th centesimal dilution of Lung his) and 10 Apis mel (10th centesimal dilution of Apis mel). An alternance of inhibition, inactivity and stimulation was observed when basophils were incubated in the presence of serial dilutions of Lung his and Apis mel.
3 The investigation of the clinical efficacy of high dilutions of Lung his and Apis mel should be envisaged in allergic diseases in parallel with *in vitro* and *ex vivo* biological assays.

Keywords basophils degranulation IgE homeopathic drugs

Introduction

One of the characteristics of homeopathic drugs is the dilution procedure they undergo. What is actually administered to the patient contains little, if any, of the starting substance. This casts some doubts on the real efficacy of homeopathic practice. Several biological effects of very highly diluted substances were recently reported on the immune response in mice (Doucet-Jaboeuf *et al.*, 1982), the synthesis of PAF-acether by mouse peritoneal macrophages (Davenas *et al.*, 1987) and the human basophil degranulation test (Poitevin *et al.*, 1986). The latter test, a simple *in vitro* model for immediate hypersensitivity studies, has been extensively used in our and other laboratories to explore allergic sensitivity at the cellular level through IgE-dependent basophil sensitisation (Egido *et al.*, 1977; Benveniste, 1981; Pirotzky *et al.*, 1982; Yeung Laiwah *et al.*, 1984). The test is based on a one-step method of basophil staining after exposure to the specific allergen.

Using this test, we have measured the effect of an homeopathic drug, Apis mellifica, on the *in vitro* degranulation of basophils sensitized to common allergens (Poitevin *et al.*, 1986). After incubation of the cells with high dilutions of Apis mellifica, a significant inhibition of allergen-induced degranulation was observed. More recently, we have observed an inhibitory effect of very high dilutions of histamine on the *in vitro* anti-IgE-induced degranulation of human basophils. This inhibitory effect was observed not only for usual concentrations of anti-IgE antibody (1.66×10^{-9} M) but also for concentrations of this antibody well-beyond what is routinely used in immunological studies (1.66×10^{-16} to 1.66×10^{-18} M). We also studied the human basophil degranulation induced by high and low concentrations of anti-IgE antiserum after incubation of the cells with serial dilutions of Apis mellifica and Lung histamine, another substance used in the homeopathic treatment of allergic diseases.

Methods

Products

Lung histamine, an extract from guinea-pig Lungs after anaphylactic shock, and Apis mellifica, an extract from honey bee, were purchased from the Laboratoires Homeopathiques de France (LHF, Asnières, France).

For preparation of Lung histamine, a lung fragment (approximately 3 g) was obtained from a guinea pig that was submitted to anaphylactic shock. The fragment was first ground with fine sand and washed with saline (1:9, w/v). The supernatant was again diluted with saline (1:9, w/v). The latter mixture, corresponding to the 1st centesimal dilution of the starting substance, was named 1 Lung histamine (1 Lung his). It contained 150 nM histamine as detected using an automated version of the Shore technique (Shore *et al.*, 1959; Siraganian & Brodsky, 1976).

For preparation of Apis mellifica, entire honey bees were first crushed in a grinder and 65% ethanol was then added (1:20, w/v). This stock solution was again diluted with 65% ethanol (1:10, v/v). The latter solution was finally diluted with saline (1:10, v/v), thus obtaining 1 Apis mellifica (1 Apis mel) that corresponded to the 1st centesimal dilution of the stock solution. It contained 90 nM histamine.

One Lung his and 1 Apis mel were 20 times serially diluted 100-fold in saline by thorough mixing using a Vortex for 10 s under a laminar flow hood. The obtained solutions were designated by the abbreviations Apis mel and Lung his prefixed by the number of 100-fold dilutions the starting substance underwent. For example, 5 Apis mel corresponded to the 5th 100-fold dilution of the stock solution. Control solutions were prepared exactly as above except that Lung his and Apis mel were omitted in saline and in 65% ethanol respectively at the first step of the dilution procedure. Just before use, all solutions were diluted once more 1/100 (v/v) in Tris- or HEPES-buffered Tyrode's (see below) and received an arbitrary code number except for the first set of experiments conducted with Lung his. The technicians did not know the composition of the tested solutions until the end of the study.

Goat anti-human IgE antiserum was purchased from Nordic Immunology (Tilburg, The Netherlands). The initial solution (1 mg antibody ml^{-1} i.e. 6.66×10^{-6} M) was serially diluted 10-fold in Tris- or HEPES-buffered Tyrode's down to 6.66×10^{-22} M.

Human basophil degranulation test

It was performed using the method described in Benveniste (1981) with slight modifications. Venous blood (20 ml) from healthy donors was collected using heparin 1 U ml^{-1} and a mixture of EDTA-Na_4 2.5 mM/EDTA-Na_2 2.5 mM (final concentrations) as anticoagulants and allowed to sediment. The leucocyte-rich plasma was harvested, twice washed by centrifugation (400 *g*, 10 min) and finally resuspended in an aliquot of 200 to 500 µl of Tris-buffered Tyrode's (in g l^{-1}: NaCl, 8; KCl, 0.915; Tris, 0.605; EDTA-Na_4, 1.040; glucose, 1; human serum albumin, 2.5; heparin, 5000 U l^{-1}; pH, 7.4). In some comparative experiments, cells appeared morphologically better in HEPES buffer. Thus, in the last experiments, HEPES 2.6 g l^{-1} was substituted for Tris.

Cell suspension (10 µl) was layered on the bottom of each well of a microtitre plate that contained 10 µl of Lung his, Apis mel or control dilutions. The plates remained at room temperature in a moist chamber for 30 min. Ten µl of Ca^{2+} (5 mM final) and 10 µl of goat anti-human IgE antiserum at indicated concentrations (both in Tyrode's) were then added. To a control well was added Ca^{2+} in 20 µl Tyrode's but no anti-IgE antiserum. The plates were then incubated at 37°C for another 30 min. Ninety µl of the staining solution (100 mg toluidine blue (Merck, Darmstadt, GFR) and 280 µl glacial acetic acid in 100 ml of 25% ethanol; pH 3.2 to 3.4) were added in each well and the suspension was thoroughly mixed. Specifically red-stained basophils (i.e. non degranulated basophils) were counted under a microscope using a Fuchs-Rosenthal haemocytometer.

No variations were observed when leucocytes were incubated either in the absence of anti-IgE antiserum or in the presence of Lung his or Apis mel dilutions alone. A mean ± s.e. mean of 89 ± 6 (n = 53) basophils was counted for the controls.

Data analysis

Results were expressed as percent degranulation of human basophils calculated using the following formula:

$$\frac{\text{basophils in control} - \text{basophils in sample}}{\text{basophils in control}} \times 100$$

For statistical studies the Wilcoxon test was used when $n > 5$, and the Wilcoxon-Mann-Whitney test when $n \leq 5$, (n = number of experiments). They were performed using the actual basophil counts and not the percentages of degranulation.

Table 1 Human basophil degranulation induced by 1.66×10^{-9} M (final concentration) anti-IgE antibody in the presence of dilutions of Lung his as compared with saline submitted to the same dilution procedure as 15 Lung his (15 sal). Data are means of percent degranulation ± s.e. mean of *n* experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$.

Nature of the sample	*Series of experiments* I (n = 10)	II (n = 11)	III (n = 9)
Tris-buffered Tyrode's	40.3 ± 2.6	35.9 ± 3.8	44.7 ± 3.4
15 sal	39.5 ± 3.8	37.6 ± 4.2	43.4 ± 2.5
1 Lung his	50.1 ± 2.3**	41.7 ± 4.7	51.0 ± 3.0*
5 Lung his	24.3 ± 4.8***	20.9 ± 5.7**	33.3 ± 5.3*
9 Lung his	40.1 ± 4.4	42.6 ± 4.2*	51.4 ± 3.8***
15 Lung his	27.9 ± 2.8****	29.0 ± 4.9**	30.6 ± 4.7***

Results

Effect of Lung his and Apis mel on basophil degranulation induced by 1.66 × 10^{-9} M anti-IgE antibody (final concentration)

Lung histamine A first set of 10 experiments was performed in Tris-buffered Tyrode's using 1, 5, 9 and 15 Lung his (Table 1, column I). As compared to saline, basophil degranulation induced by goat anti-human anti-IgE (1.66×10^{-9} M) was significantly decreased in the presence of 5 Lung his ($P < 0.005$) and 15 Lung his ($P < 0.001$) whereas a significant increase was seen with 1 Lung his ($P < 0.01$). In a second set of 11 experiments conducted as above, the decreased degranulation observed with 5 Lung his and 15 Lung his was confirmed and a slight but significant increase appeared with 9 Lung his ($P < 0.05$) (Table 1, column II). Nine experiments were then undertaken to study the effect of a very large scale of Lung his dilutions from 1 to 15 Lung his on basophil degranulation induced by the same concentration of anti-IgE antiserum (Table 1, column III). This study confirmed the modifications previously observed and also showed two zones of inhibited degranulation around 5 Lung his and 15 Lung his in alternance with two peaks of potentiated degranulation at 1 Lung his and at 9 Lung his. No variation in anti-IgE-induced basophil degranulation was observed following preincubation of cells with either Tris-buffered Tyrode's or the dilution corresponding to 15 Lung his from which Lung his was omitted in the first step.

Apis mellifica In five preliminary experiments, basophil degranulation induced by anti-human anti-IgE (1.66×10^{-9} M) in the presence of 9 Apis mel was significantly decreased from 50.1 ± 2.1% to 17.0 ± 2.9% ($P < 0.02$) as compared with saline submitted to the same dilution procedure as 9 Apis mel. Basophil degranulation was then performed in four experiments in the presence of serial dilutions of Apis mel from 1 to 20 Apis mel (Figure 1). As compared with control (46.5 ± 1.6%), an inhibition occurred with 8 Apis mel (26.6 ± 4.0%, $P < 0.02$), 9 Apis mel (24.8 ± 2.4%, $P < 0.02$) and 10 Apis mel (20.8 ± 4.8%, $P < 0.02$). Again no effect was seen when preincubating cells either with the control dilutions or Tyrode's alone.

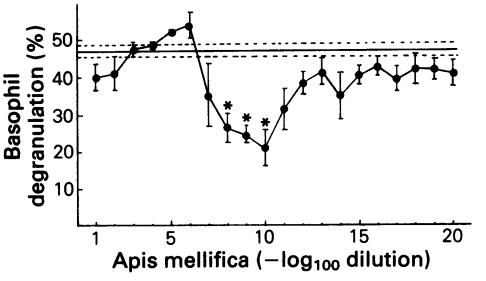

Figure 1 Basophil degranulation induced by 1.66×10^{-9} M (final concentration) anti-IgE antibody in the presence of serial dilutions of Apis mel from 1 to 20 Apis mel. Control degranulations in the presence of HEPES-buffered Tyrode's alone or the dilution corresponding to 9 Apis mel without adding Apis mel in the starting solution were 47.1 ± 1.0% and 46.5 ± 1.6% respectively (mean ± s.e. mean, $n = 4$). Control numbers were pooled in the figure (⁼⁼⁼⁼, mean ± s.e. mean, $n = 8$).* $P < 0.02$.

Effect of Lung his and Apis mel on basophil degranulation induced by very low concentrations of anti-IgE antibody

Lung histamine The effect of 15 Lung his was studied on basophil degranulation induced by 1.66×10^{-16} to 1.66×10^{-22} M anti-IgE antibody.

The control dilution for 15 Lung his was saline prepared exactly as 15 Lung his except that Lung his was omitted in the first step. All experiments were performed in HEPES-buffered Tyrode's. Significant inhibition of maximal anti-IgE degranulation was obtained with 15 Lung his (Figure 2).

In three experiments, the effect of the pre-incubation of cells with a range of 1 to 18 Lung his was explored on basophil degranulation induced by the dilution of anti-IgE antibody that was given the maximal degranulation (either 1.66×10^{-16}, 1.66×10^{-17} or 1.66×10^{-18} M). Two zones of inhibition were observed from 3 Lung his to 5 Lung his ($P < 0.05$) on the one hand and from 12 Lung his to 18 Lung his on the other hand. For 18 Lung his, degranulation was nearly zero ($P < 0.05$) (Figure 3). No significant difference in the anti-IgE-induced degranulation could be observed when comparing cells pre-incubated with Tris-buffered Tyrode's *vs* the control dilution.

Apis mellifica The effect of 9 Apis mel was studied on basophil degranulation induced by 1.66×10^{-16} to 1.66×10^{-22} M anti-IgE antibody. It was compared with a control solution where 65% ethanol was diluted in saline as far as 9 Apis mel according to the same protocol. All five experiments were performed in HEPES-buffered Tyrode's. Significant inhibitions were obtained with 9 Apis mel (Figure 4).

Basophil degranulation either induced by 1.66×10^{-16}, 1.66×10^{-17} or 1.66×10^{-18} M anti-IgE antibody (see above) was then studied in three experiments in the presence of serial dilutions from 1 to 20 Apis mel (Figure 5). One zone of inhibition was observed corresponding to the dilutions of Apis mel from 6 Apis mel to 10 Apis mel with a total inhibition of the degranulation for 10 Apis mel ($P < 0.05$). A significant inhibition appeared also for 13 Apis mel and 20 Apis mel ($P < 0.05$). No significant difference in the anti-IgE-induced degranulation could be observed when comparing incubation of cells in HEPES-buffered Tyrode's alone *vs* incubation in the control dilution.

Discussion

High dilutions of homeopathic drugs used for the treatment of allergic diseases are efficient in inhibiting the *in vitro* anti-IgE-induced degranulation of human basophils. This effect can be observed not only on the degranulation induced by usual concentrations of anti-IgE antibody (1.66×10^{-9} M) but also on that induced by low concentrations of this antibody (1.66×10^{-16}, 1.66×10^{-17} or 1.66×10^{-18} M) where the actual number of IgG anti-IgE molecules is from 1×10^3 to 1×10 per well. These numbers are to be compared with the number of basophils per well (about 3000). Therefore the number of IgG anti-IgE molecules per basophil (0.3 to 0.03) is too low to trigger this process, according to classical pharmacology.

The two homeopathic drugs we studied contain in the first dilution (1 Lung his and 1 Apis mel) approximately 1×10^{-7} M histamine. According to the Avogadro number ($N = 6.023 \times 10^{23}$ molecules mol^{-1}), there was in our assays less than one molecule of histamine per ml of 8 Lung his or 8 Apis mel solution. However, we have shown that 15 Lung his and 10 Apis mel (where the probability of the presence of histamine or any other substance is near zero), were the most active solutions inhibiting the 1.66×10^{-9} and the 1.66×10^{-16}, 1.66×10^{-17} or 1.66×10^{-18} M anti-IgE-induced degranulation. Such an inhibition was already observed with high dilutions of histamine (Beauvais *et al.*, submitted for publication) but the better effectiveness obtained with 10 Apis mel and 15 Lung his as compared to histamine itself suggests that the action of Apis mel and Lung his does not depend only on the presence of histamine but on unidentified substances, also present in concentrations well below the usual pharmacological range.

The inhibitory effect of the high dilutions of Apis mel and Lung his appears to be more significant on degranulation induced by 1.66×10^{-16}, 1.66×10^{-17} or 1.66×10^{-18} M anti-IgE than on that induced by 1.66×10^{-9} M antibody. It is known in pharmacology that drugs are more inhibitory when the stimulation is weak. In our experiment, the same phenomenon is observed with high dilutions of the homeopathic drug.

We have reported (Poitevin *et al.*, 1986) the inhibition by 9 Apis mel and 15 Apis mel of human basophil degranulation induced by low dilutions of allergens. Nine Apis mel inhibited degranulation by about 60%, a result that is very similar to that observed in the present work on the 1.66×10^{-9} M anti-IgE-induced degranulation. By contrast, 15 Apis mel did not show any effect on such an anti-IgE-induced degranulation.

Of interest is also the presence of two zones of inhibition observed around 10 Apis mel and 20 Apis mel on the degranulation induced by 1.66×10^{-16}, 1.66×10^{-17} or 1.66×10^{-18} M anti-IgE and around 5 Lung his and 15 Lung his on the degranulation induced by 1.66×10^{-9} M as well as 1.66×10^{-16}, 1.66×10^{-17} or 1.66×10^{-18} M

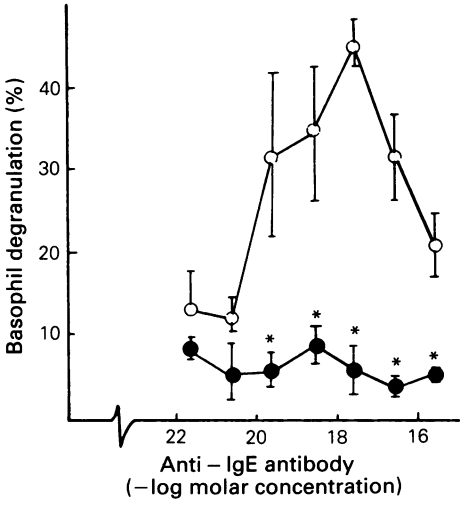

Figure 2 Basophil degranulation induced by 1.66×10^{-16} to 1.66×10^{-22} M anti-IgE antibody in the presence of either 15 Lung his (•) or saline serially diluted in saline as far as 15 Lung his (○) (mean ± s.e. mean, $n = 3$). $^*P < 0.05$.

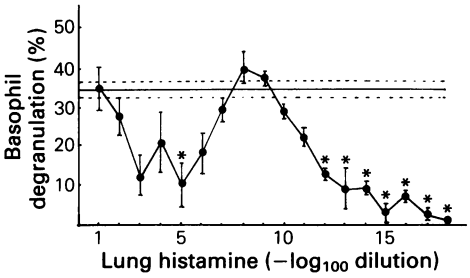

Figure 3 Basophil degranulation either induced by 1.66×10^{-16}, 1.66×10^{-17} or 1.66×10^{-18} M anti-IgE antibody in the presence of serial dilutions of Lung his from 1 to 18 Lung his. Control degranulations in the presence of Tris-buffered Tyrode's alone or saline serially diluted as 15 Lung his were 34.2 ± 2.1% and 35.0 ± 1.7% respectively (mean ± s.e. mean, $n = 3$). Control numbers were pooled in the figure (▭, ± s.e. mean, $n = 6$). $^*P < 0.05$.

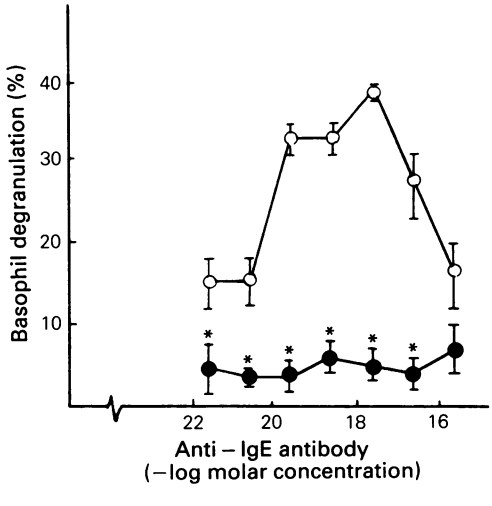

Figure 4 Basophil degranulation induced by 1.66×10^{-16} to 1.66×10^{-22} M anti-IgE antibody in the presence of either 9 Apis mel (•) or ethanol 65% serially diluted in saline as far as 9 Apis mel (○) (mean ± s.e. mean, $n = 5$). $^*P < 0.02$.

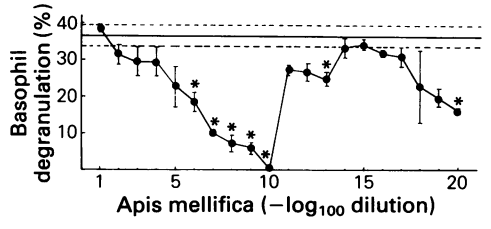

Figure 5 Basophil degranulation either induced by 1.66×10^{-16}, 1.66×10^{-17} or 1.66×10^{-18} M anti-IgE antibody in the presence of serial dilutions of Apis mel from 1 to 20 Apis mel. Control degranulations in the presence of HEPES-buffered Tyrode's alone or the dilution corresponding to 9 Apis mel without adding Apis mel in the starting solution were 35.9 ± 3.8% and 36.7 ± 2.4% respectively (mean ± s.e. mean, $n = 3$). Control numbers were pooled in the figure (▭, mean ± s.e. mean, $n = 6$). $^*P < 0.05$.

anti-IgE. The alternance of inhibition, inactivity and stimulation has already been observed in other assays (Boyd, 1954; Pelikan & Unger, 1971) but has never been explained.

Generally, biologists do not use concentrations where the molecule number is thought to be too low to exhibit a biological effect. How the high dilutions of anti-IgE antibody on the one hand and of Apis mel and Lung his on the other hand are capable of inducing basophil degranulation and inhibition of the degranulation respectively, remains unexplained until now. However, contaminants from plastic glass or air possibly diluted into water during the shaking procedure were irrelevant to the results observed since there was no effect with ethanol 65% or saline that underwent the same dilution procedure as Apis mel or Lung his, respectively. Also, we have repeatedly verified that the dilution procedures were correct by measuring, up to 3 CH, the dilution slope of various radioactive substances including histamine.

The results obtained in our laboratory with high dilutions of various compounds (Poitevin *et al.*, 1986; Davenas *et al.*, 1987; Davenas *et al.*, submitted for publication) indeed suggest that a 'biological information' has been transmitted to cells from a solution where no molecule could possibly be present. Only sophisticated biophysical and chemical means could yield a valid explanation to the type of interaction taking place between substance and solvent during the serial dilution procedure. However, no results are available up to now (see review in Scofield, 1984).

The present work demonstrates the biological effect of high dilutions of Apis mel and Lung his on human basophil degranulation but is not evidence for their clinical efficacy. In this way, some well-controlled human studies are necessary. Recently, the clinical effect of homeopathic drugs have been tested in two assays which demonstrated the efficacy of individualised homeopathy in rheumatoid arthritis (Gibson *et al.*, 1980) and the activity of high dilutions of mixed grass pollen in active hayfever (Reilly *et al.*, 1986). The clinical effect of high dilutions of Apis mel and Lung his should be investigated in allergic diseases in parallel with *in vitro* and *ex vivo* biological assays. More experimental assays on animal models or man are necessary to envisage the clinical application of highly diluted biological substances and precise the mode of action of homeopathic drugs.

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