

The human basophil degranulation test as an *in vitro* method for the diagnosis of allergies

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Summary

We have designed a human basophil degranulation test based on a one-step method of basophil staining after exposure to a specific allergen. This very simple test is now commercially available as a ready-to-use kit. It explores the allergic sensitivity at the cellular level, probably through IgE-dependent sensitization of the basophil. It positively correlates with histamine release and radioallergosorbent test (RAST). It detects sensitivity to common allergens and to chemical and food products as well. In its present state, the test provides a simple, inexpensive specific means for *in vitro* diagnosis of allergic diseases. Recently, it has been further developed to be performed on the automatic Hemalog D Technicon machine.

Introduction

The availability of several different methods for measuring total and specific serum IgE represents an important improvement in the diagnosis of allergic states. However, immediate hypersensitivity reactions in man or in experimental animals do not depend directly upon circulating IgE antibodies, but upon the release of mediators from IgE-sensitized mastocytes and basophils. Therefore, *in vitro* methods developed for diagnostic purposes should reflect the end stage of the reaction, i.e. the degranulation of the cells, as closely as possible. It is therefore not surprising that the diagnosis of allergies based on cellular reactivity has been the subject of research for many years (Shelley & Juhlin, 1961; Klopstock, Schwartz & Grinberg, 1962; Hirsch & Zastrow, 1972; Shelley, 1974; Leynadier, Luce & Dry, 1977; Siraganian, 1977; Moneret-Vautrin, Grilliat & L'Hoste, 1978; Dry, Leynadier & Luce, 1979). In our laboratory, we have developed a human basophil degranulation test (HBDT) which meets the following specifications: (i) it detects basophil IgE-dependent degranulation with a high antigenic specificity; (ii) utilizes whole blood, without previous purification of the baso-

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phils, which simplifies the technique and prevents the loss of cells caused by cell fragility; (iii) utilizes very common technical apparatus available in any laboratory or even to doctors; (iv) is very simple to perform and does not require a long training period for routine use by the laboratory technician.

This test is based on a one-step specific method of staining of blood basophils. This is accomplished by simply mixing the blood with a toluidine blue solution that lyses the erythrocytes, fixes the leucocytes, and stains the basophils metachromatically (Benveniste *et al.*, 1977). If the blood has been exposed to the antigen before staining, the degranulated basophils do not stain and cannot be counted. The apparent loss of cells eliminates the necessity to examine cells individually to evaluate the degree of degranulation: those which degranulated during exposure to the antigen are the uncounted basophils. Therefore, variations due to individual interpretation of the results are greatly reduced as compared to previously published methods. We have already published the experimental data obtained from using this test in rabbits (Benveniste *et al.*, 1977). We present herein the first clinical results obtained in allergic and non-allergic patients, and compare the HBDT with other *in vitro* and *in vivo* diagnostic parameters. The materials necessary to perform this test are now commercially available in France for ninety-nine allergens (Baso-kit, Stallergènes, Joinville-le-pont, France).

Materials and methods

Clinical evaluation

Patients examined in allergy clinics were evaluated according to medical history and intradermal skin test, with the exception of patients with drug sensitivities who were diagnosed by medical history alone. Most of these patients had never undergone hyposensitization therapy. The skin tests were performed utilizing allergens of the same origin as those used for the *in vitro* studies. For intradermal skin tests, the highest dilutions were 1/5000 (w/v) of house dust allergen and 1/10 000 w/v of animal danders and pollen. Allergens (0.02 ml) were injected in the upper arm with a disposable syringe and the reactions were observed after 15 min. Skin test grading was based on measurements of the weal utilizing the following criteria: the site was called negative when it was no different from a diluent control and positive when the weal was above 0.5 cm.

Allergens

Glycerinated allergens were obtained from either the Institut Pasteur (Paris) or the Laboratoire des Stallergènes. Cat, dog and horse dander, house dust and grass pollens were utilized in starting concentrations of 10 mg/ml. The drugs which were used to cause reactions in the patients were furnished by the manufacturers or purchased from pharmacies. We purposely did not use pure substances.

Allergens were diluted in an aqueous Ca^{++} and Mg^{++} -containing solution (usually from 10^{-1} to 10^{-4} $\mu\text{g}/\text{ml}$, final concentration in blood) and distributed in each well of microtitre Cooke plates in the following order: the first and last wells (A and H) received diluting solutions without allergens; all those in between (B to G) received serial tenfold dilutions of the allergens. Other plates, designated as 'control plates' received identical concentrations of allergens diluted in a solution containing neither calcium nor magnesium. The solutions were left to evaporate for 12 hr at 37°C and the

plates were then stored on the laboratory shelves for periods of up to 2 years. Recently, we have used the commercially available kits with results identical to those obtained using the 'home-made' plates.

Counting of basophils

Basophils were counted on the basis of their metachromatic staining with 0.025% toluidine blue in 30% ethyl alcohol adjusted to pH 3.4–3.6 with 4 μ l/ml glacial acetic acid. Staining was achieved by mixing 90 μ l of this solution with 10 μ l of whole blood. The technique has been previously described using rabbit blood (Benveniste *et al.*, 1977). However, for human blood we used double-chamber Fuchs-Rosenthal haemocytometer with a volume of 3.2 mm³ each instead of the Malassez. Utilizing this technique, the basophils were the only cells with stained cytoplasm. They appeared red and were very easily identifiable against a clear background. The nuclei of the other leucocytes faintly stained light blue which allowed us to obtain the total leucocyte count within the same chamber used for basophil counts. We took counts from both chambers of the haemocytometer. Given the volume of the chamber and the tenfold dilutions we counted between 15 and 20 basophils per chamber. The average of the two basophil counts obtained on each haemocytometer was multiplied by 3.13, in order to calculate the number contained per mm³ of blood. The total number of leucocytes in each well was counted and this number used as an index for the reliability of the dilutions. In healthy individuals, the average number of basophils counted per mm³ on 121 samples was $44 \pm 8/\text{mm}^3$ (mean $\pm$ 1 s.d.). When less than 20 basophils/mm³ were counted, it was usually impossible to perform the test. However, we have recently used the buffy-coat obtained by 10 min centrifugation at 200 *g* instead of whole blood (Gérard, Legras & Monneret-Vautrin, 1980). This increased three to tenfold the number of basophils counted in the chambers, thereby notably decreasing the number of rejected patients (now less than 1%).

Degranulation of basophils (Fig. 1)

This technique was performed either on whole blood or on blood which had been centrifuged and washed with tris-buffered Tyrode's to remove the plasma constituents as described in Benveniste *et al.* (1977). One sample was resuspended in plasma and the other in Tyrode's solution to reconstitute the original volume. They were then subjected to the degranulation procedure which was performed as follows: the cells suspended in plasma or in Tyrode's solution were gently placed in the bottom of two parallel rows of wells on the microtitre plates. Adequate mixing with the pre-evaporated antigen was obtained by repeatedly aspirating the cells up and down the micropipette. The plates were then incubated for 10 min at 37°C. After incubation, 90 μ l of the staining solution was added to each well and immediately agitated. The contents of the wells were transferred to the counting chambers and allowed to settle for 5–10 min. During the counting procedure, the plates were covered with a wet filter paper to minimize evaporation. Each sample was counted twice. The maximum degranulation (i.e. the well with the lowest count) was calculated and the test was then repeated with the same concentration of antigen in one well of the 'no calcium' control plate. The percentage of degranulation was calculated using the following formula:

$$\frac{\text{basophil no. in control sample} - \text{basophil number in test samples}}{\text{basophil no. in control sample}} \times 100$$

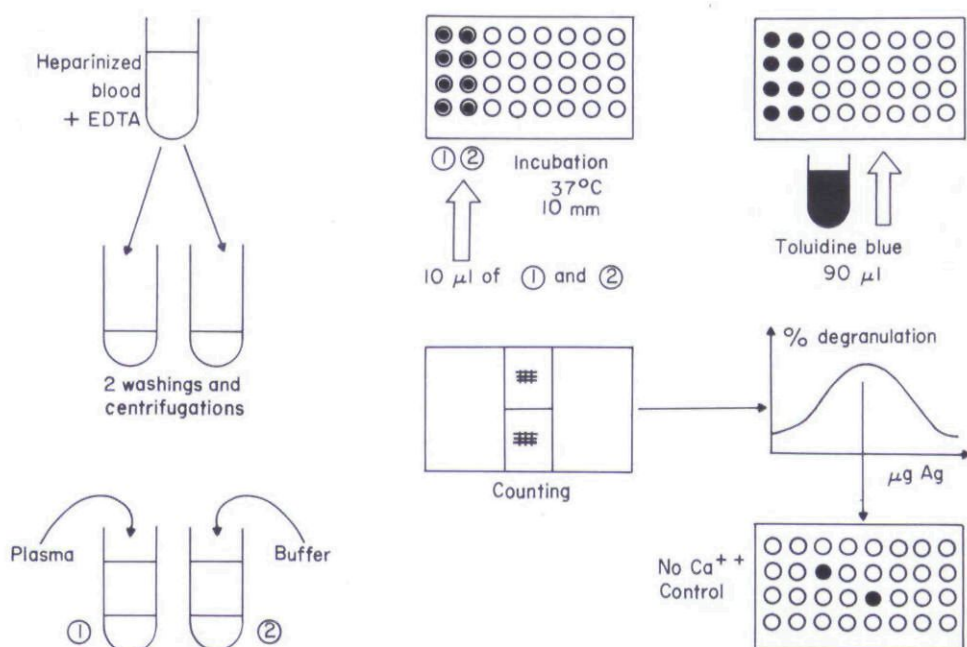


Fig. 1. Procedure for performing the manual HBDT. The blood drawn on a mixture of heparin and ethylene-diamine-tetra-acetate (EDTA), can be used either as such, or after two washings and centrifugations. Aliquots (10 μ l) of 'whole' (1) and 'washed' (2) blood are distributed into the eight wells of a vertical row of the microtitre plate (only four wells are shown in the fig.). After agitation and incubation at 37°C for 10 min, 90 μ l of the staining solution are added in each well. The 100 μ l of blood/solution mixture are transferred into the Fuchs-Rosenthal haemocytometer. When the concentration inducing maximal degranulation is determined, a test is performed with the same concentration in a control plate, without Ca^{++} . Results are expressed as percentage maximal degranulation and the corresponding concentration of allergen.

When the 'no antigen' control differed by more than 30% from the 'no calcium' one, the test was rejected. According to Poisson's law, differences between a test well and the mean of the two control wells were significant above 40% at the $P \leq 0.05$ level when 30 basophils were counted in the control wells, at 40% when 40 basophils were counted, and at 30% when there were 60 basophils (statistical study by J. Macario & C. Clauss). These limits were the basis for determining whether a test was positive or not. In fact, the distribution of the basophils on the haemocytometer is gaussian, especially when buffy-coat is used instead of whole blood.

Preliminary studies have shown that the degranulation never reached significant levels in fifty-seven 'non-atopic' patients tested, except in the case of house dust allergen in which three patients with positive tests ultimately demonstrated allergic symptoms. We systematically tested any new antigens on a minimum of six patients allergic to substances other than the tested antigens. Their basophils never degranulated in the presence of the non-specific antigen. The specificity of the test was illustrated in this preliminary study by the non-cross reaction to bee and wasp antigens, or to different species of grass pollen.

Comparison of HBDT with histamine release

Thirteen experiments were performed upon six patients clinically allergic to grass

pollens in order to compare the HBDT with the release of histamine from blood leucocytes. Fifty ml of blood were collected and the leucocytes were separated according to published techniques (Lichtenstein & Osler, 1964). HBDT was performed on 'whole' and 'washed' blood, and on aliquots of the leucocyte preparations used for histamine release. The amount of histamine was measured by the spectrofluorometric automated method (Ruff *et al.*, 1967). These experiments were performed in cooperation with Dr B. David, Institut Pasteur, Paris.

Radioallergosorbent test RAST

This test was performed according to routine technique using commercially available kits (Pharmacia, Uppsala, Sweden).

Results

An example of the actual data obtained with the HBDT is given in Table 1. A positive correlation was found between the percentage of histamine released from purified leucocyte preparations and the degranulation observed in the same cell preparations, both in the presence of grass pollens (Fig. 2). In this experiment, six patients were studied, three thrice, one twice and two once, at four antigenic concentrations. Next, the numbers of positive HBDT were compared to those obtained using the skin test and RAST (Table 2). In the case of dust allergy, the HBDT was a little more sensitive than the RAST. We obtained significant (over 35%) degranulation in thirty-seven out of forty-one patients but, in fact, the test was not performed on four patients because of technical limitations due to low basophil counts (before use of the buffy-coat technique). The test was positive in all the thirty-seven HBDTs actually performed. In the grass pollen-sensitive patients, the HBDT did as well as the RAST; one patient was RAST-negative and HBDT-positive. Again, the HBDT was positive in all twenty-six tests actually performed. In the sixty-three positive patients, the overall percentage of

Table 1. Actual results of four HBDT

Antigen concentration in wells ($\mu\text{g/ml}$)	Patient 1		Patient 2 <i>Candida albicans</i>	Patient 3 Aspirin*
	House dust	Grass pollens		
None	24†	22	51	30
10^2	20	24	20	19
10^1	10	22	18	19
10^0	10	20	24	9
10^{-1}	4	20	29	4
10^{-2}	16	16	26	19
10^{-3}	12	16	20	29
'No calcium'‡ well	20	24	47	25
Degranulation	82% at 0.1 ($\mu\text{g/ml}$)	· 0	63% at 10 ($\mu\text{g/ml}$)	85% at 0.1 $\mu\text{g/ml}$)

* Lysin acetylsalicylate (Aspegic).

† Number of basophils actually counted.

‡ Performed with the concentration of antigen inducing maximal degranulation.

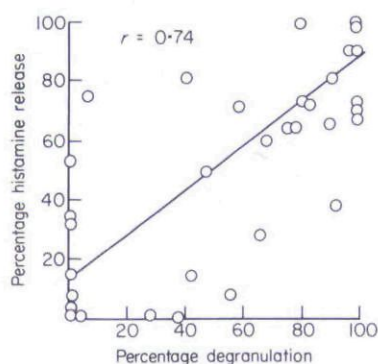


Fig. 2. Correlation between percentage of histamine released from purified leucocyte preparations and of degranulations observed in the presence of grass pollens.

basophil degranulation as a result of exposure to grass pollen and house dust was $64 \pm 17.6\%$ (mean of the maximal degranulation—whatever the concentration of the antigen—in individual patients ± 1 s.d.).

A double-blind study was performed on fifteen volunteers with or without sensitivity to ragweed (in cooperation with Dr Gavin, Miles Laboratories, Elkhart, Indiana, U.S.A.) (Table 3). The presence of a clinical sensitivity was related to skin tests, RAST

Table 2. HBDT and RAST in patients with positive skin tests to dust and grass pollens

Allergens	Number of patients	Not done*	RAST	HBDT
Dust	41	4	24/29 (82)†	37/41 (90)
Pollens	29	3	25/26 (96)	26/29 (90)

* In this and the following Tables, 'not done' indicates that the number of basophils was insufficient to perform the test.

† Number of positive HBDT over number of performed tests (%).

Table 3. Double-blind study on seven ragweed sensitive and eight ragweed non-sensitive volunteers

Patient no.	Clinical sensitivity	Skin test	RAST	HBDT
7	—	—	—	—
5	+	+	+	+
1	+	—	—	—
1	+	+	—	+
1	—	+	—	—

Table 4. Study on forty-five grass pollen sensitive patients

Number of tests	Not done	Number/done	Positive	Blocking factors	
			Number/total	Partial	Complete
Number	64*	16	42/48	42/64	9/48
Percentage		25	87	67	18
					5/48
					10

* Nineteen patients were tested twice.

Table 5. HBDT in sixty allergic patients; presence of blocking factors

Allergens	Patient no.	Not done	'Whole' blood	'Washed' blood	Difference % 'washed-whole blood'
Pollens	15	3	8/12 (66)*	11/12 (91)	25
Dust	15	3	8/12 (66)	8/12 (66)	0
Cat	10	1	7/9 (77)	8/9 (89)	12
Dog	13	2	8/11 (73)	10/11 (91)	18
Horse	7	0	6/7 (86)	7/7 (100)	14

* Number of positive HBDT over number of performed tests (%)

and HBDT. One patient negative for HBDT was also negative for RAST and skin tests. One patient negative for RAST was positive for HBDT and skin tests.

Sixty-four HBDTs were performed upon forty-five patients sensitive to a mixture of grass pollens (Table 4). In this series, which was performed at a very early stage in the test's development, we encountered a very high (25%) rejection rate because of insufficient basophil counts. Positive HBDT were obtained in 87% of the actually performed tests. We found a partial inhibition of degranulation in 18% of the patients, i.e. more than 1 log of antigen concentration was needed to obtain the same degranulation in plasma as compared to buffer. Inhibition was complete in plasma at all antigen concentrations in 10% of the patients. When the test was repeated twice (nineteen patients, usually within 2 months) the results were very similar with 2 log variations of the antigen concentration causing maximum degranulation.

The presence of blocking factors was further investigated in fifty-seven patients sensitive to various allergens as indicated by their medical histories and skin testing (Table 5). The number of patients tested for each allergen is too small to draw definitive conclusions but this study is indicative of the high sensitivity of the test and of the importance of performing the test on washed blood in order to increase this sensitivity and detect plasma blocking factors.

One-hundred and nine patients demonstrating reactions to various drugs (Table 6) were explored with the HBDT. The results indicate a high percentage of positive degranulation, even in patients in whom the reaction could not be identified as a typical type I reaction.

Table 6. 104 Patients with drug reactions

	Number of drug reactions	Not done	Number of reactions —type I	Positive HBDT
Penicillin	34	11	7/23	19/23 (83)*
Ampicillin	26	4	4/22	10/22 (45)
Aspirin	40	8	9/32	23/32 (72)
Iodine	4	0	3/4	3/4 (75)

* Number of positive HBDT over number of performed tests (%).

Discussion

Diagnostic tests based on the study of effector cells—namely the basophil in humans—explore three important parameters in the onset of allergic symptoms: (i) the production of IgE antibodies; (ii) their subsequent fixation on the cell membrane; and (iii) the capacity of the cell to degranulate in the presence of the allergen. RAST explores only the first step, and it is known experimentally and clinically that allergy can develop without detection of circulating specific IgE (Benveniste *et al.*, 1977; Perelmutter & Potvin, 1977; Samsoen, 1978) and that the presence of these antibodies is not a positive indicator of an allergic state (Samsoen, 1978; Saurat *et al.*, 1976). Skin and provocation tests explore these three parameters plus the reactivity of the vasculature to the mediators released during cell degranulation. Although these tests retain a useful role, they are subject to several practical limitations and are not totally innocuous, hence the efforts of several authors to develop a diagnostic test based on cell reactivity. It has also been proposed to measure the amount of histamine liberated in the presence of the antigen *via* an automated spectrofluorometric method but the complexity of the technique and the costs of the apparatus has hampered its widespread use (Siraganian, 1977). The first attempts to use degranulation itself as a diagnostic procedure were unsuccessful due to the complexity of the technique and variability of the counting procedure (Shelley & Juhlin, 1961). It was necessary to examine the basophils present in the microscopic field one by one in order to establish a degranulation score. Other techniques have been proposed and were successfully applied to the allergic diagnosis, but did not reach the routine stage (Klopstock *et al.*, 1962; Hirsch & Zastrow, 1972; Leynadier *et al.* 1977; Shelley, 1974). More recently, others have successfully combined the enrichment procedure as described (Moneret-Vautrin, Gerard & Grilliat, 1978) with our staining technique (Mumcuoglu & Wortmann, 1979). The test described in this article was developed with the goal of marketing it as a ready-to-use kit. It has been commercially available in France for 3 years and approximately 1000 tests are performed each month.

The positive correlation established between clinical symptoms, skin and provocation tests, and HBDT shows that the latter test represents a valid procedure for the diagnosis of immediate hypersensitivity in patients. This correlation reached 80% for common allergens like pollens, dust, and animal danders. The correlation was not as good when the medical history was incomplete which is frequently the case in drug allergies. The test detects immediate hypersensitivity with accurate specificity: we have

yet to observe a positive degranulation caused by an allergen to which the patient was not clinically sensitive. This, however, does not seem to be the case for allergic reactions to food (Moneret-Vautrin *et al.*, 1979). Our study also showed that HBDT and histamine release positively correlated with each other. Similar results had been previously obtained in rabbits (Benveniste *et al.*, 1977). The test can detect sensitivity to any allergens and has proved invaluable when other procedures for allergic diagnosis were not possible. We use it for very young children, patients with poor skin conditions, when the skin tests are not specifically positive, and especially when the clinician does not want to utilize a skin test containing 'dangerous' antigens. We frequently observed degranulation with aspirin, both in washed and whole blood. This type of hypersensitivity has been described as non-immunological in nature (Amos *et al.*, 1971; Parker, 1974). Our results indicate that it could indeed be related to basophil degranulation, probably mediated by cell-bound IgE antibody. Basophil degranulations were routinely obtained with food and insect antigens in our own and several other laboratories (Moneret-Vautrin *et al.*, 1979; Weill *et al.* 1978). The same method has been applied to the detection of antigens other than the classic ones. Degranulations were observed in the presence of DNA in the blood of systemic lupus erythematosus patients (Egido *et al.*, 1980) and also in the presence of *Staphylococcus albus* in shunt nephritis (Martini *et al.*, 1978), as well as in the presence of *Streptococcus* antigens in post-streptococcal glomerulonephritis and parasitic antigens (J. Benveniste, unpublished observations; Fleury *et al.*, 1980). Suggesting this simple technique could be applied to the diagnosis of various auto-immune and infectious diseases.

In all, we detected in 20% of the patients a difference when using whole *vs* washed blood, i.e. more antigen was needed to reach the same degranulation in whole blood as compared with washed leucocytes. In 10% of the patients, the test was completely negative for whole blood, but degranulation occurred after removal of the plasma. This result probably indicates the presence of blocking antibodies. We have not been able to conduct follow-up studies exploring the relationship between hyposensitization, clinical improvement, and the appearance of these antibodies.

However, the HBDT has several limitations. Obviously, it cannot be used when the number of basophils is too low. Our usual minimum was 20/mm³ which occurred in about 20% of our patients, half of whom were testable a few days later. The new concentration technique brings this percentage down to 1. A patient should not be tested during an allergic manifestation, but rather at least 3 or 4 days after the disappearance of symptoms. The rejection rate due to low basophil counts is minimized when the blood is tested unwashed immediately after collection, as in some patients basophils disappear after washing. This may be an indication of the peculiar fragility of these patient's basophils.

We have now automatized the counting procedure after the incubation period, basophils are counted on a Hemalog D (Technicon). Results are strictly identical to the manual technique (Fig. 3) with the obvious advantages of speed, homogeneity of results, and ability to count a large number of cells. The main disadvantage is the cost of the machine and the relative complexity of its maintenance.

The above-described test may well represent progress in that it is simple, inexpensive, available to the clinician and capable of detecting immediate hypersensitivity, not only to common allergens, but also to drugs, chemicals, and food allergens. In our experience it has very often represented the only means of diagnosis, notably in the

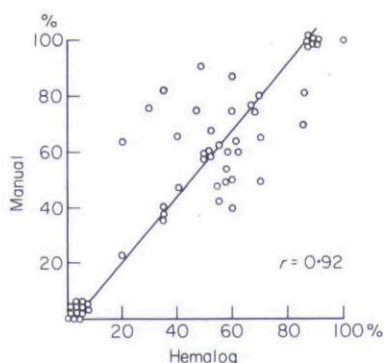


Fig. 3. Correlation between degranulations to various allergens observed with the manual technique and the automated one (Hemalog).

case of drug allergy. At present it cannot replace the commonly used skin testing procedures, but it is used more and more frequently in many clinical situations in which the skin test is impractical or leaves unspecific results. The HBDT can provide semi-quantitative information on the presence of blocking factors and could be used as a method of determining the class of the sensitizing or blocking antibodies.

Acknowledgment

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Addendum

'Report of a Meeting on Allergic Diseases. Geneva 24-28 July, 1978'. *Clinical Allergy* (1980) **10**, 1-20.

Tables 1 and 2 in this report are based on Tables 1 and 3 from: Turner, Kevin J. (1978). The Conflicting role of parasitic infections in modulating the prevalence of asthma, *Papua New Guinea Medical Journal*, **21**, 86.

Erratum

In the paper 'Serial IgE levels in allergic farmers related to the mite content of their hay' by O.D. Cuthbert *et al.* (*Clinical Allergy*, 1980, **10**, 601-607), the last paragraph on page 603, beginning 'Thus over one-third . . .' was inserted in error, and should be ignored.

European Academy of Allergy and Clinical Immunology. Annual Meeting, 1981

This meeting will be held in Clermont-Ferrand on 24-26 September 1981. Further information can be obtained from Professor Claude Molina, Hôpital Sabourin, B.P. 12 - La Plaine, 63018 Clermont-Ferrand, France.

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