Human basophil degranulation is not triggered by very dilute antiserum against human IgE

S. J. Hirst*, N. A. Hayes*, J. Burridge†, F. L. Pearce‡ & J. C. Foreman*§

Departments of * Pharmacology, † Statistical Science and ‡ Chemistry, University College London, Gower Street, London WC1E 6BT, UK

We have attempted to reproduce the findings of Benveniste and co-workers, who reported in 1988 that degranulation of human basophil leukocytes is triggered by very dilute (10^2–10^{120}) antiserum against IgE. The results were contrary to conventional scientific theory and were not satisfactorily explained. Following as closely as possible the methods of the original study, we can find no evidence for any periodic or polynomial change of degranulation as a function of anti-IgE dilution. Our results contain a source of variation for which we cannot account, but no aspect of the data is consistent with the previously published claims.

We examined the effects of dilutions of anti-IgE from 10^2 to 10^{60} on human basophil degranulation. The original paper[1] stated that anti-IgE dilutions were effective only if vigorously shaken (succussed) during preparation. We have compared the effects of succussed anti-IgE, unsuccussed anti-IgE and succussed buffer. The 10^2 dilution of anti-IgE produced consistent, significant degranulation, but dilutions from 10^{12} to 10^{60}, whether succussed or not, failed to cause degranulation. In Fig. 1, the data from all three treatment types (succussed anti-IgE, unsuccussed anti-IgE and succussed buffer) have been pooled. The only apparent and significant effect of treatment on cell density occurs at the 10^2 dilution of anti-IgE. Any effect of the higher dilutions would have to be very subtle.

Results from the three different treatment types are shown separately in Fig. 2. Separate controls were included in each experimental session and the means for each of the dilution ranges (ranges 1, 2 and 3; see Fig. 1 legend) examined are shown in Fig 2. Mean cell density appears to depend on dilution of anti-IgE, but the control cell density also varies between experiments, which demonstrates the need for appropriate controls. Without

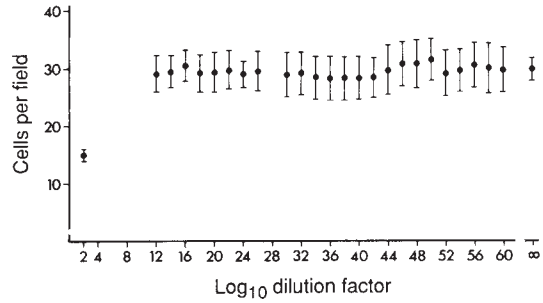

FIG. 1 Mean cell densities (with standard errors) as a function of dilution for all the data. The data for succussed anti-IgE, unsuccussed anti-IgE and succussed buffer have been combined. For the buffer control 10^{∞}) and anti-IgE dilution 10^2, $n=108$; for the other anti-IgE dilutions, $n=36$.

METHODS. Blood (120 ml) collected by venepuncture from a superficial ante-cubital vein was anticoagulated with heparin (1U ml^{-1}), EDTA-Na_2 and EDTA-Na_4 (each 2.5 mM) in saline (154 mM) containing 6% dextran T-70. Red cells were sedimented for 90 min at room temperature (21 °C), and the leukocyte-rich plasma (~70 ml) was recovered and centrifuged at 400g for 10 min. Pellets were resuspended in HEPES-buffered Tyrode solution (in g per l: NaCl, 8.0; KCl, 0.195; HEPES, 2.6; EDTA-Na_4, 1.04; glucose, 1.0; human serum albumin, 1.0) containing heparin (5,000 units per litre) at pH 7.4 and recentrifuged. Pellets were resuspended in HEPES-buffered Tyrode solution and pooled before further centrifugation. The final pellet was resuspended in 1.5 ml HEPES-buffered Tyrode solution. Anti-IgE (goat anti-human IgE (Fc) from Nordic Immunology, UK; stored frozen at −75 °C in 10-μl aliquots of 1 mg ml^{-1} in sterile deionized water) was serially diluted in triplicate by 100-fold in the range 10^2 to 10^{60} in HEPES-buffered Tyrode solution in polystyrene tubes. Between each dilution, solutions were mixed as required by the protocol by vigorous mechanical shaking (succussion) for 10 s. Mechanical shaking was standardized using a device (on loan from Nelson's Homeopathic Pharmaceutical Co.) with which the tube containing the solution would strike a rubber bung roughly 20 times per second. In some experiments, dilutions of HEPES-buffered Tyrode solution were shaken in the same way. The prepared solutions were taken to a separate room away from the laboratory and an independent person working alone applied a code to each tube using random number tables. The key to the code was sealed in an envelope and retained until the experiment was completed. The numbered tubes were then shuffled and returned to the person conducting the experiment. It was not possible for the person performing the experiment to know the contents of the tubes. To each coded tube, 5 μl $CaCl_2$ was added to give a final concentration of 5 mM, followed by 30 μl of the cell suspension prepared as described. The tubes were incubated at 37 °C for 30 min, then centrifuged at 400g for 10 min at room temperature. Pellets were resuspended in 100 μl staining solution (100 mg toluidine blue and 280 μl glacial acetic acid in 100 ml 25% ethanol in deionized distilled water). Dense purple-staining (non-degranulated) basophils were counted using an improved Neubauer haemocytometer at ×500 magnification. Cells were counted by a single, trained individual who recorded the number of cells in each high-power field and the number of fields, to provide a total cell count of ~100 per sample. To ensure that cells were counted within a reasonably short time after preparation, experiments were conducted in sessions, which consisted of 30 samples comprising triplicates of 10 different treatments. Each session was completed in a single day. The 10 treatments in a session were: the control of buffer alone (10^{∞}), the control low dilution of anti-IgE (10^2) and 8 different high dilutions. These high dilutions were either succussed anti-IgE, unsuccussed anti-IgE or succussed buffer. A total of 36 sessions were conducted as follows. Succussed anti-IgE (treatment A): 10^{12}–10^{26} (range 1), repeated 5 times; 10^{30}–10^{44} (range 2), repeated 5 times; and 10^{46}–10^{60} (range 3), repeated 5 times; unsuccussed anti-IgE (treatment B): same three dilution ranges, repeated 4 times; succussed buffer (treatment C): same three dilution ranges, repeated 3 times.

§ To whom correspondence should be addressed.

TABLE 1 Results of tests on mean differences and mean cell densities

Treatment type‡	Dilution range	*t*-Tests* on mean differences: Maximum *t*-statistic	Bonferroni bounds for *P* value	*F*-tests† on mean cell densities: d.f. n_1	n_2	Fn_1, n_2	*P* value§
	1	2.003	$0.116 < P < 0.926$			0.269	0.972
A	2	0.888	$0.425 < P < 1$	8	32	0.317	0.954
	3	2.970	$0.041 < P < 0.329$			1.849	0.104
	1	1.722	$0.184 < P < 1$			0.806	0.604
B	2	1.089	$0.356 < P < 1$	8	24	0.792	0.615
	3	1.986	$0.141 < P < 1$			0.567	0.794
	1	2.041	$0.138 < P < 1$			0.686	0.698
C	2	3.050	$0.093 < P < 0.742$	8	16	0.683	0.701
	3	2.696	$0.114 < P < 0.915$			1.551	0.216

* The *t*-tests test separately for each dilution whether the mean difference is significantly different from zero. There are 8 such tests within each dilution range and the value given (mean control cell count − mean treatment cell count) is the maximum of these. The degrees of freedom are 4, 3 and 2, respectively, for treatment types A, B and C.

† The *F*-tests allow for an additive 'session' effect and test whether there are differences among the high-dilution and control means. The tests are based on 45, 36 and 27 observed means for treatment types A, B and C, respectively.

‡ Treatments: type A, succussed anti-IgE; type B, unsuccussed anti-IgE; type C, succussed buffer.

§ The *F*-tests are based on independent groups of sessions and their combined (Fisher) *P* values for treatment types A, B and C are all between 0.5 and 0.9.

these controls, the data could be interpreted in terms of an effect of high dilution on cell density. Such variation in controls has resulted in the customary practice of presenting the data as percentage degranulation rather than cell density. Davenas *et al.*[1] reported data in terms of percentage degranulation without showing the unmanipulated cell density data.

Figure 3 shows the same data as Fig. 2, but the ordinate is now percentage degranulation. As expected from the definition of percentage degranulation, values are distributed on either side of zero, in contrast to the data of Davenas *et al.*[1], in which there were no values of percentage degranulation below zero. The data in Fig. 3*a* do appear to reveal an effect of the higher dilutions of succussed anti-IgE. In these experiments there is a natural null hypothesis to test, namely the treatment applied to the cells produces a response which is not different from the response in the absence of a treatment. Because of this, statistical evaluation of the data relies on *P* values. Percentage degranulations do not have the expectation of zero even when the null hypothesis is true and so *t*-tests have been performed on differences defined as mean cell density of the control (buffer) minus the cell density of the treated sample.

The *t*-tests applied to the mean differences for the 10^2 dilution of anti-IgE revealed that the differences from zero were all highly significant: $P \ll 0.0001$. The *t*-tests for the mean differences for the high dilutions are given in Table 1. None of the *P* values reaches the level of statistical significance, with the exception of the high dilution range for the succussed anti-IgE, for which the *P* value indicates borderline significance. Two-way ANOVA *F*-tests were performed on the high-dilution mean cell densities (Table 1). This test examines the hypothesis of no difference between dilutions, and in this case none of the *P* values reached the level of statistical significance. It therefore seems likely that the 'significant' *t* statistic in the highest dilution range for succussed anti-IgE is a chance result. This 'significant' effect was much smaller than anything reported by Davenas *et al.*[1]. The nature of the degranulation assay is such that variability is large: it depends on counting a small number of cells (basophils constitute about 1% of circulating leukocytes) and requires a subjective assessment of stained cells. Also, for any given set of random numbers, it is expected that there will be occasions when the examination of randomness by statistical tests fails.

Davenas *et al.*[1] found effects that seemed to disappear and reappear at different dilutions. We therefore examined the data for polynomial trends or periodic cycles in mean percentage

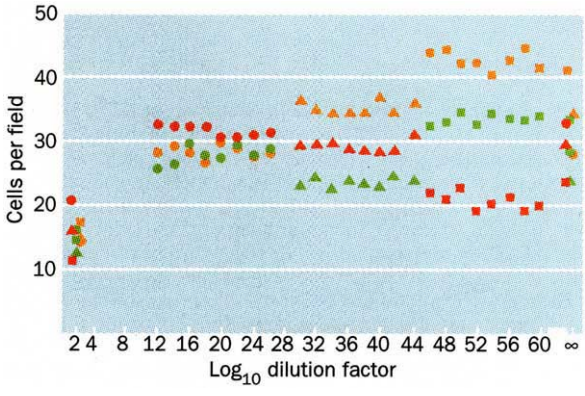

FIG. 2 Mean cell densities as a function of dilution for the separate treatments. Red, Succussed anti-IgE (treatment A), $n = 15$; Green, unsuccussed anti-IgE (treatment B), $n = 12$; Yellow, succussed buffer (treatment C), $n = 9$. ●, range 1 data; ▲, range 2 data; ■, range 3 data. The standard error bars have been omitted for clarity, but for the high dilutions the standard errors were about ±9, which is fairly wide mainly because of between-session variation.

TABLE 2 ANOVA tests (*P* values) for differences between dilutions separately for each session

Treatment type*	High-dilution range 10^{12}–10^{26}	10^{30}–10^{44}	10^{46}–10^{60}
A	0.34	0.028	0.38
(combined 'Fisher'	0.066	0.018	0.21
P value = 0.0027)	0.14‡	0.42	0.17
	0.0043§	0.42	0.21
	0.70†	0.80	0.40
B	0.56	0.25	0.27
(combined	0.27	0.97	0.27
P value = 0.086)	0.0073‖	0.76	0.16
	0.084	0.48	0.44
C	0.92	0.80	0.66
(combined	0.25	0.25	0.21
P value = 0.85)	0.65	0.91	0.71

ANOVA tests used the one-way *F*-test with (8, 18) degrees of freedom, using control and high-dilution treatments in each session; that is, the 10^2 dilutions were excluded.

* See legend to Table 1 for treatment types.

† The missing value in this session means that the *F*-test used (8, 17) degrees of freedom.

‡ See Fig. 4, Yellow. § See Fig. 4, Red. ‖ See Fig. 4, Green.

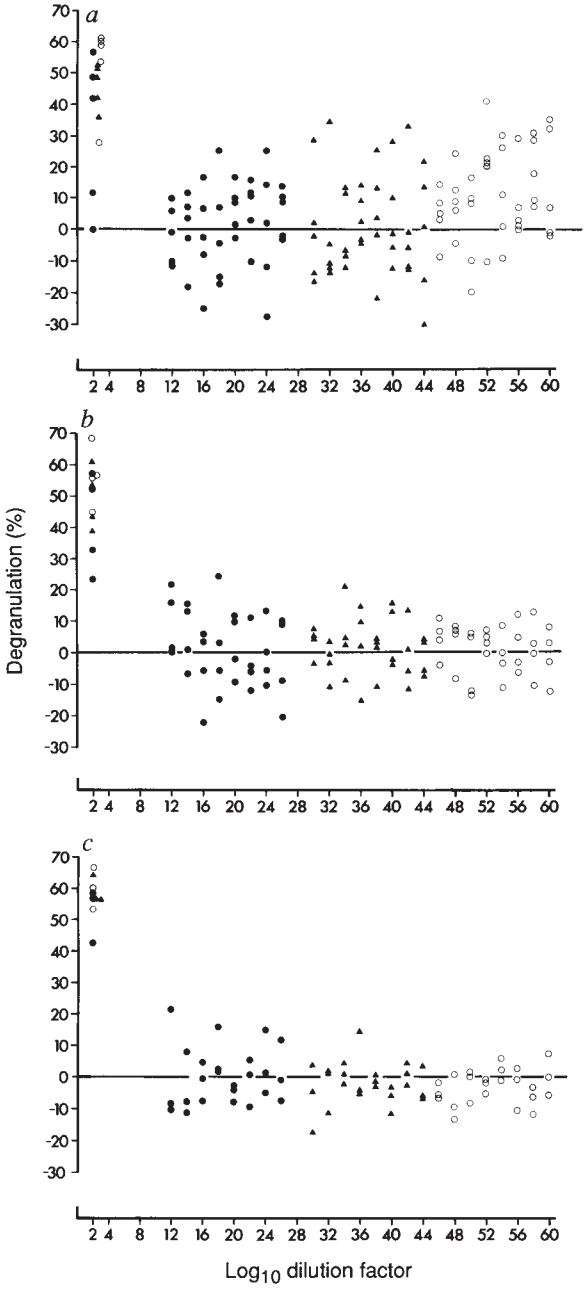

FIG. 3 Percentage degranulation of basophils as a function of dilution. Each point is the mean of triplicate determinations in a single experiment. *a*, Successed anti-IgE (treatment A); *b*, unsuccessed anti-IgE (treatment B); *c*, successed buffer (treatment C). ●, Range 1 data; ▲, range 2 data; ○, range 3 data. The percentage degranulation was calculated as 100 × (1 − treatment sample cell density/mean control sample cell density).

degranulations. With one exception, the P values for constant, linear, quadratic, cubic and periodic events with two, three or four cycles were greater than 0.1. The only convincingly significant feature ($P = 0.0015$) was the linear component of trend for the highest dilution range of successed buffer, which is inconsistent with Davenas *et al.*[1] and with conventional scientific theory.

Cell counts for fixed volumes of cell suspension should exhibit Poisson variation, possibly with some 'overdispersion' resulting from sources of variation such as counting or data recording errors, variations in volumes, and so on. In our data, each triplicate corresponding to a single dilution level within a particular experiment (session) might be expected to exhibit such extra-Poisson variation. This was quantified by using the χ^2 statistic to

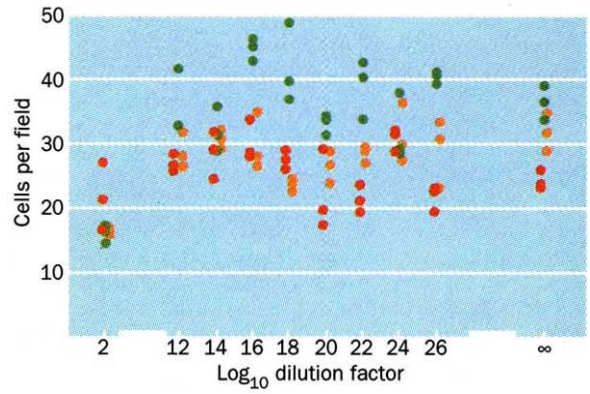

FIG. 4 Basophil densities as a function of dilution from single experiments showing the scatter of the triplicate values. Red, Successed anti-IgE (treatment A); Green, unsuccessed anti-IgE (treatment B); Yellow, successed anti-IgE (treatment C). See Table 2 and text for explanation.

compare the observed counts in each triplicate with the expected counts. The expected counts were calculated from the estimated mean count per field. Ten χ^2 statistics (with 2 degrees of freedom) were calculated for each 'session' (one for each triplicate) and summed to give a χ^2 statistic with 20 degrees of freedom. The 36 χ^2 values (one for each 'session') were generally in excess of 20, suggesting that the anticipated overdispersion was indeed present. Overall, the data suggest that the counting variance was about 1.5 times the pure Poisson variation.

According to conventional scientific theory, there should be no differences within a session between the control treatment and the eight high-dilution treatments. This can be tested separately for each session by applying the conventional one-way ANOVA F-test to the mean counts (cells per field) for each tube. The resulting P values in Table 2 are curious because the P values should, if the null hypothesis is correct, be uniformly distributed between 0 and 1. This is not the case for treatments A (successed anti-IgE) and B (unsuccessed anti-IgE), for which the P values are collectively too small (Table 2). The triplicates appear to differ from one another. Figure 4 (red and green points) shows the data with the most significant P values of 0.0043 and 0.0073. These differences detected by the F-test are small, and the most obvious ones are shown in red and green. In contrast, the yellow points in Fig. 4 show the more typical case, in which the differences were not significant ($P = 0.14$). There is no regular or reproducible pattern in this 'between triplicate' variation and it is a feature of both unsuccessed and successed anti-IgE treatments. We do not think that this can be explained by the method of preparation of the dilutions and it is not attributable to repeated dilution effects. Although it is possible that these observed effects are a statistical artefact, some unidentified part of our experimental procedure might account for them. It is an interesting feature of our data but it does not, of course, lend any support to the findings of Davenas *et al.*[1], and serves once again to underscore the complexity of the analysis of variance in an assay of this type.

We have been unable to find any evidence that very high dilutions of anti-IgE, successed or unsuccessed, cause any reproducible effects on the degranulation of human basophil leukocytes. □

Received 16 April; accepted 22 October 1993.

1. Davenas, E. *et al. Nature* **333,** 816–818 (1988).

ACKNOWLEDGEMENTS. We thank the Research Council for Complementary Medicine for financial support of this work, Nelson's Homeopathic Pharmaceutical Company for loan of the succussion apparatus, and the volunteers who generously gave their blood for these experiments.