Human basophil degranulation triggered by very dilute antiserum against IgE

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When human polymorphonuclear basophils, a type of white blood cell with antibodies of the immunoglobulin E (IgE) type on its surface, are exposed to anti-IgE antibodies, they release histamine from their intracellular granules and change their staining properties. The latter can be demonstrated at dilutions of anti-IgE that range from 1×10^{2} to 1×10^{120}; over that range, there are successive peaks of degranulation from 40 to 60% of the basophils, despite the calculated absence of any anti-IgE molecules at the highest dilutions. Since dilutions need to be accompanied by vigorous shaking for the effects to be observed, transmission of the biological information could be related to the molecular organization of water.

THE antibodies responsible for human immediate hypersensitivity belong to the IgE isotype[1]. The most salient feature of IgE is its capacity to bind to mast cell and polymorphonuclear basophil membranes through receptors with high affinity[2]. Human basophils are specifically challenged by immunological stimuli such as allergens or anti-IgE antiserum that can bridge IgE molecules in membrane[3]. This process triggers transmembrane and intracellular signals followed by granule exocytosis with the release of histamine and loss of metachromatic staining of basophil granules by a basic dye such as toluidine blue. Optical basophil degranulation is well correlated with other *in vitro* and *in vivo* procedures for the diagnosis of allergy[4-7].

In preliminary experiments, degranulation of human basophils contained in leukocyte suspensions was induced not only by the usual concentration of anti-IgE antibody (1×10^{3} dilution of anti-IgE antiserum, corresponding to 2.2×10^{-9}M anti-IgE antibody in the assay), but also by very low concentrations of this antibody ($2.2 \times 10^{-16/18}$M), where the number of IgG anti-IgE molecules in the assay is supposedly too low to trigger the process. We then further explored this phenomenon.

Serial tenfold dilutions of goat anti-human IgE (Fc) antiserum (1 mg specific antibody per ml) were prepared in HEPES-buffered Tyrode's solution containing human serum albumin (HSA) down to 1×10^{60} dilution, corresponding to a 2.2×10^{-66} M theoretical concentration (th) in the assay (see Fig. 1 legend for methods). The expected basophil degranulation, which was assessed by counting cells with metachromatical properties, was observed after exposure of leukocyte preparations to low antiserum dilutions with a maximum at $\sim 1 \times 10^{3}$ dilution. Successive peaks of degranulation varying between 40 and 60% were then found down to 1×10^{60} dilution, with periods of 6 to 9 tenfold dilutions (Fig. 1*a*). In other experiments, the antiserum was serially diluted a hundred-fold down to 1×10^{120} (to give 2.2×10^{-126} M th in the assay) and similar results were obtained (Fig. 1*b*). Degranulation induced by high dilutions of anti-IgE antiserum was observed in ten experiments on the full range of dilutions down to 1×10^{60}, when at least 70 similar results were obtained at one or the other part of the high dilution scale in the participating laboratories (Toronto, preliminary results). As controls, goat antihuman IgG (Fc) antiserum (Fig. 1*b*, $n = 4$) or Tyrode's solution containing HSA ($n = 5$) were diluted down to 1×10^{120} and 1×10^{30}, respectively. Cells incubated in conditions identical to those with anti-IgE antiserum gave no significant degranulation. The repetitive waves of anti-IgE-induced degranulation were reproducible, but the peaks of degranulation could shift by one or two dilutions with every fresh sequential dilution of anti-IgE and depended on the blood sample. The waves of basophil degranulation were also seen with substances other than anti-IgE anti-serum at high and low dilutions, such as monoclonal anti-human IgE antibodies, specific antigen in allergic patients or in peroxidase-immunized rabbits, phospholipase A_2 from bee venom or porcine pancreas, the Na^+ ionophore monensin (up to 90% degranulation at 1×10^{-30} M th) and the Ca^{2+} ionophores A23187 and ionomycin (1×10^{-38} M th). The specificity of the observed effects at high dilutions (already noted when comparing antiserum against IgE with antiserum against IgG) was further strikingly illustrated in the ionophore experiments, because removing the corresponding ion from the cellular environment blunted basophil

Table 1 Basophil counts after exposure to anti-IgE antiserum at low and high dilutions

Samples	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Tyrode's-HSA*	81.3±1.2†	89.0±3.1	81.7±2.2	106.7±1.8
Tyrode's-HSA	81.6±1.4	87.7±1.4	83.0±1.0	105.0±1.2
Tyrode's-HSA	80.0±1.5	88.0±2.3	81.7±1.8	105.7±0.9
aIgE 1×10^{35}*	35.5±1.8 (56)‡	42.3±4.8 (53)	27.7±0.7 (66)	40.0±1.5 (62)
aIgE 2×10^{32}	77.6±0.8 (4)	87.3±1.2 (3)	66.3±2.3 (18)	93.7±1.9 (12)
aIgE 1×10^{33}	76.0±1.1 (6)	88.7±1.8 (1)	77.7±1.8 (4)	74.7±2.8 (30)
aIgE 1×10^{34}	53.6±1.4 (33)	52.7±1.4 (41)	38.0±0.6 (53)	48.3±2.4 (55)
aIgE 1×10^{35}	45.0±0.5 (44)	35.0±1.0 (61)	41.3±1.8 (49)	49.3±1.2 (54)
aIgE 1×10^{36}	49.0±1.7 (40)	50.3±0.7 (44)	55.0±2.1 (32)	74.3±2.3 (31)
aIgE 1×10^{37}	79.0±2.3 (2)	85.3±0.7 (5)	73.3±1.7 (10)	105.3±0.7 (0)

Blind experiments: test tubes were randomly coded twice by two independent pairs of observers and assayed. The codes were simultaneously broken at the end of all experiments. Dilutions of anti-IgE antiserum were performed as described in legend to Fig. 1.
* Uncoded additional tubes for negative (Tyrode's-HSA) or positive (aIgE 1×10^{-3}) controls. † Data represent the mean ± s.e. of basophil number actually counted in triplicate (see legend to Fig. 1 for methods). ‡ Number in parenthesis indicates percentage degranulation compared with Tyrode's-HSA.

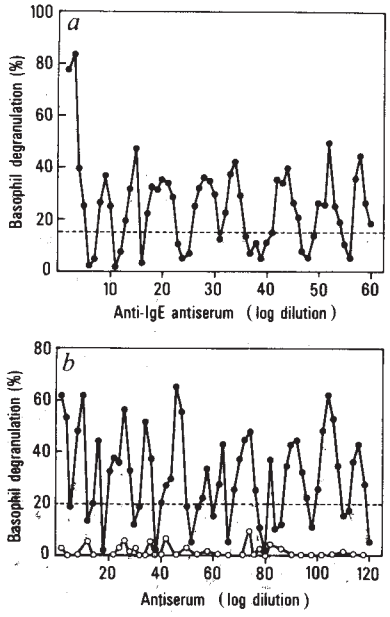

Fig. 1 Human basophil degranulation induced either by anti-IgE anti-serum (●) diluted tenfold from 1×10^2 down to 1×10^{60} (*a*) or hundredfold down to 1×10^{120} (*b*) or by anti-IgG antiserum (○) diluted hundredfold from 1×10^2 down to 1×10^{120} (representatives of at least 10 experiments for anti-IgE and 4 experiments for anti-IgG). The significant ($P < 0.05$) percentage of degranulation was 15% (*a*) and 20% (*b*). (....) relation to the number of counted basophils from control wells[15].

Methods Goat anti-human IgE (Fc) antiserum or as a control, goat anti-human IgG (Fc) antiserum (Nordic Immunology, The Netherlands) was serially diluted as indicated above in HEPES-buffered Tyrode's solution (in $g\ l^{-1}$: NaCl, 8; KCl, 0.195; HEPES, 2.6; EDTA-Na_4, 1.040; glucose, 1 human serum albumin (HSA), 1.0; heparin, 5000 U per l; pH 7.4). Between each dilution, the solution was thoroughly mixed for 10 s using a Vortex. Given the molecular weight of IgG molecules (150,000), the 1×10^{60} and 1×10^{120} dilutions correspond in the assay to 2.2×10^{-66} M (th) and 2.2×10^{-126} M (th) respectively. Venous blood (20 ml) from healthy donors was collected using heparin (1 U per ml) and a mixture of 2.5mM EDTA-Na_4/2.5 mM EDTA-Na_2 (final concentrations) as anticoagulants and allowed to sediment. The leukocyte-rich plasma was recovered, twice washed by centrifugation (400*g*, 10 min) and finally resuspended in an aliquot of HEPES-buffered Tyrode's solution. The cell suspension (10 μl) was deposited on the bottom of each well of a microtitre plate containing 10 μl $CaCl_2$ (5 mM final) and 10 μl of either of anti-IgE or anti-IgG antiserum dilutions. To a control well were added 10 μl $CaCl_2$ and 10 μl Tyrode's but no anti-IgE or anti-IgG antiserum. Plates were then incubated at 37°C for 30 min. Staining solution (90 ml; 100 mg toluidine blue and 280 μl glacial acetic acid in 100 ml 25% ethanol, pH 3.2 — 3.4) was added to each well and the suspension thoroughly mixed. Specifically redstained basophils (non-degranulated basophils) were counted under a microscope using a Fuchs-Rosenthal haemocytometer. The percentage of basophil degranulation was calculated using the following formula: Basophil no. in control − basophil no. in sample/ basophil no. in control × 100. Between 60 and 120 basophils were counted in cell suspensions from control wells after incubation either in the absence of anti-IgE antiserum, or in the presence of anti-IgG antiserum.

degranulation.

To confirm these surprising findings, four blind experiments were carried out (Table 1). In all cases the results were clear-cut, with typical bell-shaped degranulations at anti-IgE dilutions from 1×10^{32} to 1×10^{37}. The replicates were usually very close and of high significance (ANOVA test). In a fifth experiment, 7 control tubes and 3 tubes containing a dilution previously determined as active (1×10^{34}) were counted blind: basophil degranulation was 7.7 ± 1.4% for the controls, and 44.8, 42.8 and 45.7% for the tubes containing diluted anti-IgE. The random chance in all these experiments was 2% and therefore the cumulative results statistically confirm the measured effect.

Two further blind experiments were performed using the usual dilution procedure: of the 12 tubes used in the first experiment (Table 2), 2 tubes contained goat anti-human antiserum IgE at 1×10^2 and 1×10^3 dilutions, 6 tubes contained dilutions from 1×10^{32} to 1×10^{37}, and 4 tubes buffer-HSA alone. The tubes were then randomly coded twice by three parties, one of which kept the two codes. The 12 tubes were each divided into 4. Three batches of 12 tubes were lyophilized, one of which was used for gel electrophoresis, one for assay of monoclonal antibodies, and the last (with the unlyophilized sample) for gel electrophoresis and basophil degranulation. By comparing the results of the different tests it was easy to identify the tubes containing IgE at normal concentrations compared with the tubes containing highly diluted IgE and the control tubes. When the codes were broken, the actual results exactly fitted those predicted, but HSA and its aggregates were present in all solutions and complicated interpretation of the gel electrophoresis.

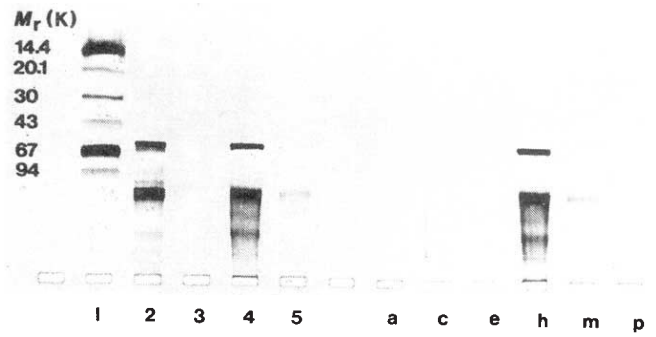

Fig. 2 Electrophoresis (polyacrylamide 7–15%, bands revealed by silver staining): samples numbered 1 to 5 are standards for the blind experiments *a*, *c*, *e*, *h*, *m*, *p*. Lane 1, Molecular weight standards for electrophoresis; lane 2, monoclonal IgG added with human serum albumin; lane 3, Tyrode's buffer without human serum albumin; lane 4, 1×10^2 anti-IgE dilution; lane 5, 1×10^3 dilution. Samples tested blind: *a* and *c*, buffer; *e*, 1×10^{36} anti-IgE dilution; *h*, 1×10^2 anti-IgE dilution; *m*, 1×10^3 anti-IgE dilution; *p*, 1×10^{35} anti-IgE dilution.

So we performed another almost identical experiment, using 6 tubes containing unlyophilized samples and buffer without HSA. Four tubes contained antibody at 1×10^2, 1×10^3, 1×10^{35} and 1×10^{36} dilutions, and 2 contained buffer alone. These tubes were coded and assayed according to the above protocol. The decoded results were clear-cut, high basophil degranulation being obtained with 1×10^2, 10^3, 10^{35} and 10^{36} dilutions, but no anti-IgE activity or immunoglobulins were detected either in the control tubes or in assays containing the 1×10^{35} and 10^{36} dilutions (Tables 2 and 3 and Fig. 2). Thus there is no doubt that there was basophil degranulation in the absence of any detectable anti-IgE molecule.

These results may be related to the recent double-blind clinical study of Reilly *et al.*[8] which showed a significant reduction of symptoms in hay-fever patients treated with a high dilution (1×10^{60}) of grass pollen versus placebo, and to our *ex vivo* experiments in the mouse[9]. We have extended these experiments to other biological systems: using the fluorescent probe fura-2, we recently demonstrated changes in intracellular Ca^{2+} levels in human platelets in the presence of the Ca^{2+} ionophore ionomycin diluted down to 1×10^{-39} M th (F. B. *et al.*, unpublished results).

Using the molecular weight of immunoglobulins and Avogadro's number, we calculate that less than one molecule of antibody is present in the assay when anti-IgE antiserum is diluted to 1×10^{14} (corresponding to 2.2×10^{-20} M). But in the experiments reported here we have detected significant basophil degranulation down to the 1×10^{120} dilution. Specific effects have also been triggered by highly diluted agents in other *in vitro* and *in vivo* biological systems[8–11], but still remain unexplained. The valid use of Avogadro's number could be questioned, but we are dealing with dilutions far below the Avogadro limit (1×10^{100} and below). It could be argued that our serial dilution procedure is subject to experimental error, but this is ruled out because: (1) pipette tips and glass micropipettes were discarded between each dilution (performed under laminar flow hood). (2) The c.p.m. in tubes containing serially diluted radioactive compounds decreased in proportion to the degree of dilution down to the background (data not shown). (3) Contamination would not explain the successive peaks of activity that evoke a periodic phenomenon and not a monotonous dose–effect curve, as usually observed when concentration of an agonist decreases. (4) To eliminate the possibility of contaminating molecules present in the highly diluted solutions, we carried out two series of experiments which can be summarized as follows. An Amicon membrane with molecular weight cut-off 10K retained the basophil degranulating IgG (150K) present at low dilutions (1×10^2, 1×10^3) in anti-IgE antiserum. By contrast, the activity present at high dilutions (1×10^{27}, 1×10^{32}) was totally recovered in the 10K Amicon filtrate. Anion or cation exchange chromatography,

Table 2 Comparison of basophil degranulation with the presence of immunoglobulins and anti-IgE activity in dilutions performed in HSA-containing Tyrode's

Samples	Basophil degranulation (%)*			Gel electrophoresis†		Anti-IgE activity
	I	II	III	*A*	*B*	μml^{-1}
Tyrode's-HSA	0	0	0	—	—	$< 1 \times 10^{-3}$
Tyrode's-HSA	0	0	0	—	—	$< 1 \times 10^{-3}$
Tyrode's-HSA	0	0	0	—	—	$< 1 \times 10^{-3}$
Tyrode's-HSA	0	0	0	—	—	$< 1 \times 10^{-3}$
aIgE 1×10^{-2}‡	53	50	33	++§	++	ND
aIgE 1×10^{-2}	51	44	37	++	++	10.6
aIgE 1×10^{-3}	65	38	45	+?	—	1.1
aIgE 1×10^{-32}	7	26	22	—	—	$< 1 \times 10^{-3}$
aIgE 1×10^{-33}	37	0	13	—	—	$< 1 \times 10^{-3}$
aIgE 1×10^{-34}	45	37	20	—	—	$< 1 \times 10^{-3}$
aIgE 1×10^{-35}	39	41	34	—	—	$< 1 \times 10^{-3}$
aIgE 1×10^{-36}	31	29	39	—	—	$< 1 \times 10^{-3}$
aIgE 1×10^{-37}	23	12	29	—	—	$< 1 \times 10^{-3}$

Blind experiments and dilution protocols as in Table 1. —, Lack of strained bands. ND, not determined. A faint band corresponding to IgG appeared after reduction by 2-mercaptoethanol.

* Basophil degranulation tests I, II, III were performed using 3 different blood samples (see Fig. 1). Percentage basophil degranulation induced by aIgE, as compared to Tyrode's HSA, was calculated from duplicates.

† Electrophoresis (polyacrylamide 7–15%, revealed by silver staining) was carried out in Rehovot (*A*) and at INSERM U 200 (*B*).

‡ Uncoded additional tube for positive control.

§ ++, + Bands correspond to IgG present in large or small amounts.

according to the type of resin used and the *p*H, did or did not retain the anti-IgE IgG at low dilutions, whereas the same activity at high dilution was always excluded from the columns and fully recovered in the first eluate. These filtration and ion-exchange experiments demonstrated that the activity of the antiserum at high dilution cannot result from contamination of the highly diluted solution with the starting material. They showed, in addition, that the high-dilution activity does not present in space the steric conformation of an IgG molecule as it acts like a 150K charged molecule, but is not retained by the 10K filter or by a charged chromatography column.

We then investigated the physical chemical nature of the entity active at high dilution. Our results can be summarized as follows. (1) The importance of agitation in the transmission of information was explored by pipetting dilutions up and down ten times and comparing with the usual 10-s vortexing. Although the two processes resulted in the same dilution (degranulations at 1×10^2 and 1×10^3 were superimposable whatever the dilution process), degranulation did not occur at high dilution after pipetting. Ten-second vortexing was the minimum time required, but vortexing for longer (30 or 60 s) did not increase high-dilution activity. So transmission of the information depended on vigorous agitation, possibly inducing a sub-molecular organization of water or closely related liquids. (2) The latter is possible as ethanol and propanol could also support the phenomenon. In contrast, dilutions in dimethylsulphoxide did not transmit the information from one dilution to the other, but increasing the proportion of water in dimethylsulphoxide resulted in the appearance and increment of the activity at high dilutions. (3) Heating, freeze-thawing or ultrasonication suppressed the activity of highly diluted solutions, but not the activity of several active compounds at high concentrations. A striking feature was that molecules reacted to heat according to their distinctive heat sensitivity, whereas all highly diluted solutions ceased to be active between 70 and 80°C. This result suggests a common mechanism operating at high dilution, independent of the nature of the starting molecule.

Therefore we propose that none of the starting molecules is present in the dilutions beyond the Avogadro limit and that specific information must have been transmitted during the dilution/shaking process. Water could act as a 'template' for the molecule, for example by an infinite hydrogen-bonded network[12], or electric and magnetic fields[13,14]. At present we can only speculate on the nature of the specific activity present in the highly diluted solutions. We can affirm that (1) this activity was established under stringent experimental conditions, such as

Table 3 Comparison of basophil degranulation with the presence of immunoglobulins and anti-IgE activity in dilutions performed in Tyrode's without HSA.

Samples	Basophil degranulation (%)		Gel electrophoresis		Anti-IgE activity
	I	II	*A*	*B*	(μml^{-1})
Tyrode's	0	0	—	—	$< 1 \times 10^{-3}$
Tyrode's	0	0	—	—	$< 1 \times 10^{-3}$
aIgE 1×10^{-2}*	85	48	++	++	ND
aIgE 1×10^{-2}	81	47	++	++	32.6
aIgE 1×10^{-3}*	ND	ND	+	+	ND
aIgE 1×10^{-3}	75	53	+	+	ND
aIgE 1×10^{-35}	35	31	—	—	$< 1 \times 10^{-3}$
aIgE 1×10^{-36}	40	35	—	—	$< 1 \times 10^{-3}$

* Uncoded tubes for positive control of basophil degranulation and/or gel electrophoresis.
ND, not determined.

blind double-coded procedures involving six laboratories from four countries; (2) it is specific for the ligand first introduced, as illustrated when goat antiserum (IgG) anti-human IgE, but not goat IgG anti-human IgG supported this phenomenon. The link between high and low anti-IgE dilutions is shown as we could not detect basophil degranulation at high dilutions if it did not occur within the classical range. High dilutions of histamine, but not of its carboxylated precursor histidine, inhibited IgE-dependent basophil degranulation. Finally, ionophores at high dilution did not work when the specific ion was removed from the cell suspension (F.B., unpublished results). (3) Using six biochemical and physical probes, we demonstrated that what supports the activity at high dilutions is not a molecule. (4) Whatever its nature, it is capable of 'reproducing' subtle molecular variations, such as the rearrangement of the variable region of an IgG (anti-ϵ versus anti-γ) molecule.

The precise nature of this phenomenon remains unexplained. It was critical that we should first establish the reality of biological effects in the physical absence of molecules. The entities supporting this 'metamolecular' biology can only be explored by physical investigation of agitation causing interaction between the original molecules and water, thus yielding activity capable of specifically imitating the native molecules, though any such hypothesis is unsubstantiated at present.

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Editorial reservation

READERS of this article may share the incredulity of the many referees who have commented on several versions of it during the past several months. The essence of the result is that an aqueous solution of an antibody retains its ability to evoke a biological response even when diluted to such an extent that there is a negligible chance of there being a single molecule in any sample. There is no physical basis for such an activity. With the kind collaboration of Professor Benveniste, *Nature* has therefore arranged for independent investigators to observe repetitions of the experiments. A report of this investigation will appear shortly. □